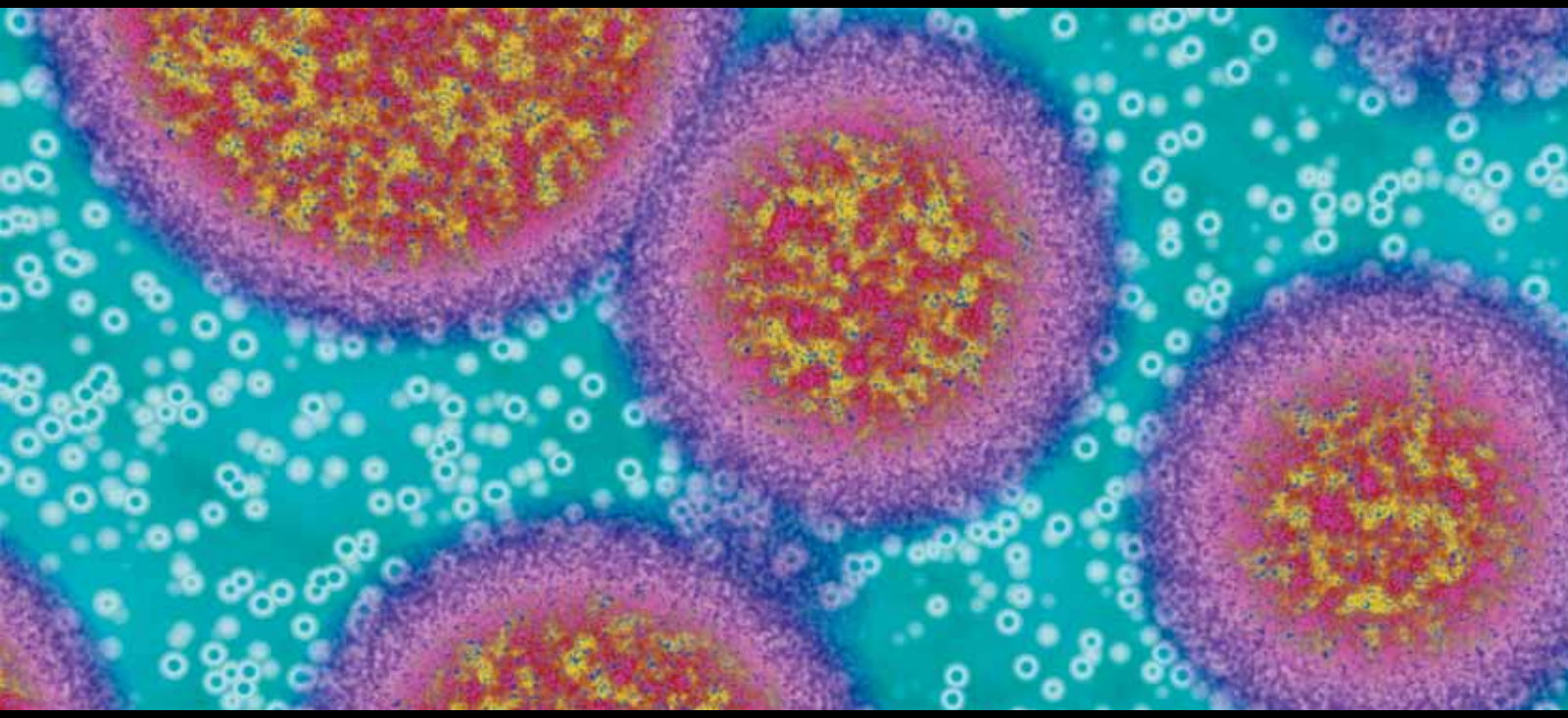
International Journal of Cell Biology

Selective Types of Autophagy

Guest Editors: Fulvio Reggiori, Masaaki Komatsu, Kim Finley, and Anne Simonsen



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Editorial Board

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 156272, 2 pages
doi:10.1155/2012/156272

Editorial

Selective Types of Autophagy

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Received 20 March 2012; Accepted 20 March 2012

The focus of this special issue of the International Journal of Cell Biology is to underscore the recent developments in the field of macroautophagy and how this degradative pathway intersects with cellular metabolism, complex physiological functions, and human diseases. During the last decade, autophagy has become an expanding field in biomedical life sciences due to its involvement with numerous intracellular processes. Autophagy also plays a role in pathology, and it has the therapeutic potential to be the target for the treatment of specific human diseases. Early studies suggested that autophagy was a nonselective process in which cytoplasmic structures were randomly sequestered into autophagosomes before being delivered to the mammalian lysosome or the plant and yeast vacuole for degradation. Now there is growing evidence that unwanted cellular structures can be selectively recognized and exclusively eliminated within cells (F. Reggiori et al., "*Selective types of autophagy*"). This is achieved through the action of specific autophagy receptors, as reviewed by C. Behrends and S. Fulda in "*Receptor proteins in selective autophagy*") and studied by K. Marchbank et al. "*MAP1B interaction with the FW domain of the autophagic receptor Nbr1 facilitates its association to the microtubule network*". Thus excess or damaged organelles including mitochondria (A. May et al., "*The many faces of mitochondrial autophagy: making sense of contrasting observations in recent research*"; Y. Hirota et al., "*The physiological role of mitophagy: new insights into phosphorylation events*"), peroxisomes (A. Till et al., "*Pexophagy: the selective degradation of peroxisomes*"), lipid droplets (R. Singh and A. Cuervo, "*Lipophagy: connecting autophagy and lipid metabolism*"), endoplasmic reticulum and ribosomes (E. Cebollero et al., "*Reticulophagy and ribophagy: regulated degradation of protein production factories*") can be specifically sequestered by autophagosomes and targeted to the lysosome for degradation.

Importantly, there is growing evidence that selective autophagy subtypes also have a wide range of physiological functions. In yeast, the cytosol-to-vacuole (Cvt) pathway transports hydrolases into the vacuole, which is reviewed by M. Umekawa and D. Klionsky in "*The cytoplasm-to-vacuole targeting pathway: a historical perspective*". In eukaryotes, autophagy plays a central role in both innate and acquired immunity. Further sequestration and elimination of invading pathogens such as *Salmonella* and *Staphylococcus aureus* have been exploited to study autophagosome biogenesis (T. Noda et al., "*Three-axis model for Atg recruitment in autophagy against Salmonella*"; M. Mauthe et al., "*WIPI-1 positive autophagosome-like vesicles entrap pathogenic Staphylococcus aureus for lysosomal degradation*"). In pancreas cells, autophagy has recently been shown to specifically turn over secretory granules, as described by M. Vaccaro in "*Zymophagy: selective autophagy of secretory granules*". Dysregulation of autophagic function has been implicated in a growing list of disease processes and has underscored the selective or substrate-specific versions of the pathway. Examples in this special issue include the clearance of aggregates associated with neurological diseases, as reviewed by T. Lamark and T. Johansen in "*Aggrephagy: selective disposal of protein aggregates by macroautophagy*" and by I. Nezis in "*Selective autophagy in Drosophila*". In terms of cancer biology, autophagy has been viewed as having

dual roles in both tumor suppression and progression. K. Hughson et al. in "*Implications of therapy-induced selective autophagy on tumor metabolism and survival*" review how activation of autophagy selective forms can be used as a potential therapeutic approach for the treatment of specific cancers. Adding to the complexity of autophagic function and regulation, the article by K. Juenemann and E. Reits "*Alternative macroautophagic pathways*" explores alternative macroautophagic pathways that are independent of key core autophagy components such as Beclin-1 or Atg5. We expect future research on the mechanism and regulation of selective autophagy, and the physiological importance of this pathway in human disease will be very exciting and expand on the findings highlighted in this issue of IJCB.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 179207, 13 pages
doi:10.1155/2012/179207

Research Article

WIPI-1 Positive Autophagosome-Like Vesicles Entrap Pathogenic *Staphylococcus aureus* for Lysosomal Degradation

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Received 14 November 2011; Revised 31 January 2012; Accepted 6 February 2012

Academic Editor: Anne Simonsen

Invading pathogens provoke the autophagic machinery and, in a process termed xenophagy, the host cell survives because autophagy is employed as a safeguard for pathogens that escaped phagosomes. However, some pathogens can manipulate the autophagic pathway and replicate within the niche of generated autophagosome-like vesicles. By automated fluorescence-based high content analyses, we demonstrate that *Staphylococcus aureus* strains (USA300, HG001, SA113) stimulate autophagy and become entrapped in intracellular PtdIns(3)P-enriched vesicles that are decorated with human WIPI-1, an essential PtdIns(3)P effector of canonical autophagy and membrane protein of both phagophores and autophagosomes. Further, *agr*-positive *S. aureus* (USA300, HG001) strains were more efficiently entrapped in WIPI-1 positive autophagosome-like vesicles when compared to *agr*-negative cells (SA113). By confocal and electron microscopy we provide evidence that single- and multiple-Staphylococci entrapped undergo cell division. Moreover, the number of WIPI-1 positive autophagosome-like vesicles entrapping Staphylococci significantly increased upon (i) lysosomal inhibition by bafilomycin A_1 and (ii) blocking PIKfyve-mediated PtdIns(3,5)P_2 generation by YM201636. In summary, our results provide evidence that the PtdIns(3)P effector function of WIPI-1 is utilized during xenophagy of *Staphylococcus aureus*. We suggest that invading *S. aureus* cells become entrapped in autophagosome-like WIPI-1 positive vesicles targeted for lysosomal degradation in nonprofessional host cells.

1. Introduction

Macroautophagy (hereafter autophagy) is a cytoprotective cellular degradation mechanism for long-lived proteins and organelles [1]. Autophagy is specific to eukaryotic cells and important for cellular survival by enabling a constitutive clearance and recycling of cytoplasmic material (basal autophagy). Crucial to the process of autophagy is the fact, that cytoplasmic material is stochastically degraded. Portions of the cytoplasm become randomly sequestered in unique, double-membrane vesicles, autophagosomes. Autophagosomes are generated by elongation and closure of a membrane precursor, the phagophore. Subsequently, autophagosomes fuse with lysosomes to acquire acidic hydrolases for cargo degradation [2]. This stochastic constitutive form of autophagy provides constant clearance of the cytoplasm. Upon stress, such as starvation, the autophagic activity is induced above basal level to compensate nutrient shortage by providing monomeric constituents, such as amino acids, and energy. Conversely, under nutrient-rich conditions autophagy is suppressed by the mTORC1 signaling circuit [3]. Importantly, autophagy is also activated in a specific manner and targets damaged organelles, protein aggregates, or pathogens for degradation [4]. Both, stochastic and specific autophagy are crucial to secure cellular homeostasis [5].

Prerequisite for the formation of autophagosomes is the generation of an essential phospholipid, phosphatidylinositol 3-phosphate (PtdIns(3)P), a result of the activity

of the phosphatidylinositol 3-kinase class III (PtdIns3KC3) in complex with Beclin 1, p150, and Atg14L [6, 7]. The PtdIns(3)P signal is decoded through PtdIns(3)P-binding effectors specific to autophagy, such as the human WIPI proteins [8]. WIPI-1 (Atg18 in yeast) specifically binds PtdIns(3)P at the phagophore and fosters the recruitment of two ubiquitin-like conjugation systems, Atg12 and LC3, involved in phagophore elongation and closure [9]. Subsequently, WIPI-1 becomes a membrane protein of autophagosomes where it localizes at both the inner and outer membrane [10, 11]. Hence the specific localization of WIPI-1 at the phagophore and at autophagosomes upon the initiation of autophagy can monitor the process of canonical autophagy, as it is dependent on the PtdIns(3)P signal [11].

The process of autophagy is closely connected with a variety of diseases such as tumor development, neurodegeneration, and with cellular responses to pathogens, including viral infection and bacterial cell invasion [5, 12]. *Staphylococcus aureus*, a major pathogen for nosocomial infectious diseases was initially characterized as an extracellular pathogen, but was later found to also target nonprofessional host cells like keratinocytes, fibroblasts, endothelial cells, and epithelial cells where invading *S. aureus* liberates from the endosomal compartment [13]. In HeLa cells, *S. aureus* was found to become sequestered and to replicate in autophagosome-like vesicles as a result of autophagosome/lysosome fusion block, which ultimately leads to cell death [14].

Here, we visualized the invasion of mCherry-expressing *S. aureus* strains USA300, HG001, SA113 in human U2OS tumor cells that stably express GFP-WIPI-1 for automated fluorescence-based high content analyses, a procedure that monitors the autophagic process and that we have established earlier [15]. We provide evidence that *S. aureus* stimulates canonical autophagy in nonprofessional host cells and becomes entrapped in noncanonical WIPI-1 positive autophagosome-like vesicles. Time course experiments showed that the number of tumor cells that contain such WIPI-1 positive autophagosome-like vesicles with entrapped *S. aureus* cells increased over time (30 min–2 h). After an infection period of 2 h, 40–50% of the cells harbored WIPI-1 positive autophagosome-like vesicles sequestering *agr*-positive *S. aureus* (USA300, HG001), and 20% of the tumor cells contained entrapped *agr*-negative *S. aureus* (SA113). Importantly, we demonstrate that the number of WIPI-1 positive autophagosome-like vesicles harboring *S. aureus* significantly increased upon lysosomal inhibition, strongly arguing for the degradation of *S. aureus* through xenophagy. In addition, by employing GFP-FYVE and a selective PIKfyve inhibitor (YM201636) we further demonstrate the requirement of PtdIns(3)P-enriched membranes during the process of entrapping invading *S. aureus*.

2. Material and Methods

2.1. Eukaryotic Cell Culture. The human osteosarcoma cell line U2OS (ATCC) was cultured in DMEM (Invitrogen) supplemented with 10% FCS (PAA), 100 U/mL penicillin/100 μg/mL streptomycin (Invitrogen), 5 μg/mL plasmocin (Invivogen) at 37°C, 5% CO_2. Monoclonal human U2OS cell clones stably expressing either GFP-WIPI-1 [15, 20] or GFP-2xFYVE [9] were cultured in DMEM (Invitrogen) supplemented with 10% FCS (PAA), 100 U/mL penicillin/100 μg/mL streptomycin (Invitrogen), 5 μg/mL plasmocin (Invivogen), 0.6 mg/mL G418 (Invitrogen) at 37°C, 5% CO_2. The following media were used for treatments: DMEM/FCS (DMEM supplemented with 10% FCS), DMEM (DMEM without FCS), and EBSS (Sigma-Aldrich).

2.2. Bacterial Strains. S. aureus USA300, HG001, SA113, or *S. carnosus* TM300 [21] (see Table 1) were electroporated with the pCtuf-*ppmch* plasmid. The pCtuf-*ppmch* plasmid encoded mCherry fused with the propeptide of lipase for fluorescence enhancement, and *ppmch* expression was controlled by the native constitutive EF-Tu promotor. Electroporated bacterial strains were grown in basic medium (1% peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, 0.1% K_2HPO_4) at 37°C to an OD_{600} of 0.8 and harvested by centrifugation.

2.3. Bacterial Infection of Eukaryotic Host Cells. GFP-WIPI-1 expressing U2OS cells were seeded in 96-well plates (Brand) in DMEM/10% FCS 20 hours before bacterial infection. *S. aureus* (USA300, HG001, SA113) or *S. carnosus* carrying the pCtuf-*ppmch* plasmid, were diluted in DMEM, DMEM/10% FCS or EBSS (Sigma-Aldrich) to an m.o.i of 100, added to the GFP-WIPI-1 U2OS cells, and incubated for 0.5, 1, or 2 hours at 37°C, 5% CO_2. Alternatively, *S. aureus* USA300 cells were diluted (m.o.i of 100) in DMEM/FCS supplemented with either bafilomycin A_1 (200 nM, Sigma-Aldrich) or YM201636 (800 nM, Cayman Chemicals) or with both and used to infect GFP-WIPI-1 expressing U2OS cells for 2 hours at 37°C, 5% CO_2. Alternatively, GFP-2xFYVE expressing U2OS cells [9] were infected with *S. aureus* USA300 (in DMEM/FCS) for 2 hours at 37°C, 5% CO_2.

2.4. Autophagy Assay. GFP-WIPI-1 expressing U2OS cells, seeded in 96-well plates, were treated with nutrient-rich culture medium (DMEM/10% FCS), culture medium lacking serum (DMEM), or medium lacking serum and amino acids (EBSS) for 0.5, 1, or 2 hours. After fixation with 3.7% paraformaldehyde for 30 minutes, autophagy was accessed by WIPI-1 puncta formation analysis [11, 22] (see below).

2.5. Confocal Laser Scanning Microscopy. Confocal microscopy was conducted as previously described [8]. Images were acquired using an LSM510 microscope (Zeiss) and a 63 × 1.4 DIC Plan-Apochromat oil-immersion objective. For each image, 8–10 optical sections (0.5 μm) were acquired. Both, single optical sections as well as projections from 8–10 optical sections are presented.

2.6. Automated Fluorescence Image Acquisition and Analysis. Stable GFP-WIPI-1 U2OS cells were automatically imaged and analysed using the *In Cell Analyzer 1000* (GE Healthcare) as described earlier [9, 15]. Cells exposed to bacteria (see above) were stained with DAPI (5 μg/mL; Applichem). Fluorescence images were automatically acquired with a Nikon

Table 1: Bacterial strains used in this study.

Bacterial strain	Relevant properties	Relevant genotype	Reference
S. aureus USA300	Pathogenic, community-associated methicillin-resistant *S. aureus* (CA-MRSA)	agr^+	[16]
S. aureus HG001	Pathogenic, methicillin-sensitive *S. aureus* (MSSA)	agr^+	[17]
S. aureus SA113	Pathogenic, methicillin-sensitive *S. aureus* (MSSA)	agr^-	[18]
S. carnosus TM300	Apathogenic, food grade staphylococcal species		[19]

40x Plan Fluor objective and the excitation/emission filter D360_40X/HQ460_40M (DAPI), HQ535_50X/HQ620_60 M (mCherry), and S475_20X/HQ535_50M (GFP). GFP-WIPI-1 puncta were automatically analysed as previously described [15] and the number of GFP-WIPI-1 puncta-positive cells as well as the number of GFP-WIPI-1 puncta per cell was determined. Red fluorescent bacteria were automatically analysed by using the *dual area object analysis*. The algorithms *inclusion* and *multiscale top hat* were applied and the total area of bacterial fluorescence within the cell was determined. To determine the number of cells containing GFP-WIPI-1 positive autophagosome-like vesicles sequestering bacteria, automatically acquired fused images (DAPI, GFP, mCherry) of 100 individual cells for each treatment were analyzed.

2.7. Electron Microscopy. Stable GFP-WIPI-1 U2OS cells were infected with *S. aureus* USA300 (m.o.i of 100) in DMEM/FCS and fixed in 2% glutaraldehyde and 0.5% osmium tetroxide in 0.1 M PBS, dehydrated with ethanol, and embedded in Epon using standard procedures as previously described [23]. Thin sections were cut using an ultramicrotome and contrasted with uranyl acetate and lead citrate. Thin sections were examined in an EM410 electron microscope (Philips) and documented digitally (DITABIS).

2.8. Statistical Analysis. Statistical significance was evaluated using two-tailed heteroscedastic t-testing and P values were calculated.

3. Results

3.1. Visualizing Basal and Induced Autophagy by Automated GFP-WIPI-1 Image Acquisition and Analysis. The WIPI-1 puncta-formation assay allows the assessment of the evolutionarily conserved, PtdIns(3)P-dependent initiation of autophagy on the basis of fluorescence microscopy, previously employed by using confocal microscopy or automated image acquisition and analysis [11, 15]. Thereby, endogenous WIPI-1 can be visualized by indirect immunofluorescence or alternatively by introducing GFP-WIPI-1 as conducted in the present study. Fluorescent WIPI-1 puncta reflect the accumulation of WIPI-1 at membranes via its specific binding to PtdIns(3)P was found to represent phagophores and autophagosomes [10, 11]. In addition, WIPI-1 binds to PtdIns(3)P at the endoplasmic reticulum and at the plasma membrane upon the induction of autophagy, indicative for membrane origins where phagophore/autophagosome formation is initiated by unknown mechanisms [10]. Here, we employed automated GFP-WIPI-1 image acquisition and analysis as follows. Human U2OS cells that stably express GFP-WIPI-1 were seeded in 96-well plates and basal autophagy, and starvation-induced autophagy was monitored in up to 3000 individual cells per treatment over time (Figure 1). After an incubation period of 0.5, 1, or 2 h with nutrient-rich culture medium (DMEM/FCS), basal autophagic activity was found in approximately 10% of the cells (Figures 1(a) and 1(d)). Serum starvation (DMEM) elevated the number of GFP-WIPI-1 puncta-positive cells to approximately 50% (Figures 1(b) and 1(d)), and both serum and amino acid starvation (EBSS) further elevated this number to approximately 85% (Figures 1(c) and 1(d)). In addition, we demonstrate that with regard to nutrient-rich medium (DMEM/FCS), the number of GFP-WIPI-1 puncta per cell also increased upon serum (DMEM) or upon both serum and amino acid starvation (EBSS) (Figure 1(e)). These culture media (DMEM/FCS, DMEM, EBSS) were used in the following experiments to infect GFP-WIPI-1 expressing U2OS cells with mCherry-expressing Staphylococci.

3.2. Formation of GFP-WIPI-1 Positive Autophagosome-Like Vesicles upon Staphylococcus aureus Infection. Upon infection of GFP-WIPI-1 U2OS cells with pathogenic Staphylococci, here *S. aureus* HG001, in nutrient-rich medium (DMEM/FCS), we identified canonical, autophagosomal GFP-WIPI-1 membranes (Figures 2(a) and 2(b)), and new GFP-WIPI-1 autophagosome-like vesicles that were larger in diameter with decreased fluorescence intensity (Figure 2(c)) when compared to the canonical GFP-WIPI-1 puncta. GFP-WIPI-1 autophagosome-like vesicles (Figure 2(c)) were rarely observed when starvation media (DMEM, EBSS) were used during the infection with *S. aureus* HG001 (Supplementary Figure 1 available online at doi:10.1155/2012/179207).

To monitor and quantify this particular GFP-WIPI-1 response upon mCherry-expressing Staphylococci infection in an automated fashion (Figure 3), cells were stained with DAPI and by using three different excitation/emission filters, DAPI, GFP, and mCherry fluorescence images were acquired (Figure 3). Up to 2723 individual cells per treatment were automatically recognized by both DAPI and the overall cellular GFP fluorescence. GFP images were used to automatically detect and determine the number of cells harboring GFP-WIPI-1 puncta by applying a decision tree as previously described [15]. Additionally, mCherry fluorescence was used to automatically determine the fluorescence area, reflecting the load of intracellular Staphylococci. For the quantification of cells harboring GFP-WIPI-1 positive autophagosome-like vesicles entrapping Staphylococci, fused images (DAPI, GFP, mCherry) of 100 individual cells were used (Figure 3).

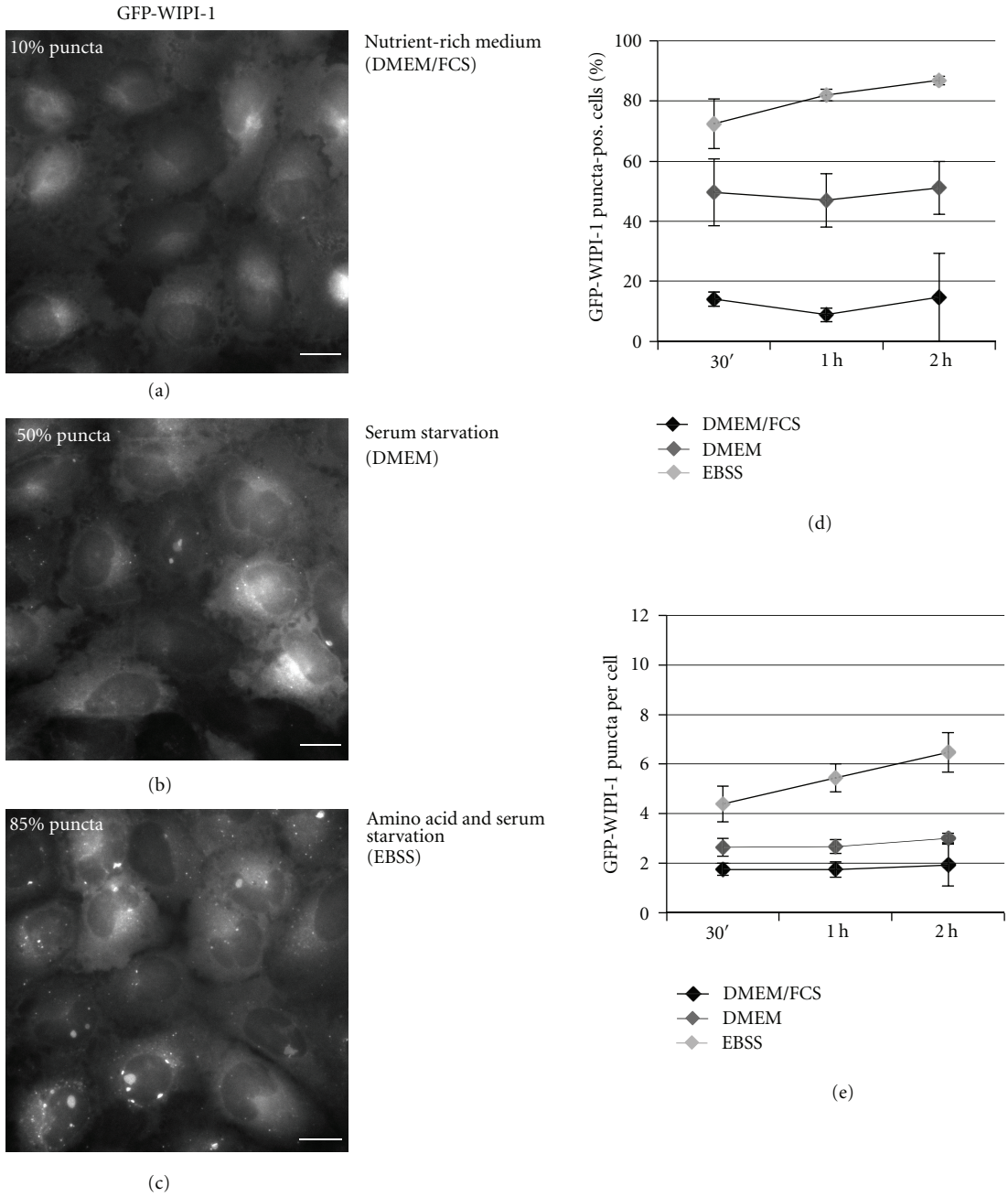

Figure 1: GFP-WIPI-1 puncta formation upon serum and amino acid starvation. GFP-WIPI-1 U2OS cells were treated with nutrient-rich culture medium (DMEM/FCS), serum-free culture medium (DMEM), or with medium lacking both serum and amino acids (EBSS) for 0.5, 1, and 2 h. Fluorescence images were automatically acquired and 2 h treatment images are shown ((a)–(c)). The number of GFP-WIPI-1 puncta-positive cells (d), and of GFP-WIPI-1 puncta per cell (e) was automatically determined. Each measure point represents mean value from up to 3000 individually analyzed cells per treatment condition ± SD ($n = 2$, each in triplicates). Scale bars: 20 μm.

3.3. Pathogenic Staphylococcus aureus USA300, HG001, and SA113 Stimulated Canonical Autophagosome Formation and Became Entrapped in GFP-WIPI-1 Positive Autophagosome-Like Vesicles. In the following experiment, GFP-WIPI-1 expressing U2OS cells were infected for 0.5, 1 and 2 h with mCherry-expressing *S. aureus* USA300 (Figure 4, Supplementary Figure 2), HG001 (Figure 5, Supplementary Figure 3), or SA113 (Figure 6, Supplementary Figure 4) either in nutrient-rich medium (DMEM/FCS), serum-free medium (DMEM), or serum and amino acid-free medium (EBSS). Subsequently, fluorescence images (approximately 2000 individual cells per treatment) were automatically acquired and analyzed as described (Figure 3). Please note that the control experiments in Figure 1 were conducted in parallel to the experiments presented in Figures 4–7 hence provide the comparison for conditions without

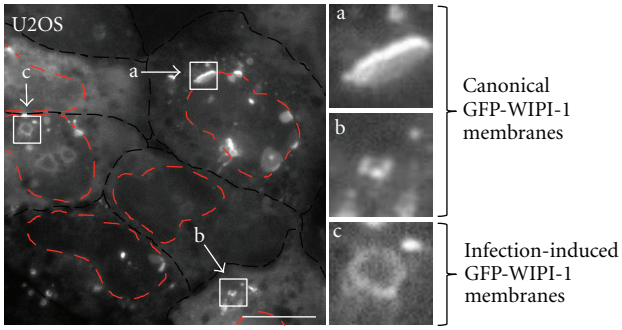

Figure 2: GFP-WIPI-1 images upon infection of U2OS cells with *S. aureus* HG001. GFP-WIPI-1 U2OS cells were infected with *S. aureus* HG001 in DMEM/FCS for 2 h and images were automatically acquired. GFP-WIPI-1 fluorescence of the cells (indicated with the black-dashed line) is shown, and cell nuclei are indicated (red-dashed line) according to DAPI staining (not shown). Highlighted are the different GFP-WIPI-1 structures observed: large perinuclear GFP-WIPI-1 positive membranes (a) and cytoplasmic GFP-WIPI-1 puncta (b), reflecting canonical autophagosomal membranes. In addition, GFP-WIPI-1 positive autophagosomal-like vesicles appeared specifically upon infection (c). Scale bars: 20 μm. Supplementary information is provided (Supplementary Figure 1).

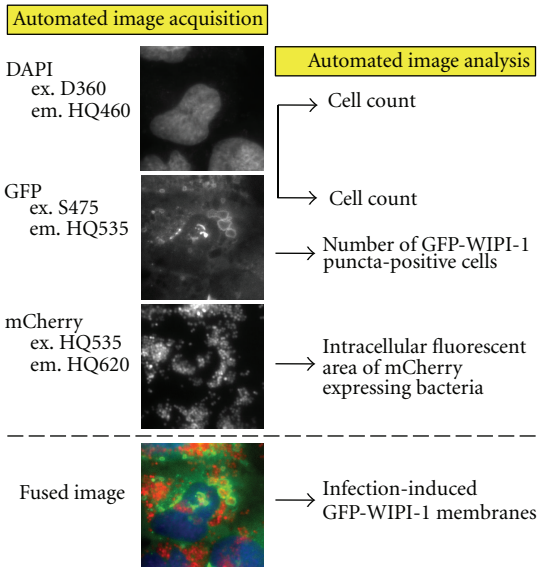

Figure 3: Automated image acquisition and analysis of stably expressing GFP-WIPI-1 U2OS cells with mCherry-expressing Staphylococci. Fluorescence images (middle panel) were automatically acquired using different emission/excitation filters for DAPI, GFP, and mCherry (left panel). DAPI and GFP images were used to automatically detect individual cells, and GFP images were used for detecting and analyzing GFP-WIPI-1 puncta formation (indicated in the right panel). Additionally, for each individual cell the bacterial area was determined (indicated in the right panel) and a fused image was further used to determine the number of cells harboring WIPI-1 positive autophagosome-like vesicles entrapping Staphylococci.

(Figure 1) and with (Figures 4–7, Supplementary Figures 2–5) Staphylococci.

As shown in Figure 1, under nutrient rich conditions (DMEM/FCS) the number of GFP-WIPI-1 puncta-positive cells is low (approximately 10%), reflecting cells that undergo basal autophagy. Interestingly, upon infection of GFP-WIPI-1 expressing U2OS cells with *S. aureus* USA300 in DMEM/FCS, a prominent increase of GFP-WIPI-1 puncta-positive cells (up to approximately 70% within 2 h of infection) was observed (Figure 4(a), in green). In addition, the number of GFP-WIPI-1 puncta per individual cell also increased upon *S. aureus* USA300 infection in DMEM/FCS (Supplementary Figure 6(B)). The elevated number of GFP-WIPI-1 puncta-positive cells and GFP-WIPI-1 puncta per cell correlated with an increase of intracellular *S. aureus* USA300 (Figure 4(a), in red). Using serum-free conditions either in the presence (DMEM, Figure 4(b), in red) or absence of amino acids (EBSS, Figure 4(c), in red), no increase of intracellular *S. aureus* USA300 was observed. However, infection of *S. aureus* USA300 in DMEM also resulted in an increase (up to approximately 70%) of GFP-WIPI-1 puncta-positive cells (Figure 4(b), in green), whereas *S. aureus* USA300 in EBSS (Figure 4(c)) did not trigger a further increase of the number of GFP-WIPI-1 puncta-positive cells when compared to EBSS treatment alone (Figure 1).

Next, we determined the number of cells displaying entrapped *S. aureus* USA300 within GFP-WIPI-1 positive

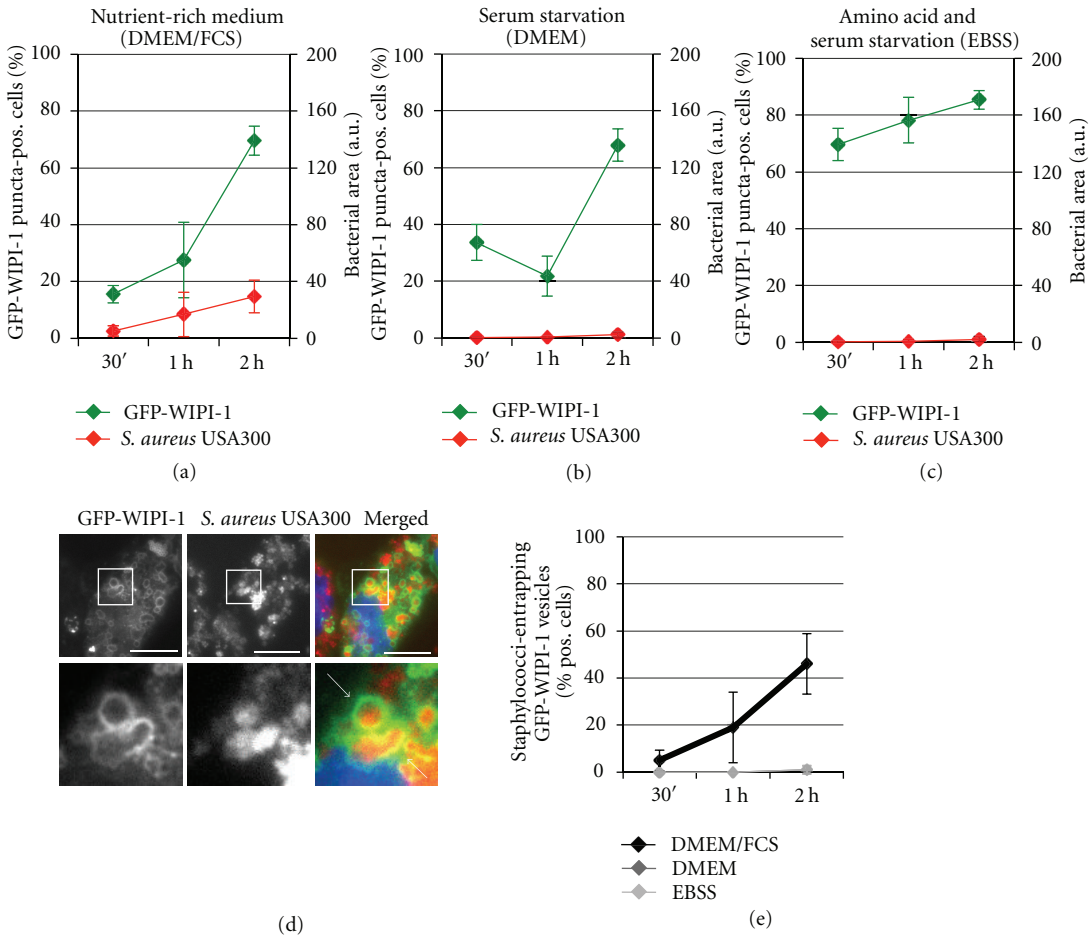

Figure 4: Pathogenic *S. aureus* USA300 induces GFP-WIPI-1 puncta formation and becomes entrapped in GFP-WIPI-1 positive autophagosome-like vesicles. GFP-WIPI-1 U2OS cells were infected with mCherry-expressing *S. aureus* USA300 for 0.5, 1, and 2 h in DMEM/FCS, DMEM, or EBSS. Automated image acquisition and analysis were conducted as described in Figure 3. The quantification of up to 2000 individual cells is presented for GFP-WIPI-1 (in green) and *S. aureus* USA300 (in red) using either DMEM/FCS (a), DMEM (b), or EBSS (c) for infection ± SD ($n = 2$, each in duplicates). Representative images (2 h infection in DMEM/FCS) are shown (d). Scale bars: 20 μm. From 100 infected cells for each of the treatment condition, the number of cells displaying GFP-WIPI-1 positive autophagosomal-like vesicles entrapping *S. aureus* USA300 was determined (e) ± SD ($n = 2$, each in duplicates).

autophagosome-like vesicles (Figures 4(d) and 4(e)). In line with the increased number of cells carrying intracellular *S. aureus* USA300 when nutrient-rich medium (DMEM/FCS) was used (Figure 4(a)), the number of cells with GFP-WIPI-1 positive autophagosome-like vesicles that entrap *S. aureus* USA300 (approximately 40%) also increased (Figure 4(e)). This was not observed by using DMEM or EBSS (Figure 4(e)). We also provide the control images corresponding to *S. aureus* USA300 infections using either DMEM or EBSS (Supplementary Figure 2).

The infection of stably expressing GFP-WIPI-1 U2OS cells with *S. aureus* HG001 in DMEM/FCS also triggered an elevation of GFP-WIPI-1 puncta-positive cells (up to 76%) (Figure 5(a), in green) and of GFP-WIPI-1 puncta per cell (Supplementary Figure 6(C)). Again, the increased number of GFP-WIPI-1 puncta-positive cells correlated with an increased bacterial load (Figure 5(a), in red) and the increase in the number of cells displaying GFP-WIPI-1 positive autophagosome-like vesicles entrapping *S. aureus* HG001 (approximately 40%) (Figures 5(d) and 5(e)). Also in this case, this feature was not observed by using DMEM or EBSS (Figure 5(e)), but DMEM conditions still triggered an increase of GFP-WIPI-1 puncta formation (Figure 5(b), Supplementary Figure 6(C)) when compared with control setting (Figure 1, Supplementary Figure 6(A)). Control images corresponding to *S. aureus* HG001 infections using either DMEM or EBSS are also provided (Supplementary Figure 3).

Next, we employed the *agr*-deficient *S. aureus* strain SA113 and infected stably expressing GFP-WIPI-1 U2OS cells. Clearly, upon infection in DMEM/FCS the number of GFP-WIPI-1 puncta-positive cells increased over time to up to 60% (Figure 6(a), in green), which correlated with an increasing bacterial load (Figure 6(a), in red). See also the increased number of GFP-WIPI-1 puncta per cell upon *S. aureus* SA113 infection in DMEM/FCS (Supplementary

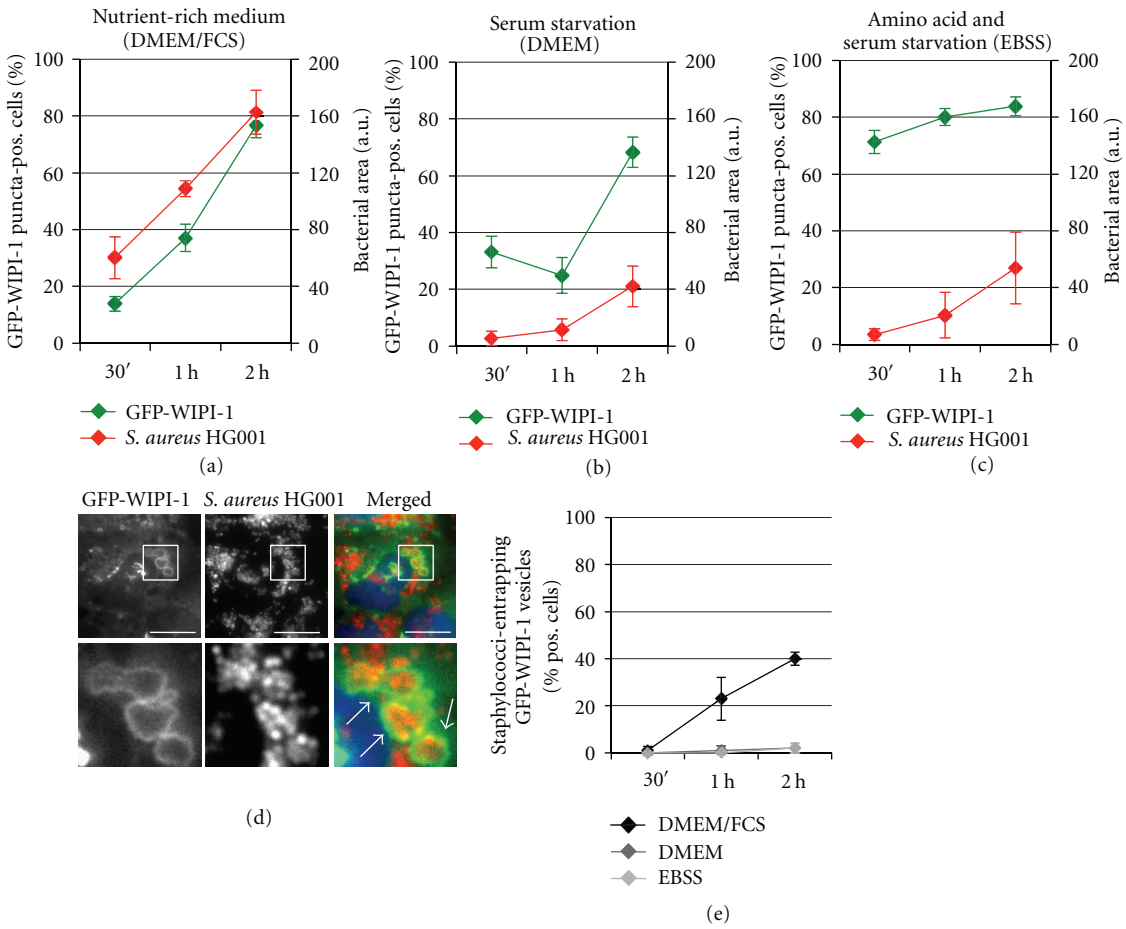

Figure 5: Pathogenic *S. aureus* HG001 induces GFP-WIPI-1 puncta formation and becomes entrapped in GFP-WIPI-1 positive autophagosome-like vesicles. According to Figure 4, GFP-WIPI-1 U2OS cells were infected with mCherry-expressing *S. aureus* HG001 in DMEM/FCS (a), DMEM (b), and EBSS (c), and up to 2000 individual cells were analyzed. Images (2 h, DMEM/FCS) are shown (d). Scale bars: 20 μm. The number of cells displaying GFP-WIPI-1 positive autophagosomal-like vesicles entrapping *S. aureus* HG001 was determined (e) ± SD ($n = 2$, each in duplicates).

Figure 6(D)). In contrast to the effect of the employed *agr*-positive *S. aureus* strains USA300 (Figure 4) and HG001 (Figure 5), the number of cells displaying *S. aureus* SA113 entrapped in GFP-WIPI-1 positive autophagosome-like vesicles was prominently lower (approximately 18%) (Figures 6(d) and 6(e)). However, the presence of *S. aureus* SA113 in DMEM also triggered an increase of GFP-WIPI-1 puncta-positive cells (Figure 6(b)) when compared to control settings (Figure 1), whereas in EBSS no further elevation was achieved (Figure 6(c)), and in both cases, cells did not display entrapped *S. aureus* SA113 (Figure 6(e)). Control images of *S. aureus* SA113 infections with either DMEM or EBSS are also provided (Supplementary Figure 4).

3.4. Apathogenic Staphylococcus carnosus TM300 Cells Were Not Entrapped in Intracellular GFP-WIPI-1 Positive Autophagosome-Like Vesicles. In contrast to the pathogenic *S. aureus* strains (see above), infection of stably expressing GFP-WIPI-1 U2OS cells with the apathogenic *S. carnosus* TM300 did not result in an invasion of host cells in either of the used media (Figures 7(a)–7(c)). In line, GFP-WIPI-1 positive autophagosome-like vesicles were not induced (Figures 7(d) and 7(e)). Control images for *S. carnosus* TM300 in DMEM or EBSS are provided (Supplementary Figure 5). Interestingly, within 2 h of incubation with *S. carnosus* TM300 in DMEM/FCS, the number of GFP-WIPI-1 puncta-positive cells increased (approximately 45%) (Figure 7(a)) when compared to the control settings (Figure 1), which was not observed by using DMEM (Figure 7(b)) or EBSS (Figure 7(c)). However, the number of GFP-WIPI-1 puncta per individual cell did not increase upon infection of *S. carnosus* TM300 in DMEM/FCS (Supplementary Figure 6(E)) when compared to uninfected conditions (Supplementary Figure 6(A)).

3.5. Inhibition of PtdIns(3,5)P_2 Production and Lysosomal Inhibition Increased the Number of WIPI-1 Positive Autophagosome-Like Vesicles Entrapping Staphylococcus aureus. Next, we questioned whether pathogenic *S. aureus* cells entrapped in GFP-WIPI-1 positive autophagosomal-like vesicles are degraded in the lysosome. We employed

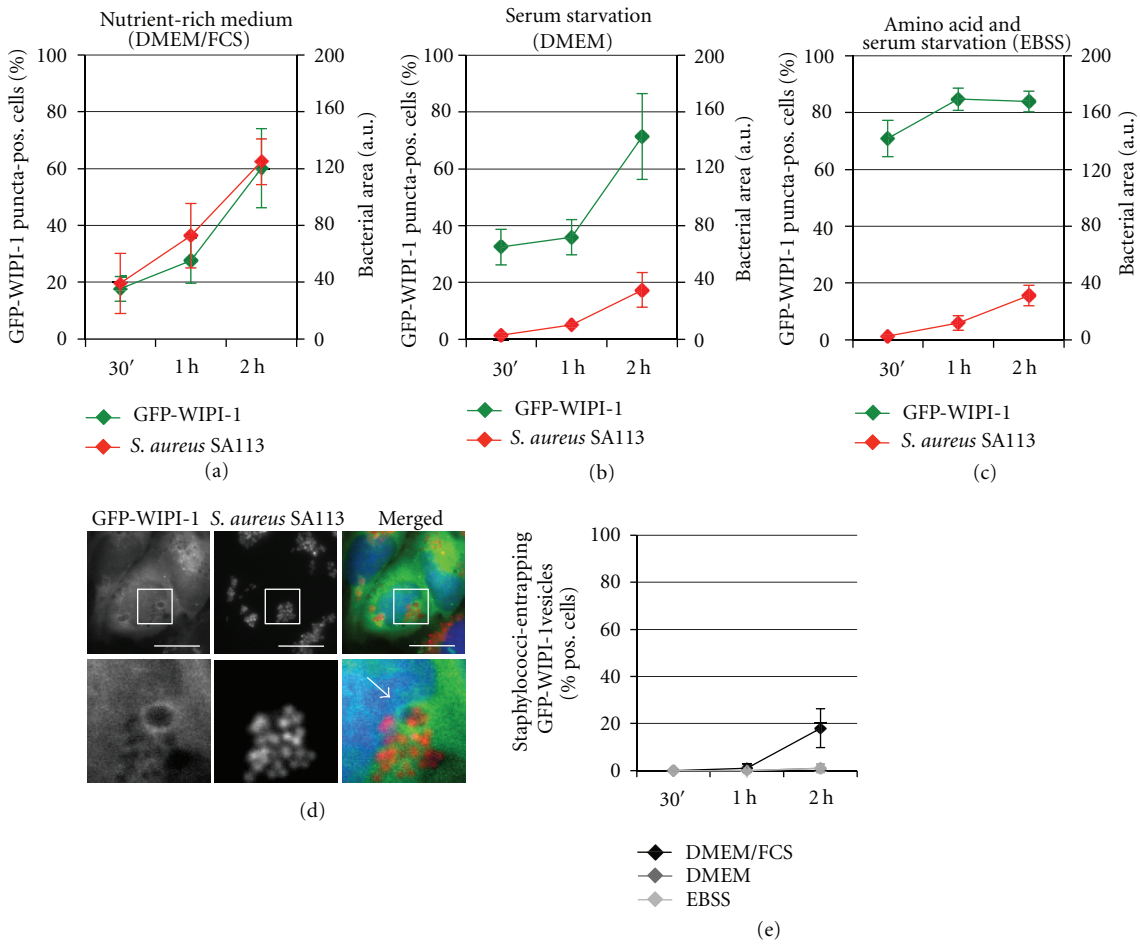

Figure 6: Pathogenic *S. aureus* SA113 induces GFP-WIPI-1 puncta formation and becomes entrapped in GFP-WIPI-1 positive autophagosome-like vesicles. According to Figures 4 and 5, GFP-WIPI-1 U2OS cells were infected with mCherry-expressing *S. aureus* SA113 in DMEM/FCS (a), DMEM (b), and EBSS (c) and analyzed (up to 2000 individual cells), representative images (2 h, DMEM/FCS) are shown ((d), scale bars: 20 μm), and the quantification of cells displaying GFP-WIPI-1 positive autophagosomal-like vesicles entrapping *S. aureus* SA113 is presented (e) $\pm$ SD ($n = 2$, each in duplicates).

the lysosomal inhibitor bafilomycin A_1 (Baf A_1) to block autophagosome/lysosome fusion events upon infection of GFP-WIPI-1 expressing U2OS cells with *S. aureus* USA300 in DMEM/FCS. Upon Baf A_1 addition the number of cells harboring GFP-WIPI-1 positive autophagosomal-like vesicles entrapping *S. aureus* USA300 (Figure 8(a), left panel) significantly increased. And, the number of GFP-WIPI-1 positive autophagosomal-like vesicles per individual cell also significantly increased (Figure 8(b), left panel). In this situation (Figure 8(a), left panel; Figure 8(b), left panel) we found that the bacterial load did not significantly change (Supplementary Figure 7).

Further, during infection of GFP-WIPI-1 expressing U2OS cells with *S. aureus* USA300 in DMEM/FCS we employed YM201636 (YM), a specific PIKfyve inhibitor that blocks PtdIns(3,5)P_2 production from PtdIns(3)P [24]. Upon YM treatment the number of cells harboring GFP-WIPI-1 positive autophagosomal-like vesicles (Figure 8(a), left panel) and the number of the vesicles per cell (Figure 8(b), left panel) significantly increased. Again, the intracellular bacterial load within the cells did not change (Supplementary Figure 7). Baf A_1/YM cotreatment had an additive effect (Figures 8(a) and 8(b) left panels). The corresponding automated GFP-WIPI-1 puncta formation analysis is also provided (Figures 8(a) and 8(b) right panels).

3.6. Confocal and Electron Microscopy of Intracellular Staphylococcus aureus USA300. To achieve more image resolution, we infected GFP-WIPI-1 expressing U2OS cells with *S. aureus* USA300 in DMEM/FCS followed by confocal laser scanning microscopy (Figure 9(a)). Clearly, GFP-WIPI-1 positive autophagosome-like vesicles harbored multiple *S. aureus* USA300 cells and the analysis of individual confocal sections confirmed that these vesicles are found in the cytoplasm (Figure 9(a), 1–4).

It has been shown that *S. aureus* invading HeLa cells become sequestered in Rab7-positive endosomes [14]. As Rab7 marks late endosomes, we here used GFP-2xFYVE to visualize early endosomes. We used GFP-2xFYVE expressing U2OS cells for infection with *S. aureus* USA300 in

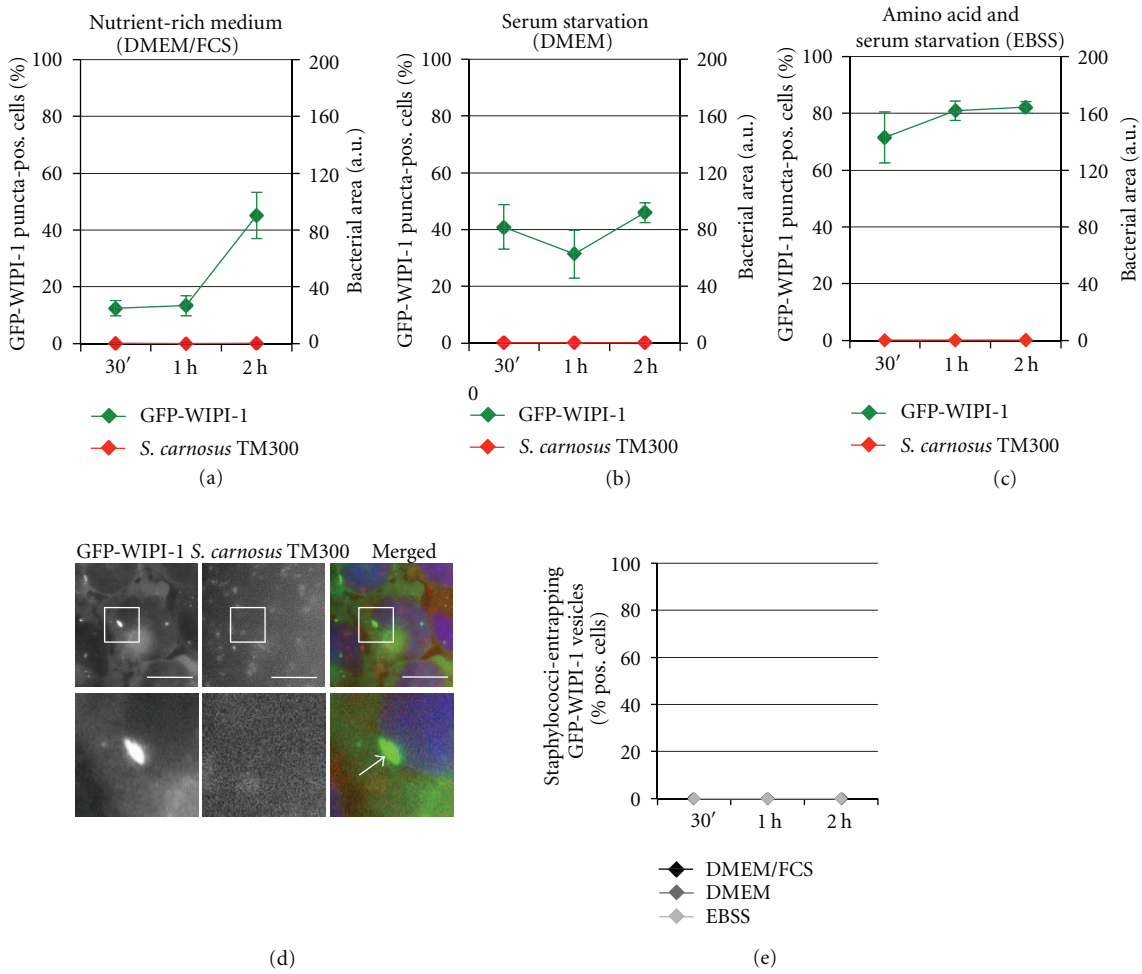

Figure 7: Apathogenic *S. carnosus* TM300 cells are not entrapped in GFP-WIPI-1 positive autophagosome-like vesicles. According to Figures 4–6, GFP-WIPI-1 U2OS cells were infected with mCherry-expressing *S. carnosus* TM300 in DMEM/FCS (a), DMEM (b), and EBSS (c) and analyzed (up to 2000 individual cells). Representative images (2 h, DMEM/FCS) are presented ((d), scale bars: 20 μm). The number of cells with GFP-WIPI-1 positive autophagosomal-like vesicles entrapping *S. carnosus* TM300 is presented (e) ± SD ($n = 2$, each in duplicates).

DMEM/FCS. Indeed, we also found that *S. aureus* USA300 cells were entrapped in GFP-2xFYVE positive endosomes (Figure 9(b), 1–4).

Further, by electron microscopy we found that intracellular *S. aureus* USA300 cells are entrapped in vesicles with a single *S. aureus* USA300 cell (Figure 3.6), or in vesicles harboring multiple *S. aureus* USA300 cells (Figure 3.6). In both cases, intracellular *S. aureus* USA300 cells showed clear signs of ongoing cell division (red arrows).

4. Discussion

Autophagy is considered an ancient eukaryotic pathway for cellular self-digestion that evolved with the endomembrane system [25]. As the endomembrane system provided an opportunity for invading pathogens to manipulate the host cell, it is further considered that the autophagic response to pathogen invasion may have also evolved as an early host defense program of eukaryotic cells [25, 26]. Interestingly enough, this hypothesis explains that (i) autophagy is in part a stochastic degradation pathway to clear the cytoplasm, thereby securing the functionality of both proteins and the endomembrane system, but is also (ii) a specific response triggered by certain stress exposures, such as pathogen invasion. In fact, the autophagic response to pathogen invasion has been identified because autophagy-related proteins (ATG) essential to the stochastic process of autophagy, such as Atg5 and LC3, have also been found to decorate membranes harboring intracellular pathogens and to be functionally involved in the cellular response to pathogens [4, 27]. Still, molecular mechanisms of autophagic responses to pathogen exposure are insufficiently understood.

Bacterial pathogens employ a variety of mechanisms to manipulate host cell membranes [28, 29]. Commonly, many bacteria interfere with the phosphoinositide metabolism that is often targeted by bacterial virulence factors [30]. Among the phosphoinositides, PtdIns(3)P is the essential variant for the forming autophagosomal membrane, hence it can be anticipated that PtdIns(3)P might commonly interconnect bacterial infection with the autophagic pathway. In fact, it

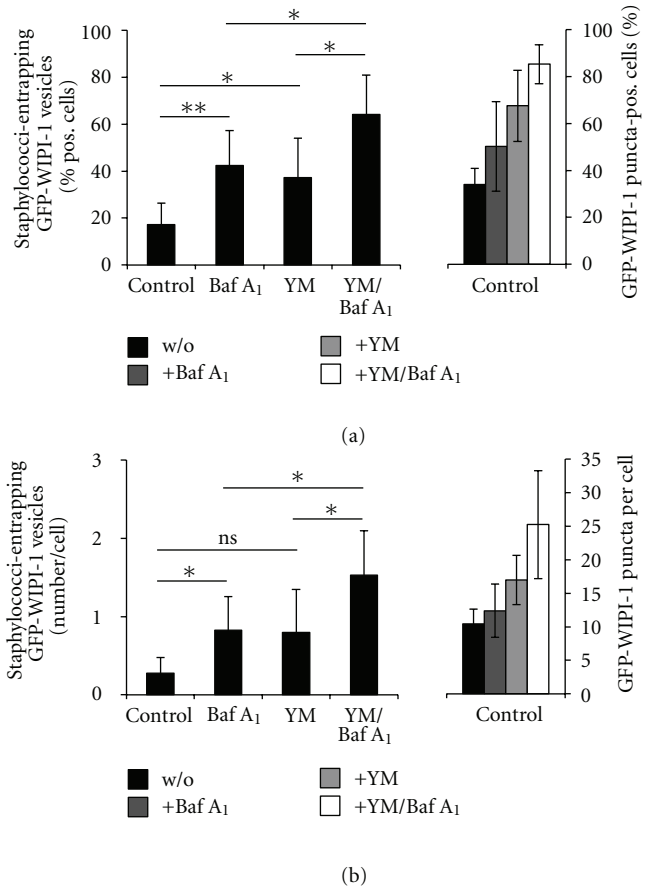

Figure 8: Bafilomycin A_1 and YM201636 treatments increased the number of GFP-WIPI-1 positive autophagosome-like vesicles entrapping Staphylococci. GFP-WIPI-1 U2OS cells were infected with *S. aureus* USA300 in DMEM/FCS in the absence (control) or presence of 200 nM bafilomycin A_1 (Baf A_1), 800 nM YM201636 (YM), or with both (Baf A_1/YM) for 2 h. Images were automatically acquired (not shown). The number of GFP-WIPI-1 puncta-positive cells ((a), right panel) and the number of GFP-WIPI-1 puncta per cell ((b), right panel) was determined. From 100 infected cells for each of the treatment condition, the number of cells displaying GFP-WIPI-1 positive autophagosomal-like vesicles entrapping *S. aureus* USA300 ((a), left panel) and the number of GFP-WIPI-1 autophagosomal-like vesicles entrapping *S. aureus* USA300 per cell ((b), left panel) was determined ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, ns: not significant.

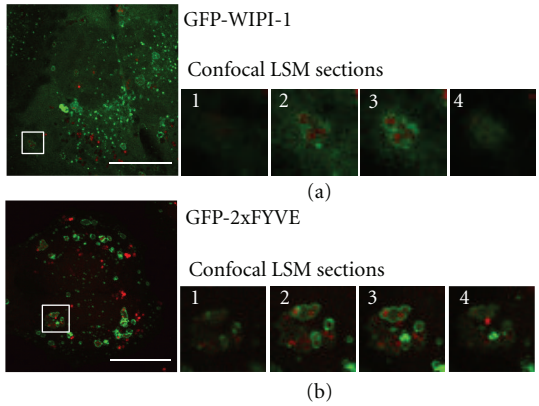

Figure 9: Confocal laser scanning microscopy of *S. aureus* USA300 infected GFP-WIPI-1 or GFP-2xFYVE expressing U2OS cells. GFP-WIPI-1 (a) or GFP-2xFYVE (b) expressing U2OS cells were infected with *S. aureus* USA300 for 2 h in DMEM/FCS. Representative images ($n = 3$) are shown. Magnifications display individual LSM sections (1–4). Scale bars: 20 μm.

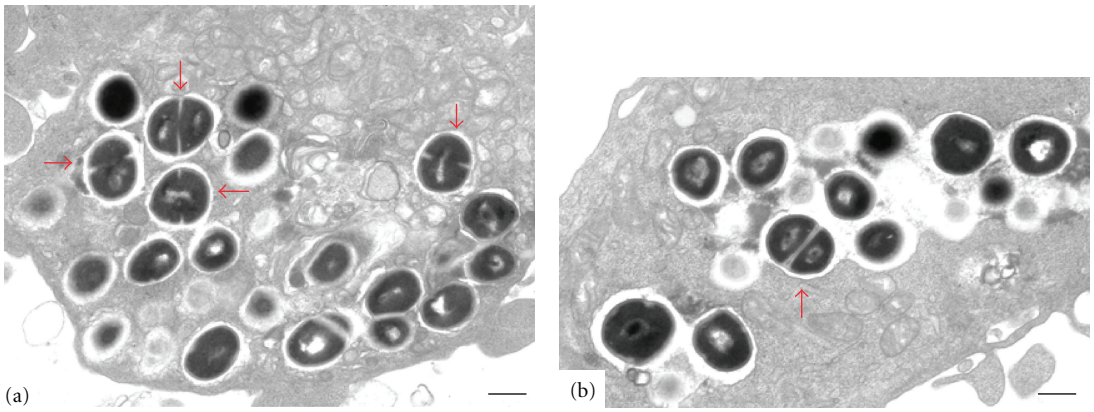

Figure 10: Electron microscopy of *S. aureus* USA300 infected GFP-WIPI-1 expressing U2OS cells. GFP-WIPI-1 U2OS cells were infected with *S. aureus* USA300 in DMEM/FCS followed by conventional electron microscopy. Either single *S. aureus* USA300 cells were found to reside within a vesicle (a), or multiple cells were found in enlarged vesicles (b). Red arrows indicate dividing Staphylococci. Scale bars: 500 nm.

has been shown that PtdIns(3)P is involved in the formation of Salmonella-containing vacuoles serving as a niche in host cells, and that PtdIns(3)P is targeted by *M. tuberculosis* to inhibit phagosome maturation [31]. Here, we addressed this question by investigating the process of *S. aureus* invasion of tumor cells.

A study by Schnaith and coworkers suggested a model that connected the autophagic response with *S. aureus* infection via the bacterial *agr*-virulence factor [14]. In this model, late phagosomes with (i) *agr*-positive *S. aureus* become entrapped in autophagosome-like vesicles, where *S. aureus* replicate and subsequently escape into the cytoplasm to promote host cell death, but (ii) *agr*-deficient *S. aureus* are subjected to lysosomal degradation [14].

We here provide evidence, that exposure of nonprofessional host cells (tumor cells) to Staphylococci stimulates the canonical WIPI-1 response at the onset of autophagy, which is to bind to PtdIns(3)P at the phagophore to foster the recruitment of downstream ATGs, such as Atg5 and LC3 [9, 32]. Interestingly, this response is attributable to the interaction of Staphylococci with the host cell membrane, as we found WIPI-1 to become stimulated upon both noninvasive and invasive Staphylococci. In line, WIPI-1 was also stimulated upon peptidoglycan treatment (data not shown). By further analyzing invasive *S. aureus* strains in this study, we identified new WIPI-1 positive autophagosome-like vesicles that entrapped multiple *S. aureus* particles. And, moreover, *agr*-positive *S. aureus* strains were more efficiently entrapped when compared to *agr*-deficient *S. aureus* cells. Our results demonstrate that WIPI-1, a principal PtdIns(3)P effector at the onset of stochastic, canonical autophagy, is also involved in selective engagement of the autophagic pathway, moreover underscored by the notion that Staphylococci prominently stimulated WIPI-1 in nutrient-rich conditions. And, our results demonstrate that *S. aureus* (i) stimulates autophagy and (ii) in addition, becomes entrapped in WIPI-1 positive autophagosome-like vesicles.

The most compelling explanation would be that WIPI-1 becomes stimulated upon *S. aureus* interaction with the plasma membrane, subsequently WIPI-1 positive phagophore membranes, for example, originated from the endoplasmic reticulum, are utilized to sequester *S. aureus* where bacterial replication occurs. In addition, we also found *S. aureus* particles sequestered in phagosomes, marked by the FYVE domain [33], which are intended for phagocytosis. Hence our results can be viewed as host cell response to *S. aureus*, critically involving PtdIns(3)P membranes that either serve as phagosome membranes, or that are utilized to further sequester *S. aureus*, thereby generating a replication niche. Evidence that bacterial replication occurs is given by our electron microscopy analysis showing dividing *S. aureus* cells within the sequestering vesicle. The importance of PtdIns(3)P-enriched membranes during sequestration of invading *S. aureus* is further emphasized by our finding that more WIPI-1 positive autophagosome-like vesicles entrap *S. aureus* cells when phosphorylation of PtdIns(3)P to PtdIns(3,5)P_2 by PIKfyve was specifically blocked.

PtdIns(3)P-enriched membranes promote vesicle fusion with lysosomes. In line, FYVE domain marked phagosomes that carry *S. aureus* would be subjected to phagocytosis as suggested [14]. If WIPI-1 positive autophagosome-like vesicles entrapping *S. aureus* identified in this study would reflect cytoplasmic sequestration of invaded *S. aureus* with PtdIns(3)P-enriched WIPI-1 positive phagophores, the resulting autophagosome-like vesicles should become subjected to fusion with the lysosomal compartment, because they are enriched in PtdIns(3)P. But it was shown that lysosomal fusion is blocked upon *S. aureus* invasion [14]. To address this question we employed bafilomycin A_1 to inhibit the functionality of the lysosomal compartment. Clearly, lysosomal inhibition significantly increased the number of WIPI-1 positive autophagosome-like vesicles harboring *agr*-positive Staphylococci. This demonstrates that nonprofessional host cells employ autophagy as a defense response

with regards to *S. aureus* infection, in line with previous suggestions [34]. However, under some circumstances [14] bacterial replication and vesicle escape might override this cellular defense program.

Acknowledgments

The authors kindly thank Marianne Opalka for technical assistance. This work was supported by grants from the Federal Ministry for Education and Science (BMBF, BioProfile) and the German Research Society (DFG, SFB 773) to TP-C.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 219625, 18 pages
doi:10.1155/2012/219625

Review Article

Autophagy: More Than a Nonselective Pathway

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Received 29 November 2011; Accepted 7 February 2012

Academic Editor: G. S. Stein

Autophagy is a catabolic pathway conserved among eukaryotes that allows cells to rapidly eliminate large unwanted structures such as aberrant protein aggregates, superfluous or damaged organelles, and invading pathogens. The hallmark of this transport pathway is the sequestration of the cargoes that have to be degraded in the lysosomes by double-membrane vesicles called autophagosomes. The key actors mediating the biogenesis of these carriers are the autophagy-related genes (*ATG*s). For a long time, it was assumed that autophagy is a bulk process. Recent studies, however, have highlighted the capacity of this pathway to exclusively eliminate specific structures and thus better fulfil the catabolic necessities of the cell. We are just starting to unveil the regulation and mechanism of these selective types of autophagy, but what it is already clearly emerging is that structures targeted to destruction are accurately enwrapped by autophagosomes through the action of specific receptors and adaptors. In this paper, we will briefly discuss the impact that the selective types of autophagy have had on our understanding of autophagy.

1. Introduction

Three different pathways can deliver cytoplasmic components into the lumen of the lysosome for degradation. They are commonly referred to as autophagy (cell "self-eating") and include chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy. CMA involves the direct translocation of specific proteins containing the KFERQ pentapeptide sequence across the lysosome membrane [1, 2]. Microautophagy, on the other hand, entails the invagination and pinching off of the lysosomal limiting membrane, which allows the sequestration and elimination of cytoplasmic components. The molecular mechanism underlying this pathway remains largely unknown. The only cellular function that so far has been indisputably assigned to microautophagy is the turnover of peroxisomes under specific conditions in fungi [3]. Recently, it has been reported the existence of a microautophagy-like process at the late endosomes, where proteins are selectively incorporated into the vesicles that bud inward at the limiting membrane of these organelles during the multivesicular bodies biogenesis [4]. In contrast to CMA and microautophagy, macroautophagy (hereafter referred to as autophagy) entails the formation of a new organelle, the autophagosome, which allows the delivery of a large number of different cargo molecules into the lysosome.

Autophagy is a primordial and highly conserved intracellular process that occurs in most eukaryotic cells and participates in stress management. This pathway involves the *de novo* formation of vesicles called autophagosomes, which can engulf entire regions of the cytoplasm, individual organelles, protein aggregates, and invading pathogens (Figure 1). The autophagosomes fuse with endosomal compartments to form amphisomes prior to fusion with the lysosome, where their contents are degraded and the resulting metabolites are recycled back to the cytoplasm (Figure 1). Unique features of the pathway include the double-membrane structure of the autophagosomes, which were originally characterized over 50 years ago from detailed electron microscopy studies [5]. Starting in the 1990s yeast mutational studies began the genetic and molecular characterization of the key components required to initiate and build an autophagosome

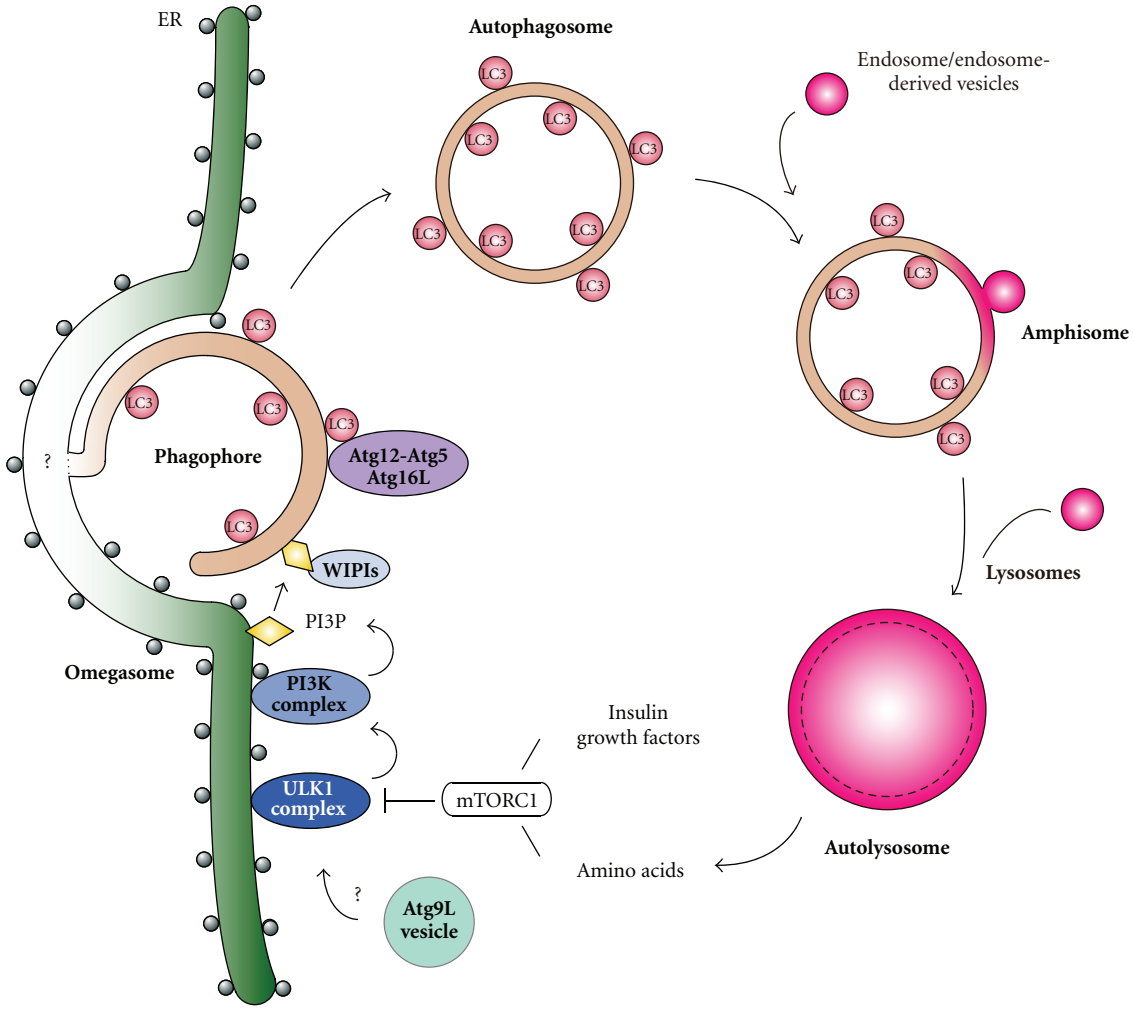

Figure 1: Multiple Atg proteins govern autophagosome formation. In response to inactivation of mTORC1 (but also other cellular and environmental cues), the ULK1 complex is activated and translocates in proximity of the endoplasmic reticulum (ER). Thereafter, the ULK1 complex regulates the class III PI3K complex. Atg9L, a multimembrane spanning protein, is also involved in an early stage of autophagosome formation by probably supplying part of the membranes necessary for the formation and/or expansion. Local formation of PI3P at sites called omegasomes promotes the formation of the phagophore, from which autophagosomes appear to be generated. The PI3P-binding WIPI proteins (yeast Atg18 homolog), as well as the Atg12-Atg5-Atg16L1 complex and the LC3-phosphatidylethanolamine (PE) conjugate play important roles in the elongation and closure of the isolation membrane. Finally, the complete autophagosome fuses with endosomes or endosome-derived vesicles forming the amphisome, which subsequently fuses with lysosomes to form autolysosomes. In the lysosomes, the cytoplasmic materials engulfed by the autophagosomes are degraded by resident hydrolases. The resulting amino acids and other basic cellular constituents are reused by the cell; when in high levels they also reactivate mTORC1 and then suppress autophagy.

[6]. Subsequently, genetic and transgenic studies in plants, worms, fruit flies, mice, and humans have underscored the pathway's conservation and have begun to unveil the intricate vital role that autophagy plays in the physiology of cells and multicellular organisms.

For a long time, autophagy was considered a non-selective pathway induced as a survival mechanism in response to cellular stresses. Over the past several years, however, it has become increasingly evident that autophagy also is a highly selective process involved in clearance of excess or dysfunctional organelles, protein aggregates and intracellular pathogens. In this introductory piece, we will briefly discuss the molecular mechanisms of selective types of autophagy and their emerging importance as a quality control to maintain cellular and organismal health, aspects that will be presented in deep in the reviews of this special issue of the *International Journal of Cell Biology* and highlighted by the research papers.

2. The Mechanism of Autophagy

2.1. The Function of the Atg Proteins. Autophagosomes are formed by expansion and sealing of a small cistern known as the phagophore or isolation membrane (Figure 1). Once complete, they deliver their cargo into the hydrolytic lumen of lysosomes for degradation. A diverse set of components

are involved in the biogenesis of autophagosomes, which primarily includes the proteins encoded by the autophagy-related genes (ATG). Most *ATG* genes have initially been identified and characterized in yeast. Subsequent studies in higher eukaryotes have revealed that these key factors are highly conserved. To date, 36 Atg proteins have been identified and 16 are part of the core Atg machinery essential for all autophagy-related pathways [7]. Upon autophagy induction, these proteins associate following a hierarchical order [8, 9] to first mediate the formation of the phagophore and then to expand it into an autophagosome [10, 11]. While their molecular functions and their precise contribution during the biogenesis of double-membrane vesicles remain largely unknown, they have been classified in 4 functional groups of genes: (1) the Atg1/ULK complex, (2) the phosphatidylinositol 3-kinase (PI3K) complex, (3) the Atg9 trafficking system, and (4) the two parallel ubiquitin-like conjugation systems (Figure 1).

The Atg1/ULK complex consists of Atg1, Atg13, and Atg17 in yeast, and ULK1/2, Atg13, FIP200 and Atg101 in mammals [12–15]. This complex is central in mediating the induction of autophagosome biogenesis and as a result it is the terminal target of various signaling cascades regulating autophagy, such as the TOR, insulin, PKA, and AMPK pathways [16] (Figure 1). Increased activity of the Atg1/ULK kinase is the primary event that determines the acute induction and upregulation of autophagy. It is important to note that ULK1 is part of a protein family and two other members, ULK2 and ULK3, have been shown play a role in autophagy induction as well [14, 17]. The expansion of this gene family may reflect the complex regulation and requirements of the pathway in multicellular long-lived organisms. Stimulation of the ULK kinases is achieved through an intricate network of phosphorylation and dephosphorylation modifications of the various subunits of the Atg1/ULK complex. For example, Atg13 is directly phosphorylated by TOR and the phosphorylation state of Atg13 modulates its binding to Atg1 and Atg17. Inactivation of TOR leads to a rapid dephosphorylation of Atg13, which increases Atg1–Atg13–Atg17 complex formation, stimulates the Atg1 kinase activity and induces autophagy [18, 19]. The mAtg13 is also essential for autophagy, but seems to directly interact with ULK1, ULK2 and FIP200 independently of its phosphorylation state [13, 14]. In addition, there are several phosphorylation events within this complex as well, including phosphorylation of mAtg13 by ULK1, ULK2, and TOR; phosphorylation of FIP200 by ULK1 and ULK2; phosphorylation of ULK1 and ULK2 by TOR [13, 14]. Additional studies are required to fully characterize the functional significance of these posttranslational modifications.

Autophagy is also regulated by the activity of PI3K complexes. Yeast contains a single PI3K, Vps34, which is present in two different tetrameric complexes that share 3 common subunits, Vps34, Vps15, and Atg6 [20]. Complex I is required for the induction of autophagy and through its fourth component, Atg14, associates to the autophagosomal membranes where the lipid kinase activity of Vps34 is essential for generating the phosphatidylinositol-3-phosphate (PI3P) that permits the recruitment of other Atg proteins [9, 21] (Figure 1). Complex II contains Vps38 as the fourth subunit and it is involved in endosomal trafficking and vacuole biogenesis [20]. There are three types of PI3K in mammals: class I, II, and III. The functions of class II PI3K remains largely unknown, but both classes I and III PI3Ks are involved in autophagy. While class I PI3K is principally implicated in the modulation of signalling cascades, class III PI3K complexes regulate organelle biogenesis and, like yeast, contain three common components: hVps34, p150 (Vps15 ortholog), and Beclin 1 (Atg6 ortholog). The counterparts of Atg14 and Vps38 are called Atg14L/Barkor and UVRAG, respectively [22–24]. The Atg14L-containing complex plays a central role in autophagy and functions very similarly as the yeast complex I by directing the class III PI3K complex I to the phagophore to produce PI3P and initiate the recruitment of the Atg machinery (Figure 1). Atg14L is thought to be present on the ER irrespective of autophagy induction [25]. Upon starvation, Atg14L localizes to autophagosomal membranes [8]. Importantly, depletion of Atg14L reduces PI3P production, impairs the formation of autophagosomal precursor structures, and inhibits autophagy [8, 24, 26, 27]. The UVRAG-containing class III PI3K complex also regulates autophagy but it appears to act at the intersection between autophagy and the endosomal transport pathways. UVRAG initially associates with the BAR-domain protein Bif-1, which may regulate mAtg9 trafficking from the trans-Golgi network (TGN) [28, 29]. UVRAG then interacts with the class C Vps/HOPS protein complex, promoting the fusion of autophagosomes with late endosomes and/or lysosomes [30]. Finally, the UVRAG-containing class III protein complex binds to Rubicon, a late endosomal and lysosomal protein that suppresses autophagosome maturation by reducing hVps34 activity [26, 31]. Importantly, both the Atg14L- and UVRAG-containing complexes interact through Beclin 1 with Ambra1, which in turn tethers these protein complexes to the cytoskeleton via an interaction with dynein [32, 33]. Following the induction of autophagy, ULK1 phosphorylates Ambra1 thus releasing the class III PI3K complexes from dynein and their subsequent relocalization triggers autophagosome formation. Therefore, Ambra1 constitutes a direct regulatory link between the Atg1/ULK1 and the PI3K complexes [32].

Together with the Atg1/ULK and the PI3K complexes, Atg9 is one of the first factors localizing to the preautophagosomal structure or phagophore assembly site (PAS), the structure believed to be the precursor of the phagophore [9, 34] (Figure 1). Atg9 is the only conserved transmembrane protein that is essential for autophagy. It is distributed to the PAS and multiple additional cytoplasmic tubulovesicular compartments derived from the Golgi [35–37]. Atg9 cycles between these two locations and consequently it is thought to serve as a membrane carrier providing the lipid building blocks for the expanding phagophore [37]. One of the established functions of Atg9 is that it leads to the formation of the yeast PAS when at least one of the cytoplasmic tubulovesicular compartments translocates near the vacuole [34]. Atg9 is also essential to recruit the PI3K Complex I to the PAS [9]. Retrieval transport of yeast Atg9 from the PAS and/or complete autophagosome is mediated by the

Atg2-Atg18 complex [38] and appears to be regulated by the Atg1/ULK and PI3K complexes [37]. Mammalian Atg9 (mAtg9) has similar characteristics to its yeast counterpart. mAtg9 localizes to the TGN and late endosomes and redistributes to autophagosomal structures upon the induction of autophagy (Figure 1) [39], further promoting pathway activity [29, 40–42]. As in yeast, cycling of mAtg9 between locations also requires the Atg1/ULK complex and kinase activity hVps34 [39, 43].

The core Atg machinery also entails two ubiquitin-like proteins, Atg12 and Atg8/microtubule-associated protein 1 (MAP1)-light chain 3 (LC3), and their respective, partially overlapping, conjugation systems [44–46] (Figure 1). Atg12 is conjugated to Atg5 through the activity of the Atg7 (E1-like) and the Atg10 (E2-like) enzymes. The Atg12–Atg5 conjugate then interacts with Atg16, which oligomerizes to form a large multimeric complex. Atg8/LC3 is cleaved at its C terminus by the Atg4 protease to generate the cytosolic LC3-I with a C-terminal glycine residue, which is then conjugated to phosphatidylethanolamine (PE) in a reaction that requires Atg7 and the E2-like enzyme Atg3. This lipidated form of LC3 (LC3-II) is attached to both faces of the phagophore membrane. Once the autophagosome is completed, Atg4 removes LC3-II from the outer autophagosome surface. These two ubiquitination-like systems appear to be closely interconnected. On one hand, the multimeric Atg12-Atg5-Atg16 complex localizes to the phagophore and acts as an E3-like enzyme, determining the site of Atg8/LC3 lipidation [47, 48]. On the other hand, the Atg8/LC3 conjugation machinery seems to be essential for the optimal functioning of the Atg12 conjugation system. In Atg3-deficient mice, Atg12-Atg5 conjugation is markedly reduced, and normal dissociation of the Atg12-Atg5-Atg16 complex from the phagophore is delayed [49]. Some evidences suggest that these two conjugation systems also function together during the expansion and closure of the phagophore. For example, overexpression of an inactive mutant of Atg4 inhibits the lipidation of LC3 and leads to the accumulation of a number of nearly complete autophagosomes [47]. While controversial [50], it has been postulated that Atg8/LC3 also possesses fusogenic properties, thus mediating the assembly of the autophagic membrane [51, 52].

It has to be noted that mammals possess at least 7 genes coding for LC3/Atg8 proteins that can be grouped into three subfamilies: (1) the LC3 subfamily containing LC3A, LC3B, LC3B2 and LC3C; (2) the gammaaminobutyrate receptor-associated protein (GABARAP) subfamily comprising GABARAP and GABARAPL1 (also called GEC-1); (3) the Golgi-associated ATPase enhancer of 16 kDa (GATE-16) protein (also called GABARAP-L2/GEF2) [53]. Although *in vivo* studies show that they are all conjugated to PE, they appear to have evolved complex nonredundant functions [54].

2.2. The Autophagosomal Membranes. The origin of the membranes composing autophagosomes is a long-standing mystery in the field of autophagy. A major difficulty in addressing this question has been that phagophores as well as autophagosomes do not contain marker proteins of other subcellular compartments [55, 56]. A series of new studies has implicated several cellular organelles as the possible source for the autophagosomal lipid bilayers. The plasma membrane and elements of the trafficking machinery to the cell surface have been linked to the formation of an early autophagosomal intermediate, perhaps the phagophore [57–61]. It is possible that early endosomal- and/or Golgi-derived membranes are also key factors in the initial steps of autophagy [34, 36, 39]. The Golgi, moreover, appears also important for autophagy by supplying at least in part the extra lipids required for the phagophore expansion [29, 62–65]. The endoplasmic reticulum (ER) is also central in this latter event. While the relevance of the ER in autophagosome biogenesis was already pointed out a long time ago [5, 55, 66, 67], recently two electron tomography studies have demonstrated the existence of a physical connection between the ER and the forming autophagosomes [68, 69]. These analyses have revealed that the ER is connected to the outer as well as the inner membrane of the phagophore through points of contact, supporting the notion that lipids could be supplied via direct transfer at the sites of membrane contact. In line with this view, it has been found that Atg14L is associated to the ER and PI3P is generated on specific subdomains of this organelle from where autophagosomes emerge under autophagy-inducing conditions [25, 70] (Figure 1). It has also been proposed that the outer membrane of the mitochondria is the main source of the autophagosomal lipid bilayers, but while the experimental evidences appear to show that mitochondria are essential for the phagophore expansion, it remains unclear whether these organelles play a key role in the phagophore biogenesis [71]. The discrepancy between the conclusions of the various studies has not allowed yet drawing a model about the membrane dynamics during autophagosome biogenesis. The different results could be due to the different experimental conditions and model systems used by the various laboratories. Alternatively, the lipids forming the autophagosomes could have different sources depending on the cell and the conditions inducing autophagy [72, 73]. A third possibility is that the source of phagophore membrane could depend on the nature of the double-membrane vesicle cargo. Additional investigations are required to shed light on these issues.

2.3. Pharmacological Manipulation of Autophagy. Despite the potential of curing, quite a substantial range of specific pathological conditions by inducting autophagy, there are currently no small molecules that allow to exclusively stimulate this pathway [74]. Nevertheless, there is a variety of chemicals that by acting on signaling cascades that also regulate autophagy permit to trigger this degradative process. These agents fall into two distinct categories based on the mechanism of action; whether they work through an mTOR-dependent (Rapamycin or Torin) or mTOR-independent pathway (e.g., lithium or resveratrol) [74]. In addition to these compounds, there are biological molecules such as interferon γ (IFNγ) and vitamin D that can be used to stimulate autophagy especially in experimental setups [75, 76].

Inhibition of autophagy can also be beneficial in specific diseases but as for the inducers there are no compounds that exclusively block this pathway without affecting other cellular processes. The small molecules inhibiting autophagy include wortmannin and 3-methyladenine, which hamper the activity of the PI3K; Bafilomycin A and chloroquine, which impair the degradative activity of lysosomes [77]. They are currently solely used in the basic research on autophagy.

3. Selective Types of Autophagy

3.1. The Molecular Machinery of Selective Autophagy. It is becoming increasingly evident that autophagy is a highly selective quality control mechanism whose basal levels are important to maintain cellular homeostasis (see below). A number of organelles have been found to be selectively turned over by autophagy and cargo-specific names have been given to distinguish the various selective pathways, including the ER (reticulophagy or ERphagy), peroxisomes (pexophagy), mitochondria (mitophagy), lipid droplets (lipophagy), secretory granules (zymophagy), and even parts of the nucleus (nucleophagy). Moreover, pathogens (xenophagy), ribosomes (ribophagy), and aggregate-prone proteins (aggrephagy) are specifically targeted for degradation by autophagy [78].

Selective types of autophagy perform a cellular quality control function and therefore they must be able to distinguish their substrates, such as protein aggregates or dysfunctional mitochondria, from their functional counterparts. The molecular mechanisms underlying cargo selection and regulation of selective types of autophagy are still largely unknown. This has been an area of intense research during the last years and our understanding of the various selective types of autophagy is starting to unravel. A recent genome-wide small interfering RNA screen aimed at identifying mammalian genes required for selective autophagy found 141 candidate genes to be required for viral autophagy and 96 of those genes were also required for Parkin-mediated mitophagy [79].

In general, these pathways appear to rely upon specific cargo-recognizing autophagy receptors, which connect the cargo to the autophagic membranes. The autophagy receptors might also interact with specificity adaptors, which function as scaffolding proteins that bring the cargo-receptor complex in contact with the core Atg machinery to allow the specific sequestration of the substrate. The selective types of autophagy appear to rely on the same molecular core machinery as non-selective (starvation-induced) bulk autophagy. In contrast, the autophagy receptors and specificity adapters do not seem to be required for nonselective autophagy.

Autophagy receptors are defined as proteins being able to interact directly with both the autophagosome cargo and the Atg8/LC3 family members through a specific (WxxL) sequence [80], commonly referred to as the LC3-interacting region (LIR) motif [81] or the LC3 recognition sequences (LRS) [82]. Based on comparison of LIR domains from more than 20 autophagy receptors it was found that the LIR consensus motif is an eight amino acids long sequence that can be written D/E-D/E-D/E-W/F/Y-X-X-L/I/V. Although not an absolute requirement, usually there is at least one acidic residue upstream of the W-site. The terminal L-site is occupied by a hydrophobic residue, either L, I, or V [83]. The LIR motifs of several autophagy receptors have been found to interact both with LC3 and GABARAP family members *in vitro*, but whether this reflects a physiological interaction remains to be clarified in most cases. It should be pointed out that not all LIR-containing proteins are autophagy cargo receptors. Some LIR-containing proteins, like Atg3 and Atg4B, are recruited to autophagic membranes to perform their function in autophagosome formation [84, 85], whereas others like FYVE and coiled-coil domain-containing protein 1 (FYCO1) interact with LC3 to facilitate autophagosome transport and maturation [86]. Others might use an LIR motif to become degraded, like Dishevelled, an adaptor protein in the Wnt signalling pathway [87]. The adaptor proteins are less well-described, but seem to interact with autophagy receptors and work as scaffold proteins recruiting and assembling the Atg machinery required to generate autophagosomes around the cargo targeted to degradation. Examples of autophagy adaptors are Atg11 and ALFY [88, 89].

The list of specific autophagy receptors is rapidly growing and the role of several of them in different types of selective autophagy will be described in detail in the reviews of this special issue. Here we will briefly discuss the best studied form of selective autophagy, the yeast cytosol to vacuole targeting (Cvt) pathway, as well as the best studied mammalian autophagy receptor, p62/sequestosome 1 (SQSTM1) (Figure 2).

The Cvt pathway is a biosynthetic process mediating the transport of the three vacuolar hydrolases, aminopeptidase 1 (Ape1), aminopeptidase 4 (Ape4) and α-mannosidase (Ams1), and the Ty1 transposome into the vacuole [90, 91]. Ape1 is synthesized as a cytosolic precursor (prApe1), which multimerizes into the higher order Ape1 oligomer, to which Ape4, Ams1, and Ty1 associate to form the so-called Cvt complex, prior to being sequestered into a small autophagosome-like Cvt vesicle. Sequestration of the Cvt complex into Cvt vesicles is a multistep process, which requires the autophagy receptor Atg19, which facilitates binding to Atg8 at the PAS, as well as the adaptor protein Atg11 (Figure 2(a)) [92]. Atg11 acts as a scaffold protein by directing the Cvt complex and Atg9 reservoirs translocation to the PAS in an actin-dependent way and then recruiting the Atg1/ULK complex [40, 93]. The PI3P-binding proteins Atg20, Atg21, and Atg24 are also required for the Cvt pathway [94, 95], but their precise function remains to be elucidated. Interestingly, Atg11 overexpression was found to recruit more Atg8 and Atg9 to the PAS resulting in more Cvt vesicles. This observation indicates that Atg11 levels could regulate the rate of selective autophagy, and maybe also the size of the cargo-containing autophagosomes in yeast [90, 96]. Indeed, a series of studies has revealed that Atg11 is also involved in other types of selective autophagy such as mitophagy and pexophagy. However, the autophagy receptors involved in the different Atg11-dependent types

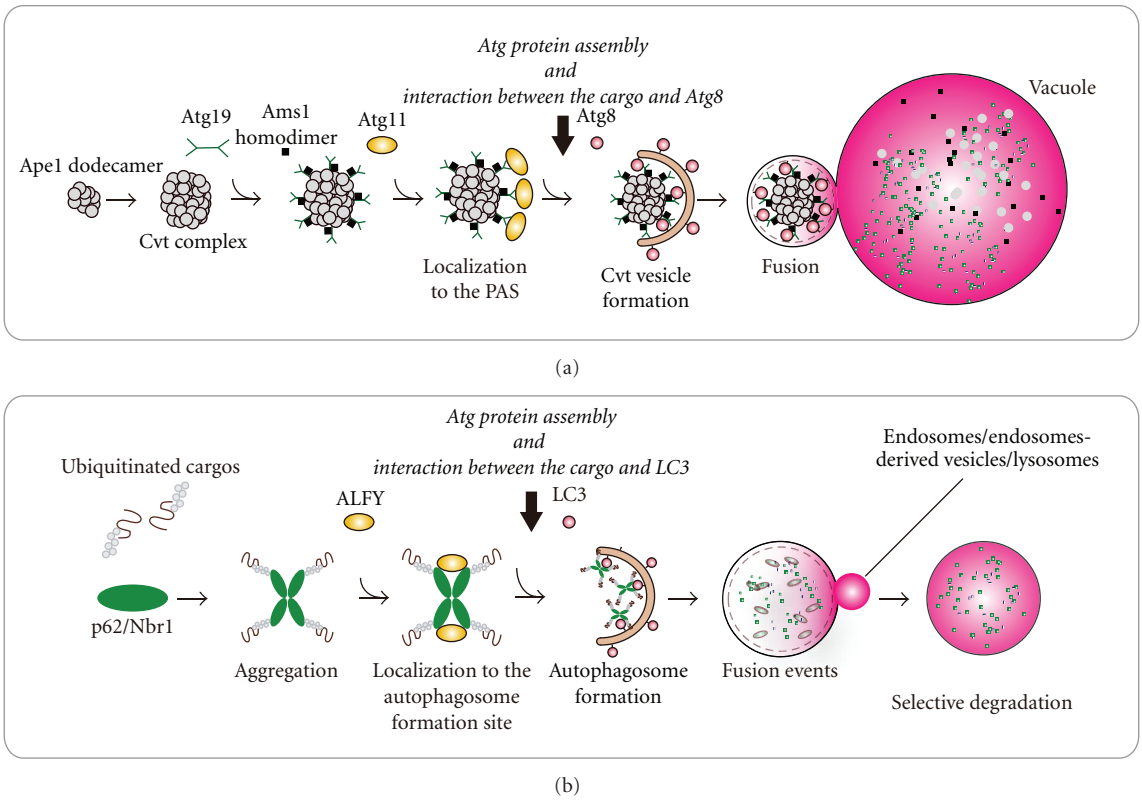

Figure 2: Representative selective autophagy. (a) The cytoplasm-to-vacuole targeting (Cvt) pathway. Ape1 is synthesized as a cytoplasmic precursor protein with a propeptide and rapidly oligomerizes into dodecamers that subsequently associate with each other to form a higher order complex. The autophagy receptor Atg19 directly binds to the complex and mediates the recruitment of another Cvt pathway cargo, Ams1, leading to the formation of the so-called Cvt complex. Atg19 also interacts with the autophagy adaptor Atg11 and this protein allows the transport of the Cvt complex to the site where the double-membrane vesicle will be generated. At this location, Atg11 tethers the Atg proteins essential for the Cvt vesicle formation and the direct binding of Atg19 to Atg8 permits the exclusive sequestration of the Cvt complex into the vesicle. (b) A model for p62 and NBR1 as autophagy receptors for ubiquitinated cargos. p62 and NBR1 bind with ubiquitinated cargos via their ubiquitin-associated (UBA) domain and this interaction triggers the aggregate formation through the oligomerization of p62 via its Phox and Bem1p (PB1) domain. Furthermore, p62 interacts with both autophagy-linked FYVE protein (ALFY), which serves to recruit Atg5 and to bind PI3P, and directly with LC3. This latter event appears to organize and activate the Atg machinery in close proximity of the ubiquitinated cargos, which allows to selectively sequester them in the autophagosomes in analogous to the Cvt pathway.

of selective autophagy are different as Atg32 is required for mitophagy [97, 98], whereas Atg30 is essential for pexophagy [99]. Like Atg19, these two proteins have an Atg8-binding LIR motif and directly interact with Atg11. Mammalian cells appear to not possess an Atg11 homologue, and further studies are necessary to delineate the molecular machinery involved in sequestration and targeting of different cargoes for degradation by autophagy in higher eukaryotes.

The mechanism of the Cvt pathway is reminiscent of the selective form of mammalian autophagy called aggrephagy, which involves degradation of misfolded and unwanted proteins by packing them into ubiquitinated aggregates. In both cases aggregation of the substrate (prApe1 or misfolded proteins) is required prior to sequestration into Cvt vesicles or autophagosomes, respectively [100–102]. Similar to Cvt vesicles, aggregate-containing autophagosomes appear to be largely devoid of cytosolic components suggesting that the vesicle membrane expands tightly around its cargo [88]. Aggrephagy also depends on proteins with exclusive functions in substrate selection and targeting [81, 88, 100, 103]. The autophagy receptors p62 and neighbour of BRCA1 gene (NBR1) bind both ubiquitinated protein aggregates through an ubiquitin-associated (UBA) domain and to LC3 via their LIR motifs and, thereby, promote the specific autophagic degradation of ubiquitinated proteins (Figure 2(b)) [81, 82, 100, 103, 104]. NBR1 and p62 also contain an N-terminal Phox and Bem1p (PB1) domain through which they can oligomerize, or interact with other PB1-containing binding partners [83]. In addition to being a cargo receptor for protein aggregates, p62 has been implicated in autophagic degradation of other ubiquitinated substrates such as intracellular bacteria [105], viral capsid proteins [106], the midbody remnant formed after cytokinesis [107], peroxisomes [108, 109], damaged mitochondria [110, 111], and bacteriocidal precursor proteins [112]. The PB1 domain was recently found to be required for p62 to localize to the autophagosome formation site adjacent to the ER [113], suggesting that it could target ubiquitinated cargo to the site of autophagosome formation or alternatively promote the assembly of the Atg machinery at this location.

The large scaffolding protein autophagy-linked FYVE (ALFY) appears to have a similar function as the specificity adaptor Atg11. ALFY is recruited to aggregate-prone proteins through its interaction with p62 [101] and through a direct interaction with Atg5 and PI3P it serves to recruit the core Atg machinery and allow formation of autophagic membranes around the protein aggregate [88] (Figure 2(b)). Interestingly, ALFY is recruited from the nucleus to cytoplasmic ubiquitin-positive structures upon cell stress suggesting that it might regulate the level of aggrephagy [114]. In line with this, it was found that overexpression of ALFY in mouse and fly models of Huntington's disease reduced the number of protein inclusions [88]. It will be interesting to determine whether ALFY, as p62, is involved in other selective types of autophagy such as the one eliminating midbody ring structures or mitochondria.

3.2. Regulation of Selective Autophagy. It is well known that posttranslational modifications like phosphorylation and ubiquitination are involved in the regulation of the activity of proteins involved in autophagy and degradation of autophagic cargo proteins, respectively. However, little is known about how these modifications may regulate selective autophagy. The fact that the core Atg machinery is required for both nonselective and selective types of autophagy gives raise to the question of whether these two types of autophagy may compete for the same molecular machinery. Such a competition could be detrimental for the cells undergoing starvation and to avoid this, there might be a tight regulation of the expression level and/or activity of the proteins specifically involved the selective autophagy. It has recently been proposed that phosphorylation of autophagy receptors might be a general mechanism for the regulation of selective autophagy. Dikic and coworkers noted that several autophagy receptors contain conserved serine residues adjacent to their LIR motifs and indeed, the TANK binding kinase 1 (TBK1) was found to phosphorylate a serine residue close to the LIR motif of the autophagy receptor optineurin. This modification enhances the LC3 binding affinity of optineurin and promotes selective autophagy of ubiquitinated cytosolic *Salmonella enterica* [115]. In yeast, phosphorylation of Atg32, the autophagy receptor for mitophagy, by mitogen-activated protein kinases was found to be required for mitophagy [116, 117].

The Atg8/LC3 proteins themselves have also been found to become phosphorylated and recent works have identified specific phosphorylation sites for protein kinase A (PKA) [118] and protein kinase C (PKC) [119] in the N-terminal region of LC3. Interestingly, the N-terminal of LC3 is involved in the binding of LC3 to LIR-containing proteins [120]. It is therefore tempting to speculate that phosphorylation of the PKA and PKC sites might facilitate or prevent the interaction of LC3 with LIR-containing proteins such as p62. It has been found that phosphorylation of the PKA site, which is conserved in all mammalian LC3 isoforms, but not in GABARAP, inhibits recruitment of LC3 into autophagosomes [118].

The role of ubiquitin in autophagy has so far been ascribed as a signal for cargo degradation. Ubiquitination of aggregate prone proteins, as well as bacteria and mitochondria, has been found to serve as a signal for recognition by autophagy receptors like p62 and NBR1, which are themselves also degraded together with the cargo that they associate with [83]. The *in vivo* specificity of p62 and NBR1 toward ubiquitin signals remains to be established under the different physiological conditions. Interestingly, it was recently found that casein kinase 2- (CK2-) mediated phosphorylation of the p62 UBA domain increases the binding affinity of this motif for polyubiquitin chains leading to more efficient targeting of polyubiquitinated proteins to autophagy [121]. CK2 overexpression or phosphatase inhibition reduced the formation of aggregates containing the polyglutamine-expanded huntingtin exon1 fragments in a p62-dependent manner. The E3 ligases involved in ubiquitination of different autophagic cargo largely remains to be identified. However, it is known that the E3 ligases Parkin and RNF185 both regulate mitophagy [122, 123]. SMURF1 (SMAD-specific E3 ubiquitin protein ligase 1) was recently also implicated in mitophagy, as well as in autophagic targeting of viral particles [79]. Interestingly, the role of SMURF1 in selective autophagy seems to be independent of its E3 ligase activity, but it rather depends on its membrane-targeting C2 domain, although the exact mechanism involved remains to be elucidated. It is also not clear whether ubiquitination could serve as a signal to regulate the activity or binding selectivity of proteins directly involved in autophagy, and whether this in some way could regulate selective autophagy. The role of ubiquitin-like proteins as SUMO and Nedd in autophagy is also unexplored.

Acetylation is another posttranslational modification that only recently has been implicated in selective autophagy. The histone de-acetylase 6 (HDAC6), initially found to mediate transport of misfolded proteins to the aggresome [124], was lately implicated in maturation of ubiquitin-positive autophagosomes [125]. The fact that HDAC6 overproduction in fly eyes expressing expanded polyQ proteins is neuroprotective further indicates that HDAC6 activity stimulates aggrephagy [126]. Furthermore, the acetylation of an aggrephagy cargo protein, muntant huntingtin, the protein causing Huntington's disease, is important for its degradation by autophagy [127]. HDAC6 has been also implicated in Parkin-mediated clearance of damaged mitochondria [128]. The acetyl transferase(s) involved in these forms of selective autophagy is currently unknown, but understanding the role of acetylation in relation to various aspects of autophagy is an emerging field and it will very likely provide more mechanistic insights into these pathways.

4. Pathophysiological Relevance of Selective Types of Autophagy

Basal autophagy acts as the quality control pathway for cytoplasmic components and it is crucial to maintain the homeostasis of various postmitotic cells [129]. While this quality control could be partially achieved by nonselective autophagy, growing lines of evidence have demonstrated

that specific proteins, organelles, and invading bacteria are specifically degraded by autophagy (Figure 3).

4.1. Tissue Homeostasis. Mice deficient in autophagy die either *in utero* (e.g., *Beclin 1* and *Fip200* knockout mice) [130–132] or within 24 hours after birth due, at least in part, to a deficiency in the mobilization of amino acids from various tissues (e.g., *Atg3, Atg5*, *Atg7*, *Atg9,* and *Atg16L* knockout mice) [49, 133–136]. As a result, to investigate the physiological roles of autophagy, conditional knockout mice for *Atg5*, *Atg7,* or *FIP200* and various tissue-specific *Atg* knockout mice have been established and analyzed [133, 137, 138]. For example, the liver-specific *Atg7*-deficient mouse displayed severe hepatomegaly accompanied by hepatocyte hypertrophy, resulting in severe liver injuries [133]. Mice lacking *Atg5*, *Atg7,* or *FIP200* in the central nervous system exhibited behavioral deficits, such as abnormal limb-clasping reflexes and reduction of coordinated movement as well as massive neuronal loss in the cerebral and cerebellar cortices [137–139]. Loss of *Atg5* in cardiac muscle caused cardiac hypertrophy, left ventricular dilatation, and systolic dysfunction [140]. Skeletal muscle-specific *Atg5* or *Atg7* knockout mice showed age-dependent muscle atrophy [141, 142]. Pancreatic β cell-specific *Atg7* knockout animals exhibited degeneration of islets and impaired glucose tolerance with reduced insulin secretion [143, 144]. Podocyte-specific deletion of *Atg5* caused glomerulosclerosis in aging mice and these animals displayed increased susceptibility to proteinuric diseases caused by puromycin aminonucleoside and adriamycin [145]. Proximal tubule-specific *Atg5* knockout mice were susceptible to ischemia-reperfusion injury [146]. Finally, deletion of *Atg7* in bronchial epithelial cells resulted in hyperresponsiveness to cholinergic stimuli [147]. All together, these results undoubtedly indicate that basal autophagy prevents numerous life-threatening diseases.

How does impairment of autophagy lead to diseases? Ultrastructural analyses of the mutant mice revealed a marked accumulation of swollen and deformed mitochondria in the mutant hepatocytes [133], pancreatic β cells [143, 144], cardiac and skeletal myocytes [140, 141] and neurons [138], but also the appearance of concentric membranous structures consisting of ER or sarcoplasmic reticulum in hepatocytes [133], neuronal axons [137, 139] and skeletal myocytes [141], as well as an increased number of peroxisomes and lipid droplets in hepatocytes [133, 148]. In addition to the accumulation of aberrant organelles, histological analyses of tissues with defective autophagy showed the amassment of polyubiquitylated proteins in almost all tissues (although the level varied from one region to another) forming inclusion bodies whose size and number increased with aging [149]. Consequently, basal autophagy also acts as the quality control machinery for cytoplasmic organelles (Figure 3(a)). Although this could be partially achieved by bulk autophagy, these observations point to the existence of selective types of autophagy, a notion that is now supported by experimental data.

4.2. Implications of Selective Degradation of p62 by Autophagy. p62/SQSTM1 is the best-characterized disease-related autophagy receptor and a ubiquitously expressed cellular protein conserved among metazoan but not in plants and fungi [83]. Besides a role of p62 as the receptor, this protein itself is specific substrate for autophagy. Suppression of autophagy is usually accompanied by an accumulation of p62 mostly in large aggregates also positive for ubiquitin (Figure 3(a)) [104, 150]. Ubiquitin and p62-positive inclusion bodies have been detected in numerous neurodegenerative diseases (i.e., Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis), liver disorders (i.e., alcoholic hepatitis and steatohepatitis), and cancers (i.e., malignant glioma and hepatocellular carcinoma) [151]. Very interestingly, the p62-positive aggregates observed in hepatocytes and neurons of liver- and brain-specific *Atg7* deficient mice, respectively, as well as in human hepatocellular carcinoma cells, are completely dispersed by the additional loss of p62 strongly implicating involvement of p62 in the formation of disease-related inclusion bodies [104, 152].

Through its self-oligomerization, p62 is involved in several signal transduction pathways. For example, this protein functions as a signaling hub that may determine whether cells survive by activating the TRAF6–NF-κB pathway, or die by facilitating the aggregation of caspase 8 and the downstream effector caspases [153, 154]. On the other hand, p62 interacts with the Nrf2-binding site on Keap1, a component of the Cullin 3-type ubiquitin ligase for Nrf2, resulting in stabilization of Nrf2 and transcriptional activation of Nrf2 target genes including a battery of antioxidant proteins [155–159]. It is thus plausible that excess accumulation or mutation of p62 leads to hyperactivation of these signaling pathways, resulting in a disease onset (Figure 3(b)).

Paget's disease of bone is a chronic and metabolic bone disorder that is characterized by an increased bone turnover within discrete lesions throughout the skeleton. Mutations in the *p62* gene, in particular in its UBA domain, can cause this illness [160]. A proposed model explaining how p62 mutations lead to the Paget's disease of bone is the following: mutations of the UBA domain cause an impairment in the interaction between p62 and ubiquitinated TRAF6 and/or CYLD, an enzyme deubiquitinating TRAF6, which in turn enhances the activation of the NF-κB signaling pathway and the resulting increased osteoclastogenesis (Figure 3(b)) [160]. If proven, this molecular scenario could open the possibility of using autophagy enhancers as a therapy to cure Paget's disease of bone.

It is established that autophagy has a tumor-suppressor role and several autophagy gene products including Beclin1 and UVRAG are known to function as tumor suppressor proteins [161]. The tumor-suppressor role of autophagy appears to be important particularly in the liver. Spontaneous tumorigenesis is observed in the livers of mice with either a systemic mosaic deletion of *Atg5* or a hepatocyte-specific *Atg7* deletion [152, 162]. Importantly, no tumors are formed in other organs in *Atg5* mosaically deleted mice. Enlarged mitochondria, whose functions are at least partially impaired, accumulate in *Atg5*- or *Atg7*-deficient hepatocytes [152, 162]. This observation is in line with the previous

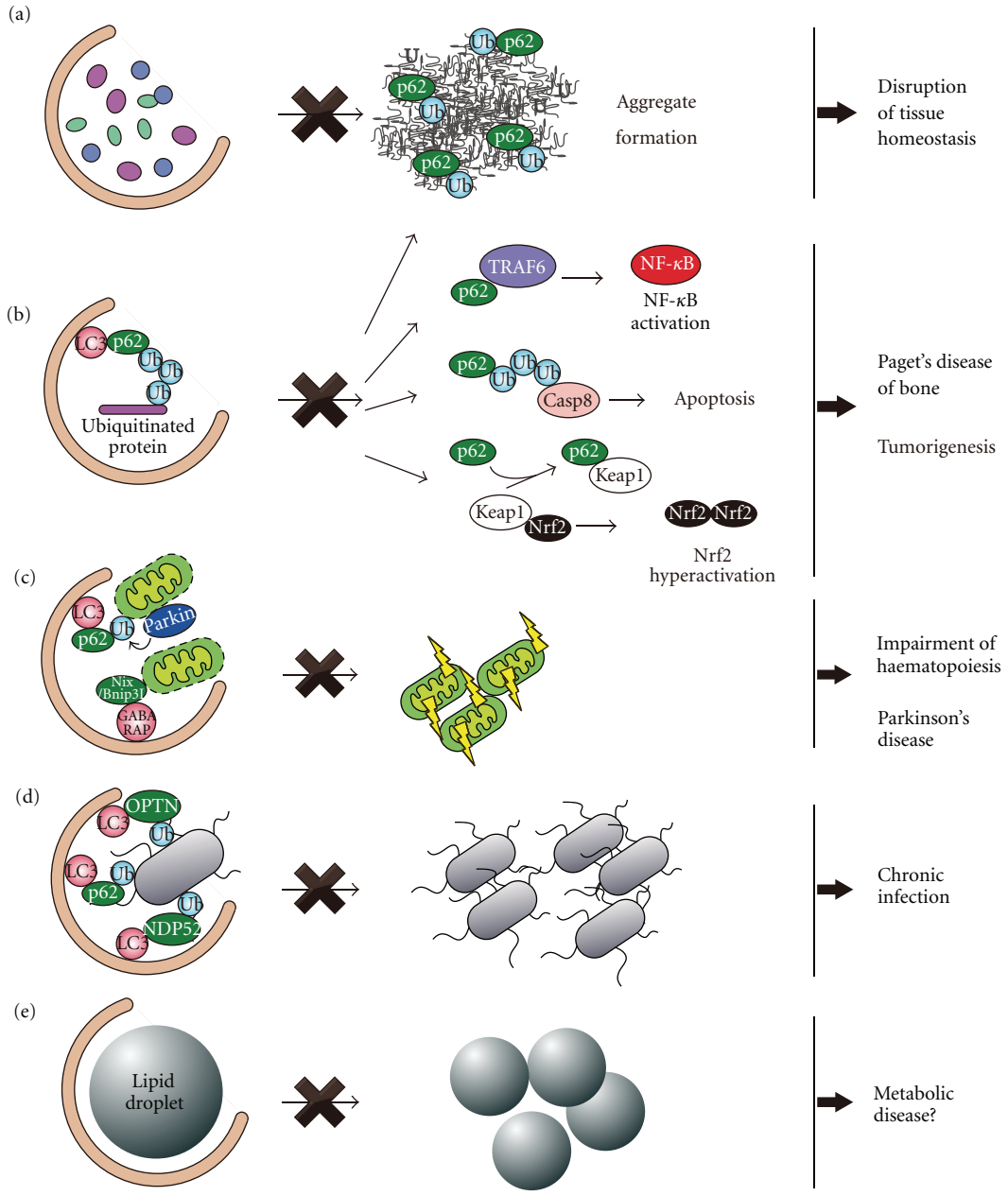

Figure 3: Pathophysiological relevance of selective autophagy. (a, b) Selective types of autophagy operates constitutively at low levels even under nutrient-rich conditions and mediates turnover of selected cytoplasmic materials through the action of autophagy receptors such as p62 and NBR1. These proteins mediate the elimination of ubiquitinated structures, including protein aggregates (a) and defects in these pathways lead to the disruption of tissue homeostasis, resulting in life-threatening diseases. Defective autophagy is usually accompanied by extensive accumulation of p62-containing aggregates, which enhances its function as a scaffold protein in several signaling cascades such as NF-κB signaling, apoptosis, and Nrf2 activation (b). Such abnormalities might be involved in tumorigenesis and Paget's disease of bone. (c) During erythroid differentiation, Nix/Bnip3L relocalization to mitochondria leads to their depolarization, which triggers mitophagy. Loss of *Nix/Bnip3L* causes an arrest in the erythroid maturation arrest, leading to severe anaemia. In response to loss of the mitochondrial membrane potential, Parkin translocates onto the damaged mitochondria in a PINK1-dependent manner, and ubiquitinated proteins present on the outer mitochondrial membrane, which induces mitophagy. Parkinson's disease-related mutations in the *Parkin* and *PINK1* genes provoke a defect in mitophagy, suggesting this selective type of autophagy has a role in preventing the pathogenesis of the Parkinson's disease. (d) Specific bacteria invading the cytosol get ubiquitinated and are recognized by autophagy receptors such as p62, NDP52, and optineurin (OPTN). This allows the specific sequestration of the microbes into autophagosomes and their delivery into the lysosomes for degradation. (e) The lipid droplets are probably degraded by autophagy selectively. This selective type of autophagy, lipophagy, supplies free-fatty acids utilized to generate energy through the β-oxidation. Impairments in lipophagy are known to cause accumulation of lipid droplets in hepatocytes and reduced production of AgRP in neurons.

data obtained in iBMK cell lines showing that both the oxidative stress and genomic damage responses are activated by loss of autophagy [163, 164]. Again, it is clear that accumulation of p62, at least partially, contributes to tumor growth because the size of the *Atg7*$^{-/-}$ liver tumors is reduced by the additional deletion of *p62* [162], which may cause a dysregulation of NF-κB signaling [165] and/or a persistent activation of Nrf2 [166].

4.3. Selective Degradation of Ubiquitinated Proteins. Almost all tissues with defective autophagy are usually displaying an accumulation of polyubiquitinated proteins [149]. Loss of autophagy is considered to lead to a delay in the global turnover of cytoplasmic components [137] and/or to an impaired degradation of substrates destined for the proteasome [167]. Both observations could partially explain the accumulation of misfolded and/or unfolded proteins that is followed by the formation of inclusion bodies.

As discussed above, p62 and NBR1 act as autophagy receptors for ubiquitinated cargos such as protein aggregates, mitochondria, midbody rings, bacteria, ribosomal proteins and virus capsids [83, 168] (Figure 3). Although these studies suggest the role of p62 as an ubiquitin receptor, it remains to be established whether soluble ubiquitinated proteins are also degraded one-by-one by p62 and possibly NBR1. A mass spectrometric analysis has clearly demonstrated the accumulation of all detectable topologies of ubiquitin chain in *Atg* deficient livers and brains, indicating that specific polyubiquitin chain linkage is not the decisive signal for autophagic degradation [169]. Because the increase in ubiquitin conjugates in the *Atg7* deficient liver and brain is completely suppressed by additional knockout of either *p62* or *Nrf2* [169], accumulation of ubiquitinated proteins in tissues defective in autophagy might be attributed to p62-mediated activation of Nrf2, resulting in global transcriptional changes to ubiquitin-associated genes. Further studies are needed to precisely elucidate the degradation mechanism of soluble ubiquitinated proteins by autophagy.

4.4. Mitophagy. Concomitant with the energy production through oxidative phosphorylation, mitochondria also generate reactive oxygen species (ROS), which cause damage through the oxidation of proteins, lipids and DNA often inducing cell death. Therefore, the quality control of mitochondria is essential to maintain cellular homeostasis and this process appears to be achieved via autophagy.

It has been postulated that mitophagy contributes to differentiation and development by participating to the intracellular remodelling that occurs for example during haematopoiesis and adipogenesis. In mammalian red blood cells, the expulsion of the nucleus followed by the removal of other organelles, such as mitochondria, are necessary differentiation steps. Nix/Bnip3L, an autophagy receptor whose structure resembles that of Atg32, is also an outer mitochondrial membrane protein that interacts with GABARAP [170, 171] and plays an important role in mitophagy during erythroid differentiation [172, 173] (Figure 3(c)). Although autophagosome formation probably still occurs in *Nix/Bnip3L* deficient reticulocytes, mitochondrial elimination is severely impaired. Consequently, mutant reticulocytes are exposed to increased levels of ROS and die, and *Nix/Bnip3L* knockout mice suffer severe anemia. Depolarization of the mitochondrial membrane potential of mutant reticulocytes by treatment with an uncoupling agent results in restoration of mitophagy [172], emphasizing the importance of Nix/Binp3L for the mitochondrial depolarization and implying that mitophagy targets uncoupled mitochondria. Haematopoietic-specific *Atg7* knockout mice also exhibited severe anaemia as well as lymphopenia, and the mutant erythrocytes markedly accumulated degenerated mitochondria but not other organelles [174]. The mitochondrial content is regulated during the development of the T cells as well; that is, the high mitochondrial content in thymocytes is shifted to a low mitochondrial content in mature T cells. *Atg5* or *Atg7* deleted T cells fail to reduce their mitochondrial content resulting in increased ROS production as well as an imbalance in pro- and antiapoptotic protein expression [175–177]. All together, these evidences demonstrate the essential role of mitophagy in haematopoiesis.

Recent studies have described the molecular mechanism by which damaged mitochondria are selectively targeted for autophagy, and have suggested that the defect is implicated in the familial Parkinson's disease (PD) [178] (Figure 3(c)). PINK1, a mitochondrial kinase, and Parkin, an E3 ubiquitin ligase, have been genetically linked to both PD and a pathway that prevents progressive mitochondrial damage and dysfunction. When mitochondria are damaged and depolarized, PINK1 becomes stabilized and recruits Parkin to the damaged mitochondria [122, 179–181]. Various mitochondrial outer membrane proteins are ubiquitinated by Parkin and mitophagy is then induced. Of note, PD-related mutations in *PINK1* and *Parkin* impair mitophagy [122, 179–181], suggesting that there is a link between defective mitophagy and PD. How these ubiquitinated mitochondria are recognized by the autophagosome remains unknown. Although p62 has been implicated in the recognition of ubiquitinated mitochondria, elimination of the mitochondria occurs normally in *p62*-deficient cells [182, 183].

4.5. Elimination of Invading Microbes. When specific bacteria invade host cells through endocytosis/phagocytosis, a selective type of autophagy termed xenophagy, engulfs them to restrict their growth [184] (Figure 3(d)). Although neither the target proteins nor the E3 ligases have yet been identified, invading bacteria such as *Salmonella enterica*, *Listeria monocytogenes,* or *Shigella flexneri* become positive for ubiquitin when they access the cytosol by rupturing the endosome/phagosome limiting membrane [185, 186]. These findings raise the possibility that ubiquitin also serves as a tag during xenophagy. In fact, to date, three proteins, p62 [105, 185, 187], NDP52 [188], and optineurin [115] have been proposed to be autophagy receptors linking ubiquitinated bacteria and LC3. An ubiquitin-independent mechanism has recently been revealed; recognition of a *Shigella* mutant that lacks the icsB gene requires the tectonin domain-containing protein 1 (Tecpr1), which appears to be a new

type of autophagy adaptor targeting *Shigella* to Atg5- and WIPI-2-positive membranes [189]. Interestingly, the *Shigella* icsB normally prevents autophagic sequestration of this bacterium by inhibiting the interaction of *Shigella* VirG with Atg5 indicating that some bacteria have developed mechanism to inhibit or subvert autophagy to their advantage [190]. This latter category of pathogens also includes viruses such as Herpes simplex virus-1 (HSV-1), which express an inhibitor (ICP34.5) of Atg6/Beclin1 [106]. However, it was recently shown that a mutant HSV-1 strain lacking ICP34.5 becomes degraded by selective autophagy in a SMURF1-dependent manner [79], suggesting that selective autophagy plays an important role in our immune system.

Recently, a different antimicrobial function has been assigned to autophagy and this function appears to be selective. During infection, ribosomal protein precursors are transported by autophagy in a p62-dependent manner into lysosomes [112]. These ribosomal protein precursors are subsequently processed by lysosomal protease into small antimicrobial peptides. Importantly, it has been shown that induction of autophagy during a *Mycobacterium tuberculosis* infection leads to the fusion between phagosomes containing this bacterium and autophagosomes, and the production of the antimicrobial peptides in this compartment kills *M. tuberculosis* [112].

4.6. Lipophagy. While the molecular mechanism is largely unknown, autophagy contributes at least partially to the supply of free fatty acids in response to fasting (Figure 3(e)). Fasting provokes the increase of the levels of free fatty acids circulating in the blood, which are mobilized from adipose tissues. These free fatty acids are rapidly captured by various organs including hepatocytes and then transformed into triglycerides by esterification within lipid droplets. These lipid droplets appear to be turned over by a selective type of autophagy that has been named lipophagy in order to provide endogenous free fatty acids for energy production through β-oxidation [148]. Indeed, liver-specific *Atg7* deficient mice display massive accumulation of triglycerides and cholesterol in the form of lipid droplets [191]. Agouti-related peptide- (AgRP-) expressing neurons also respond to increased circulating levels of free fatty acids after fasting and then induce autophagy to degrade the lipid droplets [192]. Similar to the case in hepatocytes, autophagy in the neurons supplies endogenous free fatty acids for energy production and seems to be necessary for gene expression of AgPR, which is a neuropeptide that increases appetite and decreases metabolism and energy expenditure [192].

5. Conclusions and Perspectives

Originally, it was assumed that autophagy was exclusively a bulk process. Recent experimental evidences have demonstrated that through the use of autophagy receptors and adaptors, this pathway can be selective by exclusively degrading specific cellular constituents. The list of physiological and pathological situations where autophagy is selective is constantly growing and this fact challenges the earliest concept whether autophagy can be nonselective. It is believe that under starvation, cytoplasmic structures are randomly engulfed by autophagosomes and delivered into the lysosome to be degraded and thus generate an internal pool of nutrients. In yeast *Saccharomyces cerevisiae*, however, the degradation of ribosomes, for example, ribophagy, as well as mitophagy and pexophagy, and the transport of the prApe1 oligomer into the vacuole under the same conditions requires the presence of autophagy receptors [97, 193–195]. As a result, these observations suggest that autophagy could potentially always operate selectively. This is a conceivable hypothesis because this process allows the cell to survive stress conditions and the casual elimination of cytoplasmic structure in the same scenario could lead to the lethal depletion of an organelle crucial for cell survival. Future studies will certainly provide more molecular insights into the regulation and mechanism of the selective types of autophagy, and this information will also be important to determine if indeed bulk autophagy exists.

Abbreviations

AgRP:	Agouti-related peptide
AMPK:	AMP-activated protein kinase
ALFY:	Autophagy-linked FYVE protein
Ams1:	α-mannosidase 1
Ape1:	Aminopeptidase 1
Ape4:	Aminopeptidase 4
Atg:	Autophagy-related gene
Bnip3L:	B-cell leukemia/lymphoma 2 (BCL-2)/adenovirus E1B interacting protein 3
CK2:	Casein kinase 2
CMA:	Chaperone-mediated autophagy
Cvt:	Cytoplasm to vacuole targeting
ER:	Endoplasmic reticulum
FIP200:	Focal adhesion kinase family interacting protein of 200 kD
FYCO1:	FYVE and coiled-coil domain-containing protein 1
GABARAP:	Gamma-aminobutyrate receptor-associated protein
GATE-16:	Golgi-associated ATPase enhancer of 16 kDa
HDAC6:	Histone de-acetylase 6
HOPS:	Homotypic fusion and protein sorting
HSV-1:	Herpes simplex virus-1
Keap1:	Kelch-like ECH-associated protein 1
LC3:	Microtubule-associated protein 1 (MAP1)-light chain 3
LIR:	LC3-interacting region
LRS:	LC3 recognition sequences
NBR1:	Neighbour of BRCA1 gene
NDP52:	Nuclear dot protein (NDP) 52
NF-κB:	Nuclear factor κB
NIX:	Nip-like protein X
Nrf2:	NF-E2 related factor 2
PAS:	Phagophore assembly site
PB1:	Phox and Bem1p

PE: Phosphatidylethanolamine
PD: Parkinson's disease
PI3K: Phosphatidylinositol 3-kinase
PI3P: Phosphatidylinositol 3-phosphate
PKA: Protein kinase A
PKC: Protein kinase C
ROS: Reactive oxygen species
Rubicon: RUN domain and cysteine-rich domain containing Beclin 1-interacting protein
SMURF1: SMAD-specific E3 ubiquitin protein ligase 1
SUMO: Small ubiquitin-like modifier
SQSTM1: p62/sequestosome 1
TBK1: TANK binding kinase 1
Tecpr1: Tectonin domain-containing protein 1
TRAF6: Tumour necrosis factor receptor-associated factor 6
TOR: Target of Rapamycin
TGN: *Trans*-Golgi network
UBA: Ubiquitin associated
ULK1: Unc-51-like kinase 1
UVRAG: UV-resistance associated gen
Vps: Vacuolar protein sorting.

Acknowledgments

The authors thank Shun Kageyama (Tokyo Metropolitan Institute of Medical Science) for helping in the creation of the figures used in the paper. F. Reggiori is supported by The Netherlands Organization for Health Research and Development (ZonMW-VIDI-917.76.329), by The Netherlands Organization for Scientific Research (Chemical Sciences, ECHO grant-700.59.003). M. Komatsu is supported by Funding Program for Next Generation World-Leading Researchers. K. Finley is supported by the NIH. A. Simonsen is supported by the Research Council of Norway, by the Norwegian Cancer Society, and by the South-Eastern Norway Regional Health Authority.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 208014, 11 pages
doi:10.1155/2012/208014

Research Article

MAP1B Interaction with the FW Domain of the Autophagic Receptor Nbr1 Facilitates Its Association to the Microtubule Network

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Received 14 October 2011; Revised 3 February 2012; Accepted 16 February 2012

Academic Editor: Anne Simonsen

Selective autophagy is a process whereby specific targeted cargo proteins, aggregates, or organelles are sequestered into double-membrane-bound phagophores before fusion with the lysosome for protein degradation. It has been demonstrated that the microtubule network is important for the formation and movement of autophagosomes. Nbr1 is a selective cargo receptor that through its interaction with LC3 recruits ubiquitinated proteins for autophagic degradation. This study demonstrates an interaction between the evolutionarily conserved FW domain of Nbr1 with the microtubule-associated protein MAP1B. Upon autophagy induction, MAP1B localisation is focused into discrete vesicles with Nbr1. This colocalisation is dependent upon an intact microtubule network as depolymerisation by nocodazole treatment abolishes starvation-induced MAP1B recruitment to these vesicles. MAP1B is not recruited to autophagosomes for protein degradation as blockage of lysosomal acidification does not result in significant increased MAP1B protein levels. However, the protein levels of phosphorylated MAP1B are significantly increased upon blockage of autophagic degradation. This is the first evidence that links the ubiquitin receptor Nbr1, which shuttles ubiquitinated proteins to be degraded by autophagy, to the microtubule network.

1. Introduction

Cellular turnover of damaged and misfolded proteins is mediated by two main degradation pathways; macroautophagy (hereafter referred to as autophagy) and the ubiquitin proteasome system (UPS). The UPS targets soluble, cytosolic proteins to the proteasome where they are degraded. Proteins targeted for degradation are covalently modified by the small, highly conserved, ubiquitously expressed protein ubiquitin. Ubiquitin can form chains at all seven lysine residues and typically, chains of four or more ubiquitin molecules are required for the targeting of proteins to the proteasome [1]. However, misfolded proteins can form large aggregates which render them resistant to proteasomal degradation [2]. Autophagy is an evolutionary conserved catabolic process that serves to deliver large polyubiquitinated protein aggregates and whole organelles to the lysosome for degradation [3]. A block in this process can cause the accumulation of ubiquitinated protein aggregates and ultimately cell death [4].

Autophagy requires the coordinated action of 35 to date autophagy-related genes (ATG) that mediate the formation of the double-membrane bound autophagosome which encloses a portion of the cytoplasm and delivers it to the lysosome [5, 6]. There are two ubiquitin-like conjugation systems that are required for autophagosomal formation. The Atg12-Atg5-Atg16L complex is important for elongation of the isolation membrane [7] whilst Atg8/LC3, covalently attached to phosphatidylethanolamine (PE) is essential for autophagosome biogenesis [8]. LC3 is often used

as a marker for autophagosomes and has been shown to bind and stabilise microtubules [9, 10]. The microtubule network is important for autophagosomal formation [11, 12]; however, its requirement for fusion of autophagosomes with lysosomes is still unclear [11–13]. Roles for distinct populations of microtubules have also been proposed whereby labile microtubules specifically recruit markers of the isolation membrane such as Atg5, Atg12, and LC3 to sites of autophagosomal formation whereas stable microtubules facilitate the movement of mature autophagosomes [14].

Recent evidence demonstrates that autophagy can be a selective process, whereby single proteins and cellular structures such as aggregates and organelles can be specifically targeted to autophagosomes [15, 16], but the molecular mechanism of cargo recognition is poorly understood. Recently autophagic receptors have been described which include the structurally similar proteins p62 and NBR1, as well as the TBK1 adaptor NDP52 [17–19]. These receptors are thought to bind to polyubiquitinated proteins via their C-terminal-ubiquitin-associated (UBA/UBZ) domains and sort them to sites of autophagosomal formation via their interaction with LC3 [20, 21]. Both NBR1 and p62 colocalise with ubiquitin in Mallory bodies in the liver of patients with alcoholic steatohepatitis [18] and accumulate with ubiquitin in muscle fibres of sporadic inclusion-body myositis [22]. In contrast to p62, NBR1 has not been extensively studied, however growing evidence has implicated it in a diverse range of biological functions. NBR1 interacts with the giant sarcomeric protein titin and is part of a signalling complex that regulates muscle gene expression [23]. A genetically modified mouse model expressing a C-terminally truncated form of Nbr1 identified a role for Nbr1 in bone remodelling whilst a T-cell-specific knock-out of full length Nbr1 has implicated NBR1 as a mediator of T-cell differentiation and allergic inflammation [24, 25]. NBR1 has also recently been shown to direct autophagic degradation of midbody derivatives, independent of p62 [26]. Additionally, NBR1 inhibits receptor tyrosine kinase (RTK) degradation by trapping the receptor at the cell surface [27] and via its interaction with SPRED2, mediates the lysosomal degradation of activated receptors and the attenuation of fibroblast growth factor (FGF) signalling [28]. Identification of other protein interactors of NBR1 such as calcium- and integrin-binding protein (CIB) and fasciculation and elongation protein zeta-1 (FEZ1) [29] have suggested additional roles for NBR1 in cardiac dysfunction [30] and neuronal development, respectively [31]. It has been shown that both NBR1 and p62 are recruited to autophagosomal formation sites independent of LC3; however, the mechanism is unclear [32].

In this paper, we identify NBR1 as an interaction partner of the microtubule-associated protein MAP1B. This occurs via the evolutionarily conserved FW domain. We show that whilst MAP1B is not itself a substrate for autophagosomal protein degradation, the phosphorylated form of MAP1B is stabilised by lysosomal inhibition. We propose that this interaction provides a mechanism by which NBR1 is targeted to the microtubule network to promote degradation of proteins via the autophagosome.

2. Materials and Methods

2.1. Bioinformatics. BioEdit was used to curate sequences and compile alignments. BLAST was used on various databases to identify FW-like sequences from animal, plant, fungal, protist, bacterial, and metagenome sequences. Phyre was used for structural predictions.

2.2. Primary Antibodies and Constructs. For western blot analysis and immunofluorescence the following antibodies were used: polyclonal anti-myc (A14, Santa Cruz), monoclonal anti-HA (Roche), monoclonal anti-myc (9E10, Santa Cruz), and polyclonal anti-MAP1B-HC (kindly provided by Prof. Gordon-Weeks, King's College London [33, 34], polyclonal anti-MAP1B (N19, Santa Cruz), polyclonal anti-MAP1B (C20, Santa Cruz), polyclonal anti-pThr1265-MAP1B (Novus Biologicals), monoclonal anti-Nbr1 (Abcam), monoclonal anti-p62 (Abnova), and polyclonal anti-p62 (kindly provided by Prof. Gautel, King's College London), polyclonal anti-ULK1 (Sigma), polyclonal anti-ubiquitin (Dako), polyclonal anti-EEA1 (Cell Signaling), polyclonal anti β-actin (Abcam), and monoclonal anti-His (Novagen).

Yeast two-hybrid bait for Nbr1 was amplified by PCR and cloned into pGBKT7 (Nbr1 aa346-498) (Clontech). Full length Nbr1 was cloned into pHM6 (Roche) and MAP1B aa2216- 2464 was cloned into pcDNA3.1 (Invitrogen) for the coimmunoprecipitation assay. Nbr1 aa346-498 was cloned into pGEX2T (GE Healthcare) for the GST-binding assay and MAP1B aa2227-2464 was cloned into PET6H (a modified version of pET11d-Novagen) for the recombinant binding assay.

2.3. Yeast-2-Hybrid. Yeast strain Y187 was transformed with the Nbr1 bait construct and mated with a pretransformed (yeast strain AH109) mouse neonatal calvarial cDNA library kindly supplied by Prof. Ikramuddin Aukhil, University of Florida. Resulting colonies were screened by HIS3 reporter gene activity, replated three times and inserts were sequenced. Y187 transformed with pGBKT7 Nbr1 aa346-498 was mated with yeast strain AH109 expressing the library MAP1B clone pGADT7 MAP1B aa2238-2465 and plated onto SD medium lacking leucine, tryptophan, histidine and adenine and were cultured at 30°C to verify the interaction.

2.4. Coimmunoprecipitation. COS7 cells were cotransfected with HA-Nbr1 and MAP1B-myc and after 48 hours, lysed in IP buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP-40, supplemented with protease and phosphatase inhibitors (Roche)), and cell lysates incubated with rabbit polyclonal anti-myc antibody overnight at 4°C. Protein A beads (Millipore) were then added to the lysates for a further 2 hours, beads were then washed three times in IP buffer. Proteins retained on the beads were separated by SDS-PAGE and transferred onto a nitrocellulose membrane following standard procedures. Blots were probed with mouse monoclonal anti-myc and rat monoclonal anti-HA antibodies and subsequently with a secondary antibody

(HRP-conjugated anti-mouse or anti-rat, Dako, Abcam). Detection was performed by ECL (GE Healthcare).

2.5. Bacterial Expression of Fusion Proteins. Nbr1 aa346-498 fused to GST and GST alone were expressed in Bl21(DE3) bacterial cells and proteins purified by glutathione affinity chromatography as previously described [35]. MAP1B aa2227-2464 fused to His6 was also expressed in Bl21(DE3) bacterial cells and purified in the presence of urea. Briefly, bacterial cells expressing His_6-MAP1B aa2227-2464 were lysed in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris pH 8, 6 M Urea, 5 mM Imidazole pH 8, supplemented with EDTA-free protease inhibitors (Roche)). The sample was sonicated, centrifuged, and the supernatant was incubated with Ni Sepharose 6 fast flow beads (Amersham Biosciences) for 2 hours at 4°C. Beads were then washed in low Imidazole elution buffer (100 mM NaH_2PO_4, 10 mM Tris pH 8, 6 M Urea, 20 mM Imidazole pH 8, supplemented with EDTA free protease inhibitors (Roche)) and bound proteins eluted from the beads using high Imidazole elution buffer (100 mM NaH_2PO_4, 10 mM Tris pH 8, 6 M Urea, 250 mM Imidazole pH 8, supplemented with EDTA-free protease inhibitors (Roche)). The resulting purified His-tagged protein was dialysed into 50 mM Tris pH 7.5, 150 mM NaCl and used in the GST pull-down assay.

2.6. GST Pull-Down Assays. COS7 cells were transfected with a MAP1B-myc construct and after 48 hours expression, lysed in IP buffer (as above), and lysates incubated with beads coupled with either GST-Nbr1 aa346-498 or GST alone for 2 hours at 4°C. Following incubation, beads were washed three times in IP wash buffer (50 mM Tris pH 7.5, 200 mM NaCl, 0.5% NP-40, supplemented with protease and phosphatase inhibitors (Roche)) and proteins retained on the beads were analysed by western blotting as described above, using the monoclonal anti-myc antibody, 9E10. Alternatively purified GST or GST-Nbr1 aa346-498 attached to glutathione agarose beads (Sigma) was incubated in IP buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP-40, supplemented with protease and phosphatase inhibitors (Roche)) with purified His_6-MAP1B aa2227-2464 for 2 hours at 4°C. Following incubation, beads were washed three times in IP wash buffer (50 mM Tris pH 7.5, 200 mM NaCl, 0.5% NP-40), and proteins retained on the beads were separated by SDS-PAGE and analysed by western blotting as described above, using the anti-His tag monoclonal antibody.

2.7. Cell Culture, Treatments, Transfection and Immunostaining. COS7 cells were cultured in DMEM/10% FCS by standard protocols and transfected using Fugene 6 (Roche). Cells were lysed 48 hours later in 200 μL IP buffer for pull-down and coimmunoprecipitation assays. For immunostaining PC12 cells were cultured on coverslips in DMEM/10% FCS, treated with DMSO or Bafilomycin A1 (Sigma) for 4 or 8 hours, or starved in Hanks-Balanced Salt Solution (Sigma) for 4 hours or treated with 5 μg/mL nocodazole (Sigma) for 30 minutes before or after 2 hours starvation and fixed in 4% paraformaldehyde/PBS for 10 minutes. Cells were then permeabilised in 0.1% Triton X100/PBS and incubated consecutively with primary and secondary antibodies (Dako) for one hour each prior to mounting. Cells were imaged using a Zeiss LSM 510 confocal microscope in sequential scanning mode with a Plan-Apochromat 63 x/1.4 Oil DIC objective. Quantification of MAP1B/Nbr1 colocalisation was performed using Zeiss ZEN2010 software, data represent mean ± SEM of 22 images.

3. Results

3.1. The Predicted Structure and Evolution of Nbr1 FW Domain. We used BLAST-based searches to acquire NBR1-related sequences from multiple available genomic and transcriptomic sources across a broad range of eukaryotes. These identified a region of pronounced conservation of 105 amino acids (residues 374–478 of human NBR1) which is recognisable in the single NBR1 orthologue found in most eukaryotes but is absent from p62. This novel domain has been named the NBR1 domain [36] and FW domain by Terje Johansen's group [37] after its four strikingly conserved tryptophan residues, and we will use FW nomenclature here for clarity.

Single NBR1 orthologues were found in all animals, most plants, most fungi (though notably not *Saccharomyces cerevisiae*) and some single-celled eukaryotes (such as *Dictyostelium discoideum*). In each case the NBR1-like molecule possessed an N-terminal PB1 domain, one (animals, plants) or more (fungi) ZZ domains, an FW domain, and a C-terminal UBA domain (Figure 1).

The FW domain was also found in a second, otherwise unrelated animal protein. As the human version has been named c6ORF106, we will use this name. Single c6ORF106 orthologues are found in all animal species examined, plus the single-celled metazoan sister-group choanoflagellates. No c6ORF106 orthologues were found in any other organisms. The proteins tend to be small (the human c6ORF106 is 298 amino acids long), comprising a universally conserved N-terminal α-helical domain of ~70–80 amino acids, then the FW domain and finally a poorly structured and variable length C-terminal region (Figure 1).

Intriguingly, FW domains were also found in a wide range of eubacteria. The eubacterial FW-containing proteins are strikingly diverse in domain structure, with the only common theme being that the FW domain tends to be very close to the C-terminus. Although most eubacterial genomes do not encode an FW domain-containing protein, we find that it is broadly distributed across eubacterial clades (γ-proteobacteria, chloroflexi, actinobacteria, and several unclassified metagenomes). In several of the bacterial proteins (*Halorhodospira halophila, Methylomonas methanica, Kribbella flavida, Variovorax paradoxus,* and one from a fresh water environmental metagenome), the FW domain appears immediately C-terminal to a robustly predicted "helix-turn-helix" DNA-binding motif of the XRE family. We call these XRE-FW proteins. XRE domains tend to appear either alone or with multimerisation domains (as in the *Bacillus subtilis* repressor of sporulation and biofilm formation, SinR and

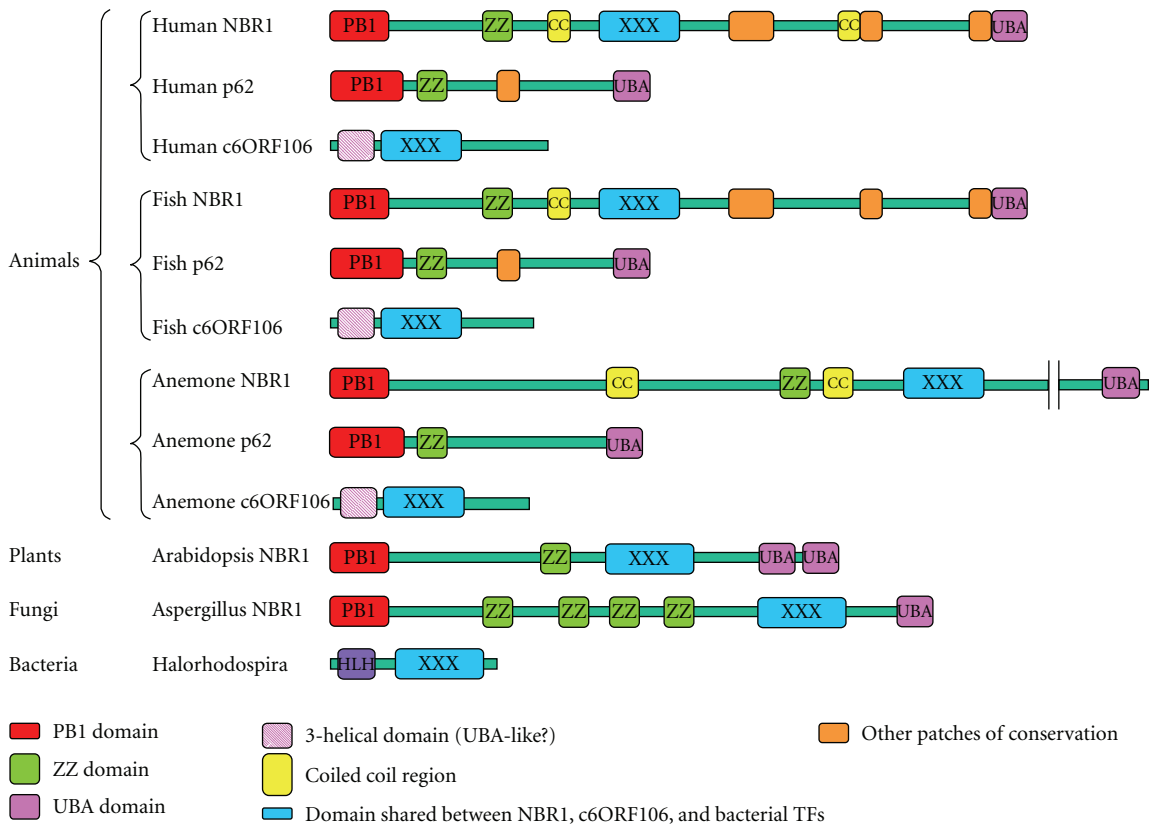

Figure 1: Schematic representation of the commonest members of the FW-containing protein families- the NBR1 proteins found in almost all eukaryotes, the c6ORF106 proteins found in almost all metazoans and the XRE-FW proteins found in some bacteria. The metazoan p62 family is also included to show its relationship to NBR1. Proteins are drawn to scale. A key to the domains appears at the bottom of the figure.

the bacteriophage repressors CI and Cro). This juxtaposition raises the possibility that the FW domain might mediate homo- and/or heterodimerisation or could bind a small signalling molecule. Some other eubacterial FW proteins consist solely of two tandem FW domains and little else (e.g., that from *Coprococcus*), while others contain transmembrane domains (e.g., that from *Streptomyces* sp.). This structural diversity suggests that the FW domain has a generically useful function that has been exploited in many ways.

We used Phyre to predict the secondary structure of all FW domains separately. This robustly predicted the same alignable structural features in every sequence, regardless of sequence divergence (Figure 2). Thus we feel that we are able to say with some confidence that the FW domain consists of two sets of three β-strands separated by a central unstructured region of more variable length. Striking sequence features include four almost invariant tryptophan residues, which lie in the middle of strand $\beta 2$, in the linker between $\beta 2$ and $\beta 3$, and in the middle of strands $\beta 5$ and $\beta 6$. These give the domain its name and are only rarely replaced by other aromatic residues. There are also several invariant glycines and prolines in some of the unstructured linkers. It is conceivable that the domain folds into a sandwich of two three-strand β-sheets with the tryptophans projecting into the hydrophobic core.

Phylogenetic analysis showed that all NBR1 FW domains clustered together, as did all c6ORF106 FW domains. Two FW sequences from metagenomic sources clustered with NBR1 sequences; one of these had a C-terminal UBA domain, and we assume that these are from eukaryotic species in the environmental metagenome sources. The reproducible monophyletic clustering of bacterial FW domains (to the exclusion of eukaryotic NBR1 and c6ORF106 sequences) argues against multiple eukaryote-to-prokaryote horizontal gene transfer events and suggests that the FW domain may be ancient, predating the split between eukaryotic and eubacterial domains.

3.2. Identification of the FW Domain of Nbr1 as an Interaction Partner of Microtubule-Associated Protein MAP1B. To identify novel protein interactors of the highly conserved FW domain of Nbr1 and therefore elucidate a function, we performed a yeast-2-hybrid screen with the FW domain of Nbr1 as bait. A neonatal calvarial cDNA library was screened and the light chain of the microtubule-associated protein 1B (MAP1B-LC1) was identified as an interaction partner of Nbr1. This interaction was verified by a directed yeast two-hybrid assay by retransforming the isolated prey vector encoding the partial MAP1B-LC1 sequence (aa2238-2465)

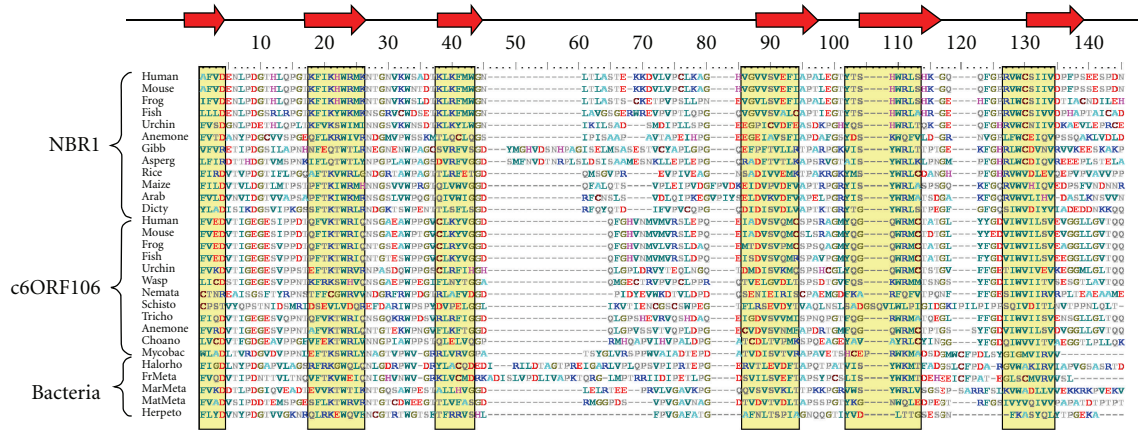

Figure 2: Alignment of NBR1, c60RFI06 and eubacterial FW domain sequences. Yellow boxes and red arrows indicate β-strands predicted by Phyre. The amino acids are colour coded as follows; red-positively charged, dark blue-negatively charged, grey-non-charged polar, dark green-aliphatic and aromatic, cyan-alanine, brown-cysteine, magenta-histidine, gold-glycine. Brackets at left indicate broad origin of FW domains (according to gross structure of host protein or phylogenetic affinity). Species are indicated as follows: Human—*Homo sapiens*; Mouse—*Mus musculus*; Frog—*Xenopus tropicalis*; Fish—*Danio rerio*; Urchin—*Strongylocentrotus purpuratus*; Anemone—*Nematostella vectensis*; Gibb—*Gibberella zeae*; Asperg—*Aspergillus nidulans*; Rice—*Oryza sativa*; Maize—*Zea mays*; Arab—*Arabidopsis thaliana*; Dicty—*Dictyostelium discoideum*; Wasp—*Nasonia vitripennis*; Nemato—*Caenorhabditis elegans*; Schisto—*Schistosoma mansoni*; Tricho—*Trichoplax adhaerens*; Choano—*Monosiga brevicollis*; Mycobac—*Mycobacterium* sp. MCS; Halorho—*Halorhodospira halophila*; FrMeta—Fresh water metagenome; MarMeta—Marine metagenome; MatMeta—Mat metagenome; Herpeto—*Herpetosiphon* sp.

into yeast strain AH109 and mating it with yeast strain Y187 that was expressing the FW domain of Nbr1 (Figure 3(a)). MAP1B is transcribed as a single mRNA, translated into a polypeptide, and subsequently cleaved producing a heavy chain (2214aa) and a light chain (250aa) [38]. Both the heavy chain (MAP1B HC) and the light chain (MAP1B-LC1) can bind to microtubules [39, 40] and to each other [41]. MAP1B has been implicated in the regulation of autophagy, as it interacts with LC3 and targets autophagosomes to axon terminals during neurodegeneration [42].

3.3. Nbr1 is Found in a Complex with MAP1B-LC1 In Vivo. To determine whether Nbr1 forms a complex with MAP1B-LC1 *in vivo*, we performed a coimmunoprecipitation experiment using COS7 cells transiently transfected with HA-Nbr1 and MAP1B-LC1-myc constructs. Using an anti-myc antibody for immunoprecipitation of MAP1B-LC1-myc, we found that HA-Nbr1 did coimmunoprecipitate with MAP1B-LC1-myc (Figure 3(b)) confirming that they are found in a complex *in vivo*. We were unable to show coimmunoprecipitation of endogenous Nbr1 and MAP1B-LC1 in PC12 cells. This is likely to be due to the levels of interacting protein being below the detection level possible by western blot analysis with the available antibodies (data not shown).

3.4. Nbr1 Interacts with MAP1B-LC1 In Vitro. The yeast-2-hybrid data suggested that the FW domain of Nbr1 interacts directly with MAP1B-LC1. To more rigorously test this hypothesis, we performed GST pull-down assays using extracts from COS7 cells overexpressing MAP1B-LC1-myc and a GST fusion of the FW domain of Nbr1 and GST alone. Indeed, the FW domain of Nbr1 interacted with MAP1B-LC1 whilst GST alone did not (Figure 4(a)).

To verify the interaction between the FW domain of Nbr1 and MAP1B-LC1 in a cell-free environment, His_6-MAP1B-LC1 was purified and incubated with the GST fusions of the FW domain of Nbr1 or GST alone. This demonstrated that the FW domain of Nbr1 interacts directly with the light chain of MAP1B (Figure 4(b)).

3.5. MAP1B Is Not Degraded by Autophagy. It has previously been observed that MAP1B-HC is not degraded by autophagy [42] however, it has not been reported whether the same is true for MAP1B-LC1. To establish if the function of the interaction between Nbr1 and MAP1B-LC1 is to facilitate the degradation of MAP1B-LC1 via autophagy, MAP1B-LC1 protein levels were analysed under conditions where autophagic protein degradation was blocked. PC12 cells, a neuronal cell line that expresses elevated levels of endogenous MAP1B, were treated with Bafilomycin A1 or DMSO for 8 hours before protein extracts were resolved by SDS PAGE and detected using antibodies that recognise p62, Nbr1, MAP1B-LC1, MAP1B-HC and β-actin. Upon blockage of autophagic protein turnover, the levels of p62 and Nbr1 were increased by 60% and 130%, respectively, demonstrating that autophagic protein degradation was blocked by Bafilomycin A1 treatment (Figures 5(a) and 5(b)). MAP1B-HC, and MAP1B-LC1 protein levels showed a negligible increase upon the blockage of autophagic protein degradation suggesting that they are not degraded by autophagy and that the function of the Nbr1-MAP1B-LC1 interaction is not to target MAP1B-LC1 for autophagic protein turnover. Surprisingly, although total MAP1B levels

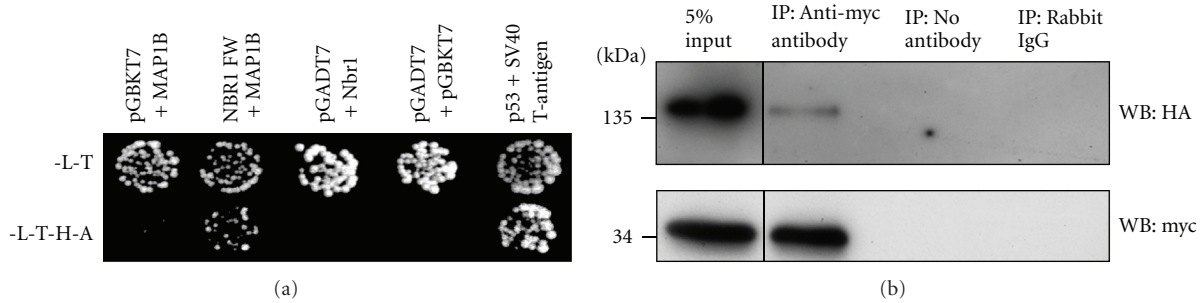

Figure 3: Nbr1 interacts with MAP1B *in vivo*. (a) Identification of Nbr1 as an interaction partner of MAP1B-LC1. Yeast-2-hybrid retransformation assay confirming the interaction between the FW domain of Nbr1 (aa346-498) and the light chain of MAP1B (aa2238-2465). Interaction was assessed by yeast growth on SD-L/-T/-H/-A medium. Empty vectors were used as negative controls, SV40 large T antigen and p53 were used as positive controls. (b) Nbr1 is found in a complex with MAP1B-LC1. Coimmunoprecipitation of HA-Nbr1 and MAP1B-LC1-myc from COS7 cells transfected with HA-Nbr1 and MAP1B-LC1-myc constructs. Extracts and precipitates were analysed by western blot using the indicated antibodies.

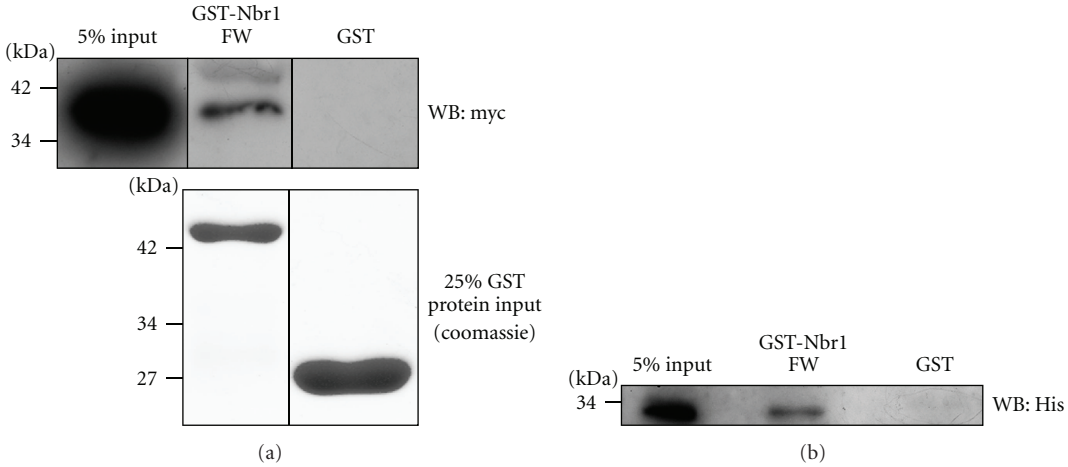

Figure 4: Nbr1 interacts with MAP1B-LC1. (a) GST pulldown assay using cell extracts from COS7 cells transfected with MAP1B-LC1-myc and immobilised GST or GST-Nbr1 FW domain. Upper panel: coprecipitated proteins were detected with an anti-myc antibody. The FW domain of Nbr1 interacts with MAP1B-LC1. Lower panel: coomassie stained SDS PAGE gel showing 25% of GST-tagged protein input. (b) GST pulldown assay using purified His-MAP1B-LC1 and immobilised GST or GST-Nbr1 FW domain. Coprecipitated proteins were detected using an anti-His antibody and demonstrated that the FW domain of Nbr1 interacts with MAP1B-LC1. The same amount of GST or GST-Nbr1 FW domain fusion protein was used as shown in (a) (lower panel).

are largely unaffected by blocking autophagic protein degradation, levels of phospho-pThr1265-MAP1B are increased following Bafilomycin A1 treatment (Figure 5(c)). This phosphorylated form of MAP1B is expressed in differentiating neurons and is a major substrate for glycogen synthase kinase-3beta (GSK-3beta) and is thought to be involved in regulating microtubule dynamics by MAP1B [43].

3.6. Nbr1 Colocalises with MAP1B upon Induction of Autophagy. Next, we analysed the subcellular localisation of endogenous Nbr1 and MAP1B by confocal microscopy. To establish if Nbr1 and MAP1B colocalise *in vivo*, PC12 cells were treated with DMSO, Bafilomycin A1 to block autophagic protein degradation or starved to induce autophagy and analysed by immunofluorescence. Under basal conditions, when levels of Nbr1 are low, there was little colocalisation between Nbr1 and MAP1B (Figure 6(A)). Upon blockage of autolysosomal protein degradation by Bafilomycin A1 treatment, Nbr1 is no longer turned over by autophagy and accumulates (Figure 6(B)) but total MAP1B is unaffected and appears excluded from Nbr1-positive vesicles. This confirms that MAP1B is not itself degraded by the autolysosomal pathway. Upon starvation and induction of autophagy, Nbr1 and MAP1B colocalise to distinct perinuclear vesicular structures (Figure 6(C)). Although this does not occur in all cells, only under starvation conditions were MAP1B-/Nbr1-positive vesicles observed. Under starvation conditions where MAP1B/Nbr1 positive punctate structures were observed, quantification of colocalisation showed a Mander's colocalisation coefficient of 64 ± 10%. These MAP1B-/Nbr1-positive vesicles also colocalise with the autophagic protein p62 (Figure 6(D)) but few colocalise with ubiquitin, suggesting that these vesicles

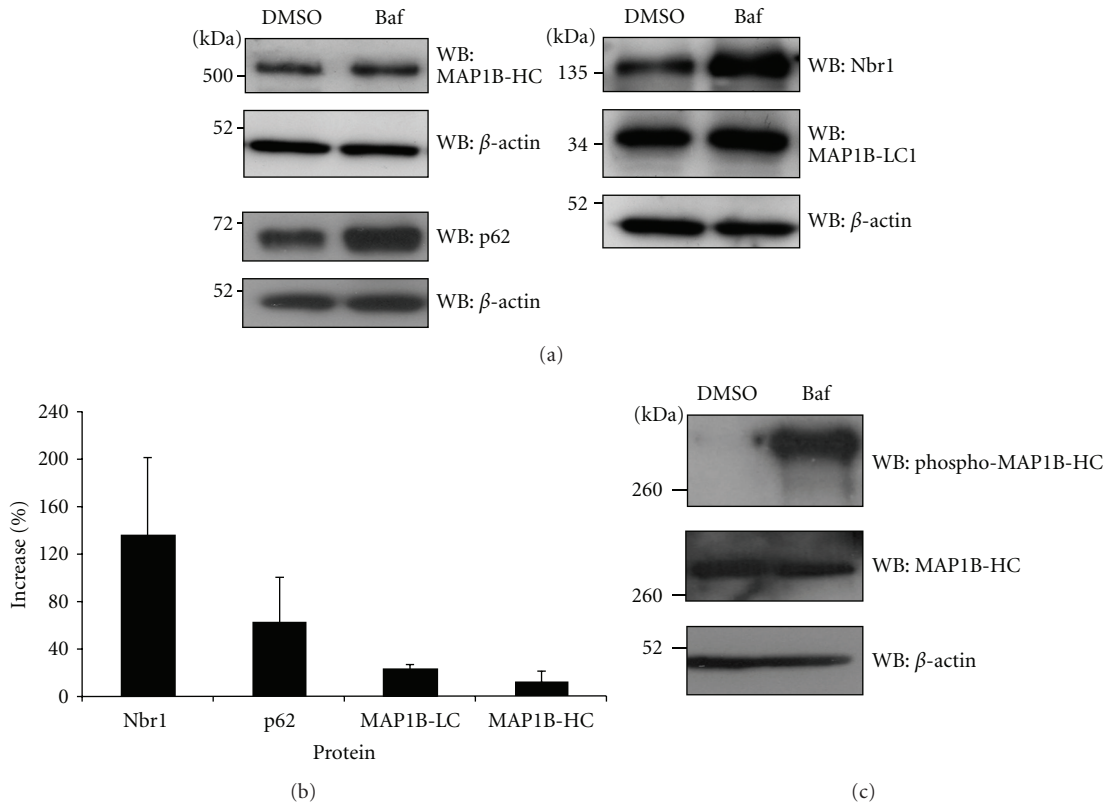

Figure 5: Western blot analysis of p62, Nbr1, MAP1B-HC, and MAP1B-LC1 protein levels following blockage of autophagic protein degradation. (a) Western blots showing protein levels in cells treated with DMSO (control) or Bafilomycin A1 (Baf). (b) Quantification of protein band intensity. MAP1B-LC1 and MAP1B-HC levels are increased by a negligable amount compared with Nbr1 and p62 upon treatment with Bafilomycin A1; Error bars represent SD, $n = 3$. (c) Phospho-MAP1B-HC is degraded by autophagy. Upon blockage of autophagic degradation with Bafilomycin A1: (Baf), levels of phospho-MAP1B-HC increase compared with levels of total MAP1B-HC.

are not aggresomes or mature autophagosomes loaded with ubiquitinated cargo (Figure 6(F)). We found little overlapping distribution with ULK1 and Nbr1/MAP1B vesicles under starvation conditions, in comparison with previous analysis of Nbr1/ULK1 colocalisation under these conditions [32] (Figure 6(E)) or with the early endosomal marker EEA1 (Figure 6(G)). This demonstrates that upon induction of autophagy, Nbr1 is recruited to MAP1B positive structures which are colocalising with p62, suggesting these may be early autophagosomes but downstream of autophagosomal formation sites.

To determine if colocalisation of Nbr1 and MAP1B in response to starvation-induced autophagy was dependent upon an intact microtubule network, PC12 cells were treated with the depolymerisation agent nocodazole under starvation conditions and examined for colocalisation. Depolymerisation of the microtubule network was confirmed by α-tubulin staining (data not shown) and resulted in loss of the punctate colocalisation of MAP1B and Nbr1 but intact Nbr1 vesicles were retained (Figure 6(H)). This suggests that MAP1B is not essential for the formation of Nbr1-positive vesicles but that an intact microtubule network is essential for colocalisation of Nbr1 and MAP1B under starvation conditions.

4. Discussion

The FW domain of Nbr1 is highly conserved throughout the eukaryotic kingdom and is also present in a number of bacterial proteins. It contains two internal repeats of ~55 residues and has a predicted secondary structure consisting of two, three β-stranded sheets. The high conservation of this region and its absence in p62 [37] suggests that it has a function that is distinct from p62. We therefore performed a yeast-2-hybrid screen with the FW domain of Nbr1 in order to determine a specific function for this region. The light chain of MAP1B (MAP1B-LC1) was identified as an interaction partner of the FW domain. As Nbr1 has previously been identified as an autophagic receptor that targets ubiquitinated proteins for degradation via its interaction with LC3 [18, 19], it was reasonable to hypothesise that the function of the interaction between Nbr1 and MAP1B-LC1 is to facilitate the autophagic degradation of MAP1B-LC1. Analysis of protein levels after autophagy blockage demonstrated that the levels of MAP1B-LC1 increased by a negligible amount suggesting that it is not degraded by autophagy (Figure 5). Blockage of autophagosomal protein degradation can also result in a reduction of protein turnover by the UPS [44] therefore, as MAP1B-LC1 is known to be degraded by the

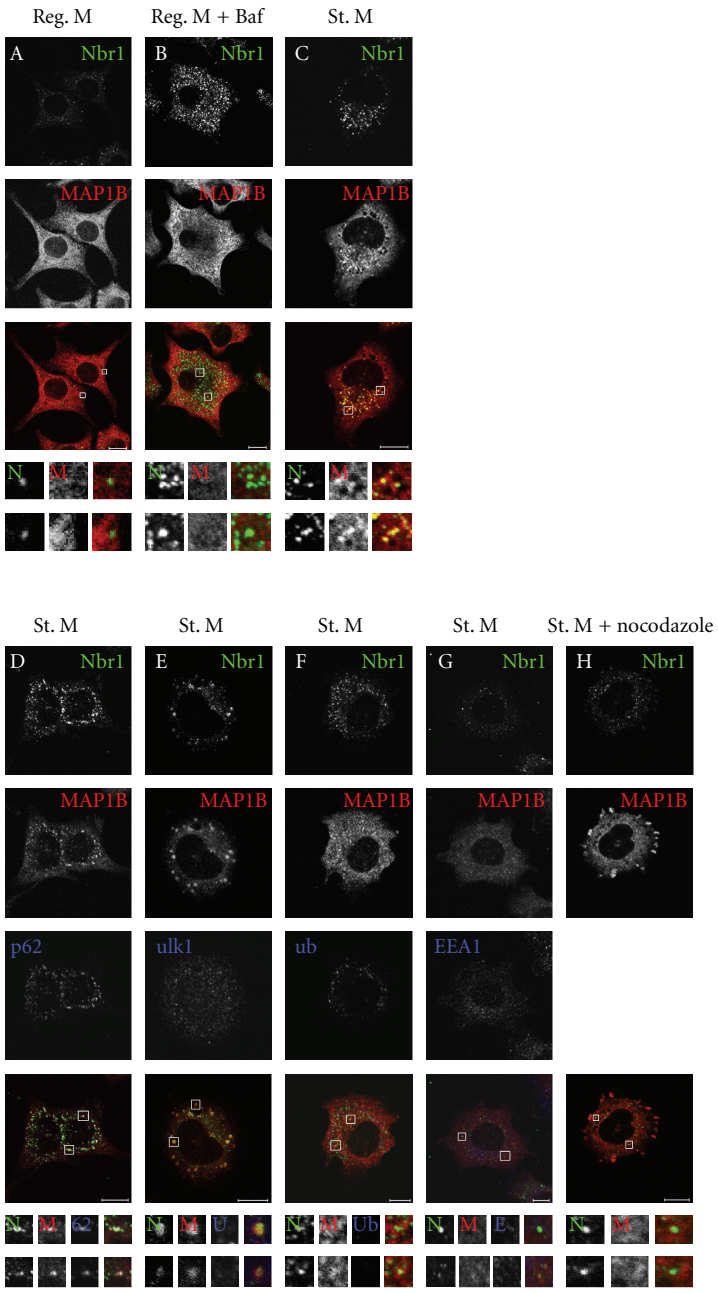

Figure 6: Nbr1 and MAP1B colocalise in discrete perinuclear vesicles upon induction of autophagy. PC12 cells were treated with DMSO, Bafilomycin or starved then fixed and stained with antibodies against the indicated proteins. Under basal conditions (A) or when autophagic degradation is blocked by Bafilomycin A1 treatment (B), very little or no colocalisation was observed between Nbr1 and MAP1B. When cells were starved to induce autophagy (C) MAP1B and Nbr1 colocalise in distinct perinuclear vesicles which are also positive for p62 (D) but are largely negative for ULK1 (E), ubiquitin (F), and EEA1 (G). Upon depolymerisation of the microtubule network and subsequent induction of autophagy by starvation, MAP1B no longer colocalised in distinct perinuclear vesicles with Nbr1 (H). Antibodies used: anti-Nbr1 (abcam), anti-MAP1B (N19, Santa Cruz), anti-p62 (M. Gautel, KCL), anti-ULKl (Sigma), anti-ubiquitin (Ub) (Sigma), and anti-EEAl (Cell Signaling). Scale bar; 10 μm.

UPS [45], this could suggest that Bafilomycin A1 treatment results in the inhibition of MAP1B-LC1 degradation via the proteasome rather than by autophagy. Interestingly, we observed that inhibition of autophagic degradation resulted in an increase in phospho-Thr1265 MAP1B, perhaps also reflected in the small increase in total MAP1B levels observed. Expression of this phosphorylated form of MAP1B is spatially regulated in differentiating neurons, and the kinase responsible for phosphorylation at this site has been identified as glycogen synthase kinase-3 beta. GSK-3 beta

inhibition has been linked to Bif-1-dependent autophagic induction under serum starvation to modulate cell survival [46].

Further biochemical analysis confirmed that the interaction between Nbr1 and MAP1B-LC1 is direct and that these proteins can be found in a complex together *in vivo*. As both Nbr1 and the microtubule network have been identified as key players in the facilitation of protein degradation via autophagy [11, 12, 18, 19], this could suggest that the Nbr1-MAP1B-LC1 interaction is important for this process. MAP1B interacts with LC3 and through this interaction it has been proposed that autophagosomes are targeted to axon terminals during neurodegeneration [47]. Additionally MAP1B has been predicted to interact with Atg12 and Atg3 suggesting that in addition to LC3, MAP1B is important for targeting other components of the autophagosomal machinery to sites of autophagosomal formation [48]. The interaction data presented here and the colocalisation of Nbr1 and MAP1B to perinuclear vesicles suggest that via its interaction with MAP1B, Nbr1 is targeted to the microtubule network, thus providing a mechanism by which proteins can be targeted to autophagosomes. The MAP1B-/Nbr1-positive vesicles do not however colocalise with ubiquitin, suggesting that these vesicles are not yet loaded with ubiquitinated cargo. Alternatively, they could represent vesicles loaded with other nonubiquitinated proteins that have been targeted for degradation. Whilst there are currently no known proteins that are targeted for autophagy by Nbr1 in a ubiquitin-independent manner, STAT5A-ΔE18 can be targeted for autophagic degradation by the PB1 domain of p62 independent of ubiquitin [49]. This suggests that Nbr1 could also be acting by a similar mechanism to target proteins for degradation independent of ubiquitin. Nbr1/MAP1B vesicles did not colocalise with EEA1, showing that these are not early endosomes. Likewise, we saw largely no colocalisation of MAP1B/Nbr1 vesicles with ULK1, suggesting that MAP1B- and Nbr1-positive structures are not present at sites of autophagosomal formation but do perhaps represent early autophagosomes that are positive for p62 and nonubiquitinated protein cargo.

This is the first evidence linking Nbr1 to the microtubule network and also demonstrates a distinct function for the FW domain of Nbr1. A similar mechanism has previously been demonstrated whereby HDAC6 is able to interact with polyubiquitinated protein aggregates and to dynein motors thereby coupling protein aggregates to the microtubule network where they can be transported to sites of autophagosomal formation [50]. Furthermore, adaptor proteins such as FYCO can interact with LC3 and microtubule motor proteins and through these interactions it has been suggested that preautophagosomal membranes are targeted to sites of autophagosomal formation [51]. Roles for MAP1S (a MAP1B homologue) in autophagic degradation of mitochondria have also been demonstrated. MAP1S interacts with LC3 and this interaction functions to target LC3, to the microtubule network. Genetic ablation of MAP1S causes the accumulation of defective mitochondria and severe defects in response to nutritive stress suggesting defects in autophagosomal biogenesis and clearance [52]. It has been suggested that recruitment of autophagosomal cargo receptors like Nbr1 and p62 to the autophagosomal formation site may be a general feature of this type of receptor, but that it is independent of Atg factors downstream of the PI3-kinase complex [32]. This study further highlights the role for microtubule associated proteins in the targeting of autophagosome machinery to the microtubule network and complements the work presented here that suggests a link between microtubule-associated proteins and autophagic receptors.

The high evolutionary conservation of the FW domain within Nbr1 homologues implicates it to have a critical role in Nbr1 function. The predicted secondary structure of the FW domain that consists of two three β-stranded sheets that form a compact "sandwich" is also present in the cholesterol-binding protein Niemann-Pick C2 (NPC2) [53, 54] suggesting additional roles for the FW domain in lipid binding.

In summary, we present the first evidence linking the autophagic receptor protein Nbr1 and the microtubule network via a direct interaction of the evolutionary-conserved FW domain of Nbr1 with MAP1B. Nbr1 is a ubiquitously expressed protein that has been implicated in several diseases [18, 22, 23], and it therefore will be of significant value to assess this interaction in tissue-specific physiological studies.

Acknowledgments

The authors would like to acknowledge Professor Gordon-Weeks (King's College London) for helpful discussion and a polyclonal MAP1B antibody and Prof. Ikramuddin Aukhil, (University of Florida) for the yeast two-hybrid calvarial cDNA library. K. Marchbank was supported by a BBSRC PhD studentship, S. Waters is supported by the Wellcome Trust, R. G. Roberts was funded by the Muscular Dystrophy Campaign, and C. A. Whitehouse is supported by an Arthritis Research UK Fellowship.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 146767, 9 pages
doi:10.1155/2012/146767

Review Article

Selective Autophagy in *Drosophila*

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Received 1 November 2011; Revised 1 February 2012; Accepted 3 February 2012

Academic Editor: Anne Simonsen

Autophagy is an evolutionarily conserved process of cellular self-eating and is a major pathway for degradation of cytoplasmic material by the lysosomal machinery. Autophagy functions as a cellular response in nutrient starvation, but it is also associated with the removal of protein aggregates and damaged organelles and therefore plays an important role in the quality control of proteins and organelles. Although it was initially believed that autophagy occurs randomly in the cell, during the last years, there is growing evidence that sequestration and degradation of cytoplasmic material by autophagy can be selective. Given the important role of autophagy and selective autophagy in several disease-related processes such as neurodegeneration, infections, and tumorigenesis, it is important to understand the molecular mechanisms of selective autophagy, especially at the organismal level. *Drosophila* is an excellent genetically modifiable model organism exhibiting high conservation in the autophagic machinery. However, the regulation and mechanisms of selective autophagy in *Drosophila* have been largely unexplored. In this paper, I will present an overview of the current knowledge about selective autophagy in *Drosophila*.

1. Introduction

Macroautophagy (from hereafter referred to as autophagy) is an evolutionarily conserved process by which a portion of the cytosol and organelles are sequestered by isolation membranes called phagophores. The phagophore engulfs portions of the cytoplasm and forms a double-membrane-layered organelle called the autophagosome. The autophagosome then fuses with a lysosome and generate the autolysosome that has a single limiting membrane, where its sequestered components are degraded [1]. Autophagy serves as a cellular response in nutrient starvation, but it is also responsible for the removal of aggregated proteins, damaged organelles, and developmental remodeling and therefore plays an important role in the quality control of proteins and organelles and in cellular homeostasis [1]. Genetic inhibition of autophagy induces degeneration that resembles degeneration observed during ageing, and physiological ageing is associated with reduced autophagic activity [2]. Autophagy is implicated in neurodegeneration, infections, tumorigenesis, heart disease, liver and lung disease, myopathies, and in lysosomal storage disorders [2]. Interestingly, it has been shown that induction of autophagy can increase longevity in multiple animal species [3]. Contrary to the belief that autophagy is a nonselective process, recent evidence suggests that degradation of proteins, protein aggregates, organelles, and bacteria can be selective through adaptor proteins [4]. It is therefore important to elucidate the role of selective autophagy in normal and pathological conditions using model organisms. The fruit fly *Drosophila melanogaster* is a genetically modifiable model organism and is an excellent model for investigating the mechanisms of selective autophagy in the context of the physiology of the cell, the system, and the living organism. This paper will summarize the current knowledge about selective autophagy in *Drosophila*.

2. Selective Autophagy in *Drosophila*

Studies in *Drosophila* so far revealed the presence of highly conserved autophagic machinery compared to yeast and mammals [5]. *atg* (autophagy-related) genes and their

regulators in *Drosophila* in many cases, in contrast to mammalian systems, have single orthologs, allowing for nonredundant genetic studies [5]. However, the regulation and mechanisms of selective autophagy have not been described in details, and there is only limited evidence for the presence of selective autophagy and autophagic cargo receptors. Additionally, cellular processes related to selective autophagy like mitophagy (selective autophagy of mitochondria), xenophagy (selective autophagy of bacteria and viruses), nucleophagy (selective autophagy of nucleus), and pexophagy (selective autophagy of peroxisomes) are largely unexplored in *Drosophila*. In the following text, I will describe what is reported so far in the literature about selective autophagy and selective autophagy-related proteins in *Drosophila*.

2.1. Selective Autophagy Receptors in Drosophila

2.1.1. Ref(2)P, the Drosophila Homologue of the Mammalian Selective Autophagy Receptor p62/SQSTM1. In mammals, six proteins have been identified as selective autophagy receptors so far: p62/SQSTM1, NBR1, NDP52, Nix, optineurin, and Stbd1 [4, 6, 7]. These proteins contain a LIR/LRS (LC3-interacting region/LC3 recognition sequence) motif and have been shown to interact with the autophagosomal membrane protein LC3 (microtubule-associated protein 1 light chain 3) [4]. The phosphatidylinositol-3-phosphate-(PI3P-) binding protein Alfy (autophagy-linked FYVE domain containing protein) was also shown to be required for selective degradation of aggregated proteins such as polyQ [8, 9] although a LIR/LRS motif has not yet been identified in Alfy sequence.

Landmark studies from Johansen's group indicated that mammalian p62/SQSTM1 is degraded selectively by autophagy and introduced the significant role of p62/SQSTM1 in autophagy [10, 11]. p62/SQSTM1 is the first identified and most studied autophagy cargo receptor. It is a multifunctional scaffold protein that serves a large variety of cellular functions [4, 12, 13]. The human p62 protein is 440 amino acids long and contains several structural and functional motifs [4] (Figure 1(a)). A Phox and Bem1p domain (PB1 domain) is located at the N-terminus and is required for di- and multimerization of the protein as well as interaction with the protein kinases MEKK3, MEK5, ERK, PKCζ, and PKCλ/ι and autophagy receptor NBR1 [4]. A zinc-finger-type (ZZ-type) domain follows the PB1 domain and is the binding site of receptor-interacting serine-threonine kinase 1 (RIP1) [12, 14]. Subsequently, there is a TRAF6-binding (TB) domain which contains the binding site of E3 ubiquitin-protein ligase TRAF6 [12, 14]. Nuclear-cytoplasmic shuttling of the protein is mediated by nuclear localization signals (NLSs) and nuclear export signal (NES) which are also present [15]. p62/SQSTM1 contains a LIR/LRS motif and a kelch-like ECH-associated protein 1 (KEAP1) interacting region (KIR) motif responsible for the interaction with LC3 and KEAP1, respectively [11, 16, 17]. The C-terminus of p62 harbors a ubiquitin-associated (UBA) domain required for its binding to mono- and polyubiquitin [4] (Figure 1(a)).

The *Drosophila* single p62 homologue, Ref(2)P (*refractory to Sigma P ref(2)P/CG10360*), has 599 amino acids and contains an N-terminal PB1 domain followed by a ZZ-type zinc finger domain and a C-terminal UBA domain (Figure 1(a)) [13, 18]. Although Ref(2)P has not been shown to be a selective autophagic substrate directly, several lines of evidence support this. First, it has been shown that Ref(2)P is a major component of protein aggregates in flies that are defective in autophagy, in flies that have impaired proteasomal function, in *Drosophila* models of human neurodegenerative diseases, and in protein aggregates formed during normal aging in *Drosophila* adult brain [18] (Figure 2). The abilities of Ref(2)P to oligo- and multimerize (through its PB1 domain) and to bind ubiquitinated proteins (through its UBA domain) were shown to be required during the *in vivo* formation of protein aggregates in the adult brain of *Drosophila* [18].

Second, bioinformatic analysis of the sequence of Ref(2)P reveals the presence of a putative LIR motif. The human p62 LIR motif is a 22 amino acid long sequence which contains an evolutionarily conserved motif of three acidic residues followed by a tryptophan (DDDW in p62) [4]. Johansen and Lamark implemented a sequence logo from 25 different LIR motifs from 21 different proteins that all have been tested for binding to ATG8 family proteins. They showed that the LIR motif seems to be eight amino acids long and proposed that the consensus LIR motif could be written as D/E-D/E-D/E-W/F/Y-X-X-L/I/V. It seems that there is a requirement for aromatic residues in the W-site (W/F/Y) and also a requirement for large, hydrophobic residues in the L-site (L/I/V) [4]. Bioinformatic analysis of Ref(2)P sequence reveals the presence of a putative LIR between amino acids 451–458 with a sequence DPEWQLID, which fits very well with the criteria for aromatic residues at W site (W) and hydrophobic residues at L site (I) (Figure 1(b)). Bioinformatic prediction also reveals the presence of a putative KIR motif spanning between the amino acids residues 484–496 (Figure 1(b)). The functional roles of putative LIR and KIR motifs of Ref(2)P have to be tested experimentally *in vitro* and *in vivo*. Taken together, the above information suggest that Ref(2)P is a selective autophagy cargo receptor in *Drosophila melanogaster*.

Ref(2)P was initially characterized in a screen for modifiers of sigma virus multiplication [19–21]. Sigma virus belongs to the family of rhabdoviruses which have two natural hosts, either insect and vertebrate or insect and plant [22]. Sigma virus is an atypical rhabdovirus, since there are no known plants or vertebrate hosts, and it only infects *Drosophila* [23]. Sigma virus is widespread in natural populations of *Drosophila*, and flies infected with the virus exhibit reduced viability of infected eggs and lower survival over winter [23–25]. ref(2)P is the best characterized locus among five host loci which are involved in the control of Sigma virus infection and multiplication, including ref(1)H, ref(2)P and ref(3)D [19, 26–28]. *Drosophila* flies in nature contain two types of alleles: the permissive alleles of ref(2)P which allow efficient sigma virus multiplication, and the restrictive alleles which reduce the replication of the virus [19, 23]. In flies having the permissive alleles, the probability of infection

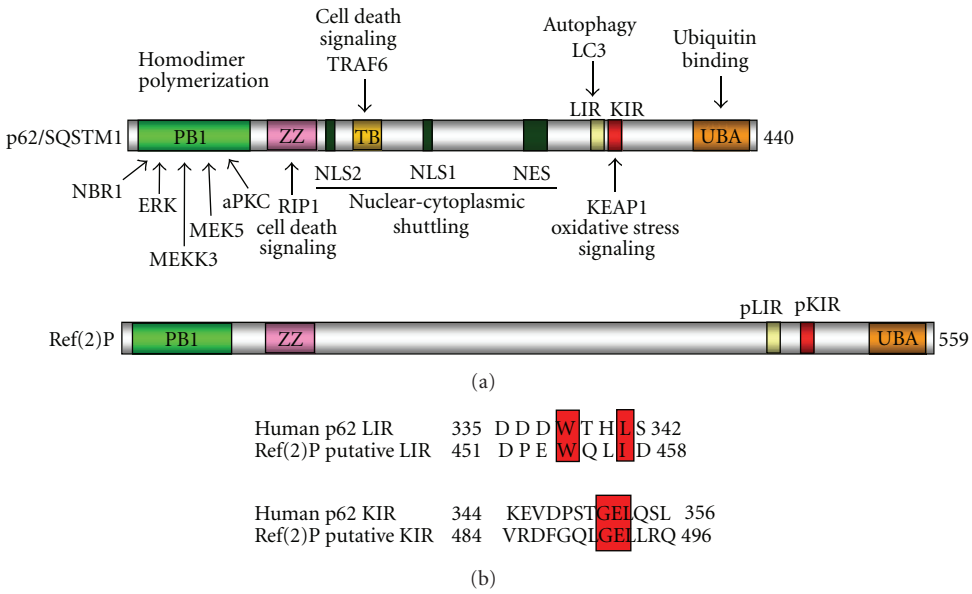

Figure 1: Schematic presentation of functional and structural domains of p62 and its *Drosophila* orthologue, Ref(2)P. (a) p62 consists of a PB1 domain (Phox and Bem1p domain) which is responsible for the interaction with the autophagy receptor NBR1 and the protein kinases ERK, MEKK3, MEK5, PKCζ, and PKCλ/ι. The PB1 domain is followed by a ZZ-type zinc finger domain which contains the binding site for RIP1 and a TB domain which harbors the binding site of TRAF6. Nuclear localization signals (NLSs) and nuclear export signal (NES) are also present. p62 contains a LIR (LC3-interacting region) and a KIR (KEAP1-interacting region) motif and a C-terminal UBA (ubiquitin associated) domain responsible for binding to ubiquitin. Ref(2)P has similar structural and functional domains compared to p62. It consists of a PB1 domain which is followed by a ZZ-type zinc finger domain and a C-terminal UBA domain responsible for binding to ubiquitin. Ref(2)P also contains putative LIR and KIR motifs. (b) Bioinformatic prediction of Ref(2)P's putative LIR and KIR motifs and alignment with human p62's motifs. The functional roles of putative LIR and KIR motifs of Ref(2)P have to be tested experimentally.

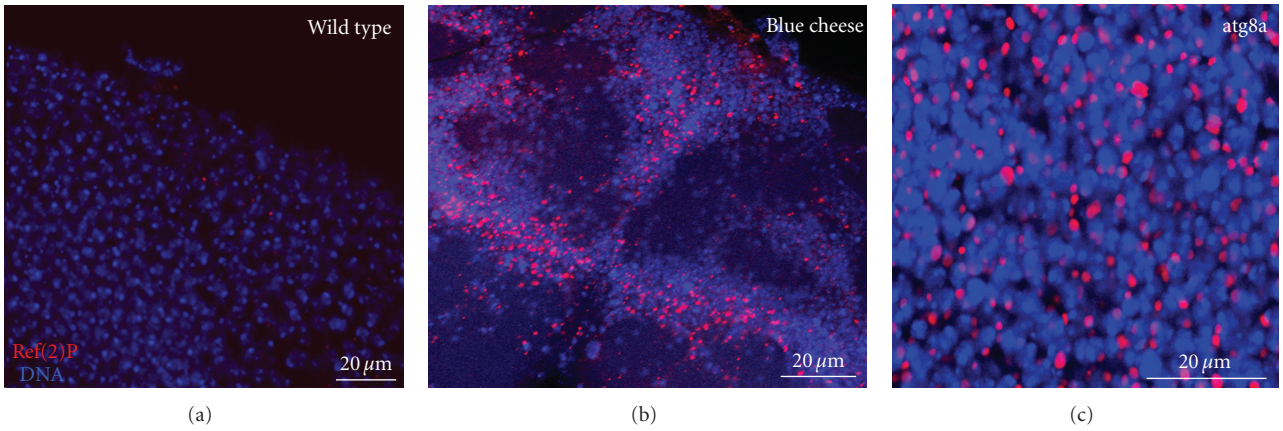

Figure 2: Ref(2)P accumulates in the adult brain of atg8a and blue cheese mutant flies. Confocal micrographs of superficial sections of the adult brain cortex of a wild-type fly (a), a blue cheese mutant fly (b), and an autophagy mutant fly (c). The tissues are stained for Ref(2)P (red) and DNA (blue). Ref(2)P accumulates ubiquitously into large sphere-shaped inclusion bodies/aggregates in blue cheese and autophagy mutants compared to wild type.

may reach 100%, whereas, in flies with restrictive alleles the infection rate drops to 0.01%, at least for some viral strains [23, 28]. It appears that the restrictive allele appeared several thousands of years ago and spread in the population as a result of natural selection since it confers a selective advantage [29]. The appearance of the sigma virus strain capable of infecting *Drosophila* flies carrying the restrictive ref(2)P alleles occurred much more recently (25 years ago) and rapidly spread in natural population across Europe [30]. Homozygous Ref(2)P null flies are fully viable but the males are sterile. The molecular mechanisms of male sterility are not clear [19, 20]. Electron microscopy studies revealed that in the testes of ref(2)P^{od1} and ref(2)P^{od3} loss-of-function mutants (where Ref(2)P protein lacks the UBA domain) and

ref(2)P^{od2} loss-of-function mutant (where Ref(2)P protein lacks the PB1 domain), characteristics of degeneration were frequently observed, such as the appearance of large myelin figures around the spermatids [20]. Additionally, the most striking difference was observed in the mitochondria, which varied in size and appeared degenerated [20]. Mammalian p62 has been shown to contribute to autophagic degradation of ubiquitinated mitochondria and to their clustering [31]. Therefore, it would be interesting to test this scenario in Ref(2)P mutant testis.

One open question is how Ref(2)P controls sigma virus multiplication at the molecular and cellular level. Work from Contamine's group suggests a direct interaction between Ref(2)P and a sigma virus protein, since Ref(2)P has been shown to interact with the sigma virus capsid P protein and to share conformation-dependent epitopes with the capsid N protein [32]. Additionally, Ref(2)P has been shown to interact genetically with DaPKC and the *Drosophila* homologue of TRAF6, dTRAF2, to participate in the Toll-signaling pathway, and to regulate the NF-κB proteins Dorsal and DIF [33, 34]. Interestingly, mammalian p62 was shown to interact with sindbis virus capsid protein, and genetic knockdown of p62 blocked the targeting of viral capsid to autophagosomes [35]. Taken together, these results suggest that Ref(2)P may target sigma virus capsid for autophagosomal degradation and also may function as a scaffolding protein during assembly of viral protein complexes. This scenario has to be tested experimentally. Intriguingly, Ref(2)P was shown to accumulate in rod-shaped structures in *Drosophila* egg chambers, structures that may represent aggregates of viruses or bacteria (Figure 3).

Another aspect of Ref(2)P function was recently reported in *Drosophila* hemocytes. Interestingly, Ref(2)P was shown to have a role in hemocyte spreading and protrusion formation [36]. This suggests that selective autophagy of an ubiquitinated substrate may function in an autophagy-dependent mechanism for cortical remodeling of hemocytes. Taken together, all the above information demonstrates that Ref(2)P, like its mammalian homologue p62, has diverse cellular functions whose molecular mechanisms have to be examined in detail.

2.1.2. Blue Cheese, the Drosophila homologue of the Mammalian Selective Aggregate Clearance Mediator Alfy. The mammalian phosphatidylinositol-3-phosphate-(PI3P-) binding protein Alfy was shown to be required for selective degradation of protein aggregates [8, 9, 37]. Alfy is a huge protein containing 3527 amino acids residues. It harbors several functional domains in the C terminus: a BEACH domain followed by a series of WD40 repeats and a PI(3)P-binding FYVE domain [8]. Despite its FYVE-domain which would suggest a localization to PI(3)P-rich endosomes, Alfy is not found on endosomes but instead localizes mainly to the nuclear envelope. Under conditions of starvation or proteasomal inhibition, Alfy relocalizes to cytoplasmic structures located close to autophagic membranes and ubiquitin-containing protein aggregates. Electron microscopy studies revealed that similar structures can be found within autophagosomes [8]. Importantly, Alfy was shown to be required for selective degradation of aggregated proteins such as polyQ-cotaining mutant huntingtin [9]. This function was proposed to be mediated by Alfy's physical interaction with PI(3)P, Atg5, and p62 [9, 37]. Therefore, Alfy functions as a scaffold receptor for recruitment of misfolded, ubiquitinated proteins to the autophagosomal membrane that become degraded by autophagy.

Blue cheese is the *Drosophila* homologue of Alfy and is highly conserved with its human homologue (~50% identity between fly and human homologs) [8, 38], and it contains similar functional domains at its C-terminal. *blue cheese* mutant flies exhibit a reduced adult life span and age-related neurodegeneration associated with accumulation of ubiquitin-conjugated protein aggregates throughout the adult central neruous system, neural atrophy, and cell death [38]. Ref(2)P accumulates in ubiquitinated inclusions in the brain of *blue cheese* mutant flies, suggesting that blue cheese is required for autophagic degradation of p62-associated ubiquitinated proteins *in vivo* [38] (Figure 2).

Finley and colleagues performed a genetic modifier screen for blue cheese genetic interactions based on alteration of the blue cheese eye phenotype. They found that recessive mutations in lysosomal trafficking genes and members of the ubiquitin and SUMO signaling pathways as well as in cytoskeletal and motor proteins have potential genetic interactions with Blue cheese [39]. They also showed that mutations of several lysosomal transport genes also alter high-molecular-weight UB-protein profiles and reduce adult life span [39]. Importantly, it was recently shown by Simonsen and Finley groups that overexpression of the C-terminal region of Blue cheese ameliorates neurodegeneation related phenotypes *in vivo* [9]. The authors tested the enhanced expression of Blue cheese in *Drosophila* eye model of polyglutamine toxicity, where UAS-polyQ127 transgene was expressed in the fly eye. It is well established that poly Q expression in the eye results in ommatidial disorganization, pigmentation loss, reduced eye size, and the appearance of necrotic regions. Enhanced expression of full-length Blue cheese (UAS-FL-Bchs) or C-terminal Blue cheese (UAS-bchs-C1000) with UAS-polyQ127 in the eye resulted in reduced number of necrotic areas and an overall improvement in eye size, morphology, and pigmentation. Taken together, these results suggest that the Alfy/Bchs proteins have a role in macroautophagic clearance of aggregation-prone proteins.

2.2. Mitophagy, Xenophagy, and Nucleophagy in Drosophila. Selective autophagy was recently shown to play an important role in the quality control of organelles and intracellular pathogens [31, 40]. However, mitophagy (selective autophagy of mitochondria), xenophagy (selective autophagy of bacteria and viruses), and nucleophagy (selective autophagy of nuclear fragments) are largely unexplored in *Drosophila*. Moreover, pexophagy (selective autophagy of peroxisomes) is not described yet in *Drosophila*. In the following lines, I will summarize what is reported so far in the literature about the processes above in *Drosophila*.

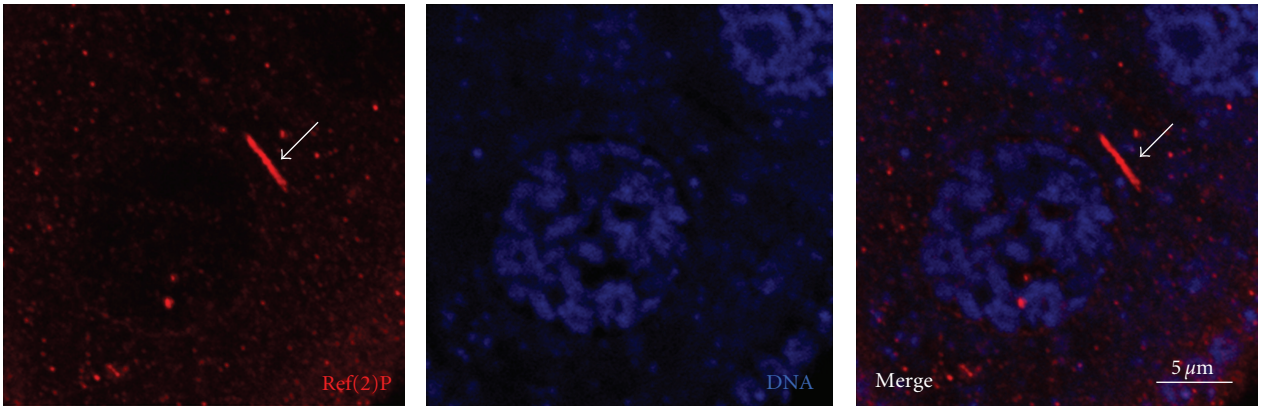

Figure 3: Ref(2)P localization in *Drosophila* egg chamber. Confocal micrograph of a middle section of a stage 8 egg chamber of wild-type fly, illustrating a portion of a nurse cell. The tissue is stained for Ref(2)P (red) and DNA (blue). Note the rod-like structure stained for Ref(2)P (arrow).

2.2.1. Mitophagy. Mitophagy has been recently described in yeast and mammals [31]. In yeast, the outer mitochondrial membrane protein Atg32 binds to the autophagosomal membrane protein Atg8 through its LIR motif [41]. In mammals, mitophagy was described during the physiological process of red blood cell differentiation and it requires the outer mitochondrial membrane protein NIP3-like protein NIX, which is also binds to LC3 through its LIR motif [42, 43]. Additionally, when mitochondria are damaged and depolarized, the kinase PTEN-induced putative kinase protein 1 (PINK1) accumulates to mitochondria and recruits the E3 ubiquitin ligase Parkin from the cytoplasm specifically to the damaged mitochondria. Subsequently, Parkin ubiquitylates mitochondrial proteins and promotes mitochondrial degradation by autophagy [31].

Genetic studies in *Drosophila* showed that the PINK1-Parkin pathway promotes mitochondrial fission or alternatively inhibit their fusion [44, 45]. It was recently shown in S2 cells that *Drosophila* PINK1 localizes to depolarized mitochondria and recruits Parkin and this promotes mitochondria degradation by autophagy [46]. Importantly, the profusion factor mitofusin (Mfn; also known as marf in *Drosophila*) was shown to be a novel substrate of Parkin [46]. Interestingly, it was also reported that activation of autophagy through Atg1 overexpression rescues PINK1 mutant phenotypes in *Drosophila* [47]. These studies suggest that, like in mammals, mitophagy also occurs in *Drosophila* and is dependent on PINK1 and Parkin, although the molecular details have to be further clarified.

Finally, it was recently reported that mitochondrial dynamics are abnormal in autophagy deficient egg chamber [48]. Dying atg1 germline mutant egg chambers exhibit abnormal mitochondrial remodeling that included the presence of mitochondrial islands suggesting that there is a cross-talk between autophagy, mitochondrial dynamics, and cell death during *Drosophila* oogenesis [48].

2.2.2. Xenophagy. Autophagy has been associated with the elimination of intracellular pathogens during mammalian innate immune responses, a process called xenophagy [40]. In *Drosophila,* xenophagy is largely unexplored. There are two reports that provide evidence for conserved mechanisms of xenophagy in *Drosophila*. In the first one, Kurata and colleagues reported that, in primary *Drosophila* hemocytes and S2 cells, autophagy prevented the intracellular growth of *Listeria monocytogenes* and promoted host survival after this infection [49]. Additionally, recognition of diaminopimelic acid-type peptidoglycan by the pattern-recognition receptor PGRP-LE was required for the induction of autophagy. Importantly, autophagy induction occurred independently of the Toll and IMD innate-signaling pathways [49].

In a second study, it was found that autophagy implements an antiviral role against the mammalian viral pathogen vesicular stomatitis virus (VSV) in *Drosophila* S2 cells as well as in adult flies [50]. The surface glycoprotein of VSV, VSVG, was shown to be the pathogen-associated molecular pattern that initiates the autophagic response. Autophagy was shown to restrain viral replication, and repression of autophagy resulted in increased viral replication and pathogenesis. Importantly, it was shown that this response was regulated by the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway which controls autophagy in response to nutrient availability [50]. These data suggest that xenophagy occurs in *Drosophila,* and the molecular mechanisms are well conserved compared to mammals.

2.2.3. Nucleophagy. Nucleophagy is the process where parts of the nucleus can be specifically degraded by autophagy [51]. Nucleophagy is best characterized in yeast *Saccharomyces cerevisiae*, and is called piecemeal microautophagy [52]. During piecemeal microautophagy of the nucleus there is formation of nucleus-vacuole junctions where parts of the nucleus are sequestered into invaginations of the vacuolar membrane, followed by fission of nuclear fragments, and its release into the vacuolar lumen, where they are degraded. A direct interaction of the nuclear membrane protein Nvj1p with that vacuole protein Vac8p of the vacuole are required for this process [51, 52]. Recently, nucleophagy was also

reported in mammals in nuclear envelopathies caused by mutations in the genes encoding A-type lamins (LMNA) and emerin (EMD) [53]. Nucleophagy was also observed rarely in wild-type cells [53].

In *Drosophila,* nuclear autophagy has been recently described during the cell death of nurse cells in late oogenesis [54]. Immunofluorescence analysis of mCherry-DrAtg8a autophagy marker in the nurse cells during the late stages of oogenesis revealed the presence of large autolysosomes adjacent to or attached to the condensed and fragmented nurse cell nucleus. Ultrastructural analysis revealed the presence of large autolysosomes which contained condensed material resembling the material of the fragmented nurse cell nucleus, suggesting that the nurse cell nuclear fragments are removed by autophagy [54].

2.3. Selective Degradation of Proteins in Drosophila. Autophagy has been shown to be responsible for the selective degradation of proteins in mammals and yeast like beta-synuclein [55], catalase [56], and acetaldehyde dehydrogenase [57]. In *Drosophila,* there is also a growing number of cases in which proteins can be preferentially degraded by autophagy.

2.3.1. Degradation of Survival Factors. Degradation of survival factors is a way of cell to die [58]. There are two recent reports that support this hypothesis in *Drosophila*. In the first study, we have demonstrated that the inhibitor of apoptosis protein dBruce was degraded by autophagy in the nurse cells during cell death in late oogenesis [54]. Genetic inhibition of autophagy in the female germline resulted in late stage egg chambers containing persistent nurse cell nuclei that did not contain fragmented DNA and in attenuation of caspase-3 activation. Importantly, we found that *Drosophila* inhibitor of apoptosis dBruce is degraded by autophagy, and this is responsible to control DNA fragmentation [54]. A second report showed that degradation of inhibitor of apoptosis protein DIAP1 during developmental dendrite pruning of *Drosophila* class IV dendritic arborization neurons is depended on Valosin-containing protein (VCP), a ubiquitin-selective AAA chaperone involved in endoplasmic reticulum-associated degradation and the maturation of autophagosomes [59, 60]. These results suggest that autophagic degradation of survival factors can cause cell death during development in *Drosophila*.

2.3.2. Degradation of Rhodopsin and Retinal Degeneration. Activated rhodopsin is degraded in endosomal pathways in normal photoreceptor cells in *Drosophila,* and accumulation of activated rhodopsin in some *Drosophila* mutants leads to retinal degeneration [61]. In a recent study, it was reported that activated rhodopsin is degraded by autophagy in order to prevent retinal degeneration [62]. Light-dependent retinal degeneration in the *Drosophila* eye is caused by silencing or mutation of autophagy genes, such as autophagy-related protein 7 and 8, or genes essential for PE (phosphatidylethanolamine) biogenesis and autophagosome formation, including phosphatidylserine decarboxylase (Psd) and CDP-ethanolamine:diacylglycerol ethanolaminephosphotransferase (Ept). Silencing of atg-7/8 or Psd/Ept resulted in an increase in the amount of rhodopsin localized to Rab7-positive late endosomes [62]. These results suggest that autophagic and endosomal/lysosomal pathways suppress light-dependent retinal degeneration and that rhodopsin is a substrate for autophagic degradation in this context.

2.3.3. Degradation of Highwire. Beyond its role in cellular homeostasis, autophagy is implicated in the regulation of developmental growth and remodeling of various cells and tissues during development [63]. One such example in *Drosophila* is the synaptic development of the larval neuromuscular junction. Shen and Ganetzky showed that autophagy promotes the synaptic development of the *Drosophila* larval neuromuscular junction, by downregulating an E3 ubiquitin ligase, Highwire, which restrains neuromuscular junction growth via a MAPKKK pathway [64, 65]. Autophagy mutants exhibit neuromuscular junction undergrowth and Atg1 overexpression, resulting in neuromuscular junction overgrowth. Moreover, overgrowth associated with Atg1 overexpression is suppressed by mutations in *atg18*, demonstrating that this overgrowth is due to elevated levels of autophagy [64, 65]. In a recent paper, *Drosophila* Rae1 was identified as a component of the Highwire complex. Loss of *Rae1* function in neurons results in morphological defects at the neuromuscular junction that are similar to those seen in Highwire mutants [66]. The authors found that Rae1 physically and genetically interacts with Highwire and limits synaptic terminal growth by regulating the MAP kinase kinase kinase Wallenda. Moreover, they found that the Rae1 is sufficient to promote Highwire protein abundance by binding to Highwire and protecting it from autophagic degradation [66]. Together, these findings indicate that Rae1 prevents autophagy-mediated degradation of Highwire and that selectively controls Highwire protein abundance during synaptic development.

3. Concluding Remarks and Future Directions

From the literature analyzed above, it is obvious that the molecular mechanisms of selective autophagy in *Drosophila* remain largely unexplored. The precise mechanisms of selective autophagy of organelles and proteins has not been directly shown in *Drosophila*, and the molecular details of the interaction of selective autophagy receptors Ref(2)P and blue cheese with the autophagic machinery have to be shown experimentally. The presence of putative LIR motif in Ref(2)P offers a fertile ground for further functional analysis *in vivo*. p62 and Ref(2)P have been proposed to collect ubiquitinated proteins and target them for degradation [4]. It would therefore be interesting to test whether induced expression of Ref(2)P ameliorates phenotypes related to neurodegeneration *in vivo*. It will also be important to elucidate in details how small or large aggregates are removed per se. Elucidation of these processes may have applications in fighting aggregation-related diseases, such as neurodegenerative diseases as well as cancer. There is emerging

evidence that mammalian p62 directly interacts with Keap1 and that p62 is a target gene for Nrf2 transcription factor implicated in oxidative stress signaling [4, 13]. It would be interesting to test the interaction of Ref(2)P with the *Drosophila* homologue of Keap1, dkeap1 [67]. It would also be interesting to test whether the *Drosophila* homologues of BNIP3-like proteins play a role in selective degradation of mitochondria.

In conclusion, *Drosophila* offers a fertile ground for studying the molecular mechanisms of selective autophagy. Future studies will hopefully uncover the molecular details of this process.

Acknowledgments

I. P. Nezis acknowledges support from the European Research Council. I. P. Nezis would like to thank the anonymous reviewers for their helpful comments on the improvement of the manuscript and Dr. Kim Finley for providing the blue cheese mutant flies.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 396705, 7 pages
doi:10.1155/2012/396705

Review Article

Zymophagy: Selective Autophagy of Secretory Granules

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Received 1 December 2011; Revised 6 February 2012; Accepted 6 February 2012

Academic Editor: Fulvio Reggiori

Timing is everything. That's especially true when it comes to the activation of enzymes created by the pancreas to break down food. Pancreatic enzymes are packed in secretory granules as precursor molecules called zymogens. In physiological conditions, those zymogens are activated only when they reach the gut, where they get to work releasing and distributing nutrients that we need to survive. If this process fails and the enzymes are prematurely activated within the pancreatic cell, before they are released from the gland, they break down the pancreas itself causing acute pancreatitis. This is a painful disease that ranges from a mild and autolimited process to a severe and lethal condition. Recently, we demonstrated that the pancreatic acinar cell is able to switch on a refined mechanism that could explain the autolimited form of the disease. This is a novel selective form of autophagy named zymophagy, a cellular process to specifically detect and degrade secretory granules containing activated enzymes before they can digest the organ. In this work, we revise the molecules and mechanisms that mediate zymophagy, a selective autophagy of secretory granules.

1. Introduction

Autophagy is an evolutionarily preserved cellular process that is responsible for the degradation of long-lived proteins and entire organelles to maintain intracellular homeostasis and to contribute to starvation and stress responses. Macroautophagy involves the formation of double-membrane autophagosomes around cargoes, including larger structures such as organelles and protein aggregates. Autophagosomes then fuse with lysosomes, where the degradation of the cargoes takes place. Both nonselective *bulk* autophagy and selective autophagy of specific proteins and organelles have been described [1]. Genetic analyses in yeast identified more than 30 conserved components that are required for different steps of autophagy (termed *Atg* genes) [2]. Recently, several lines of evidence suggest the existence of selective autophagic degradation pathways in physiology and disease, named, selective autophagy [3]. During selective autophagy, single cellular structures, such as protein aggregates and mitochondria are specifically sequestered by autophagosomes.

There is emerging evidence suggesting the involvement of ubiquitin in several forms of selective autophagy process. For example, aggregate clearance by autophagy requires ubiquitylation and ubiquitin-binding receptors such as p62 (also known as SQSTM1) [4]. Ubiquitylated artificial substrates are recognized by the autophagy machinery and are specifically degraded in lysosomes by a p62-dependent mechanism [5]. Moreover, the selective degradation of excess ribosomes during starvation depends on the deubiquitylation activity of Ubp3/Bre5 [6]. The repertoire of proteins that participate in these ubiquitin-mediated pathways during different types of autophagy is an area of intensive investigation, particularly the elucidation of the role of this specific cellular program in pathophysiological processes and complex diseases such as pancreatitis.

Zymophagy is a novel selective form of autophagy that works as a protective cell response to disease [7]. This new selective autophagic pathway is activated in pancreatic acinar cells during pancreatitis-induced vesicular transport alteration, in order to sequester and degrade potentially deleterious activated zymogen granules. In this work, we revise the molecules and mechanisms that mediate *zymophagy*, a self-eating event that protects the pancreas from self-digestion.

2. The Disease

2.1. Acute Pancreatitis. Acute pancreatitis, defined as the pancreas self-digestion, is the most frequent disease of the pancreas. During pancreatitis, ultrastructural alterations of zymogen granules are produced in a yet undefined way. These alterations are characterized by premature activation of trypsinogen to trypsin within pancreatic acinar cells leading to the progression of the disease. Most of the cases are mild acute pancreatitis resulting in a self-limiting disease, but up to 25% of the patients suffers a severe attack and around 30% of these will die [8]. The pathophysiology involves a complex cascade of events initializing in pancreatic acinar cells. An unknown trigger within the pancreas leads to conversion of digestive proenzymes (zymogens) into their active form (digestive enzymes), initiating autodigestion of the gland causing hemorrhage, necrosis, edema, and complete destruction of pancreatic parenchyma [9]. Chiari [10], more than a century ago, proposed that autodigestion by prematurely activated digestive enzymes is responsible for the onset of this debilitating disease. Although significant research effort has been expended on mechanisms responsible for this premature zymogen activation, many aspects of the pathogenesis of acute pancreatitis remain enigmatic (see [11] for a comprehensive review). The presence of autophagy has been described in dying acinar cells from human pancreatitis. Helin et al. in 1980 described the ultrastructural alterations in pancreatic acini from patients operated for acute necrotizing pancreatitis. They studied by electronic microscopy those areas of pancreatic parenchyma that show edematous inflammation under light microscopy. Their findings in acinar cells included changes in zymogen granules and an increased autophagocytosis indicated by several autophagic vesicles [12].

Cholecystokinin (CCK) is a pancreatic secretagogue that interacts with Gq-coupled receptors in the acinar cell to induce pancreatic secretion in physiological conditions. On the other hand, the hyperstimulation of CCK receptors (CCK-R) with the analogue cerulein that modifies vesicular transport leads to intracellular proteolytic enzyme activation and ultimately cell death [13]. These cellular events are characteristic of human acute pancreatitis. Moreover, autophagic morphological features were also described in cerulein-induced pancreatitis [14, 15]. Further, the secretagogue-induced model is the most commonly employed and best characterized experimental model of acute pancreatitis [16]. Interestingly, early during this experimental model, CCK-R hyperstimulation activates the selective autophagic degradation of secretory granules in the pancreatic acinar cell [7, 17].

3. The Cells

3.1. The Pancreatic Acinar Cells. The acinar cell of the exocrine pancreas produces and secretes a wide variety of potent proteolytic enzymes essential for intestinal digestion of nutrient proteins. However, these digestive enzymes are potentially harmful. Therefore, these proteases are produced as precursors (zymogens) within pancreatic acinar cells and are only activated in the duodenum. The key step in this activation process is the conversion of inactive trypsinogen to active trypsin by limited proteolysis by enteropeptidase, a highly selective trypsinogen-cleaving protease located at the luminal site of duodenal cells. The active trypsin initiates an activation cascade of digestive enzymes within the duodenum, thereby ensuring the high proteolytic capacity needed for food digestion. Under pathological conditions that cause pancreatitis, digestive zymogens undergo premature activation within the pancreatic acinar cell. High levels of activation overcome pancreas protective mechanisms, causing cell injury and acute pancreatitis [16]. However, most of the cases of acute pancreatitis are mild and autolimited. Therefore, the acinar cell may respond to the pathogenic event activating phenotypically changes that may start defense mechanisms and explain the autolimited form of the disease [17, 18].

4. The Organelles

4.1. Pathologically Activated Zymogen Granules. The pancreatic acinar cell is a highly polarized, differentiated cell whose primary function is the synthesis and secretion of digestive enzymes into the pancreatic juice. Pancreatic digestive enzymes are produced as inactive enzymes (zymogens) and stored in subcellular structures called zymogen granules, until exocytosis [16]. Zymogen granules are potentially harmful secretory granules because once activated, they release the digestive enzymes within the cell causing cell death. Thus, they are able to hydrolyze tissue parenchyma and eventually trigger a severe disease. The exact mechanisms of zymogen activation and the complete characterization of the activation compartments remain unclear [19]. However, these pathologically altered organelles containing activated zymogens may be recognized and degraded by a selective autophagic pathway that we named zymophagy [7].

5. The Molecules

5.1. VMP1. In the search for new molecules that are differentially expressed during acute pancreatitis we found a transmembrane protein that we named Vacuole Membrane Protein 1 (VMP1) [20]. The *in vivo* gen expression of VMP1 in pancreas with pancreatitis correlates with morphological features resembling autophagy [21]. VMP1 is a transmembrane protein highly activated in acinar cells early during pancreatitis-induced autophagy, and it remains in the autophagosomal membrane. We have shown that VMP1 expression is able to trigger autophagy in mammalian cells, even under nutrient-replete conditions [17]. Most of the autophagy-related proteins were described in yeast or have a yeast homologue. VMP1 does not have any known homologue in yeast, but its expression is required to start the autophagic process in mammalian cells [22]. VMP1 overexpression in mammalian cells induces the formation of numerous vesicles with ultrastructure of autophagosomes and they immunostained with LC3, the widely used marker of autophagosomes. Moreover, VMP1 expression promotes the conversion of LC3-I to LC3-II. VMP1 expression in several mammalian cell lines

induces recruitment of LC3 in punctate structures, and that recruitment is inhibited when VMP1-expressing cells are treated with the autophagy inhibitor 3-methyladenine (3-MA). VMP1 endogenous expression is induced by autophagy stimuli, and its expression is required for autophagosome development. VMP1 interacts with Beclin 1 through its hydrophilic C-terminal region, the Atg domain that is essential for autophagy [17]. Recently, Tian et al. identified EPG-3/VMP1 as one of three essential autophagy genes conserved from worms to mammals, which regulates early steps of the autophagic pathway in *C. elegans* [23]. Hierarchical analyses in mammalian cells by Itakura and Mizushima [24] show that VMP1 along with ULK1 and Atg14 localize in the ER-associated autophagosome formation sites in a PI3-kinase activity-independent manner, confirming the key role of VMP1 in the formation of autophagosomes. VMP1 expression is induced by extracellular stimuli of autophagy. For instance, rapamycin, the pharmacological inhibitor of mTOR and a well-established inductor of autophagy, is able to activate VMP1 expression, and VMP1-immunostained vesicles appear in rapamycin-treated cells [17]. Other extracellular stimuli such as gemcitabine in pancreatic cancer cells [25] and streptozotocine in pancreatic beta cells [26] induce VMP1 expression and VMP1-mediated autophagy. VMP1 autophagic vesicles are present in the pancreas of rats submitted to experimental pancreatitis, showing that VMP1 is involved in the induction of autophagy during the disease. Considering that autophagy is implicated in several pathological mechanisms operating in human diseases, the activation of the VMP1 pathway may regulate potential pathophysiological processes involved in the cell response to disease. We developed the ElaI-VMP1 mouse in which acinar cell-specific constitutive expression of a VMP1-EGFP chimera induces the formation of autophagosomes within the acinar cell [17]. Thus, the ability of VMP1 expression to induce autophagy is validated through the development of this pancreas-specific transgenic mouse. In the adult normal pancreas, autophagy and VMP1 are not detected; in contrast, in VMP1-transgenic mice, multiple VMP1 and LC3 coimmunostained autophagic structures are present in pancreas cells. We use this unique tool to investigate the VMP1 pathway in autophagy during acute pancreatitis [7, 17].

5.2. p62/SQSTM1. Dictyostelium cells lacking Vmp1 gene show accumulation of huge ubiquitin-positive protein aggregates containing the autophagy marker Atg8/LC3 and p62 homologue [27]. The polyubiquitin-binding protein p62/SQSTM1 is degraded by autophagy. It is found in cellular inclusion bodies together with polyubiquitinated proteins and in cytosolic protein aggregates that accumulate in various chronic, toxic, and degenerative diseases. It has been shown a direct interaction between p62 and the autophagic effector proteins LC3A and -B and the related γ-aminobutyrate receptor-associated protein and γ-aminobutyrate receptor-associated-like proteins. The binding is mediated by a 22-residue sequence of p62 containing an evolutionarily conserved motif. The specific interaction between p62 and LC3 is instrumental in mediating autophagic degradation of the p62-positive bodies and p62 is required both for the formation and the degradation of polyubiquitin-containing bodies by autophagy [4].

5.3. USP9x. Ubiquitination is a covalent posttranslational modification of cellular proteins involving a complex enzymatic cascade. Emerging evidence suggests that many enzymes of the ubiquitination cascade are differentially expressed or activated in several diseases, including cancer and may therefore be appropriate therapeutic targets. Protein ubiquitination is a dynamic two-way process that can be reversed or regulated by deubiquitinating enzymes (DUB). The human genome codes for hundred proteins with putative DUB activity [28], which can be broadly divided into two subgroups: ubiquitin COOH-terminal hydrolase (UCH) and the ubiquitin-specific proteases (USP) [29]. USPs comprise the largest subclass of DUBs in humans, whereas only four known UCH DUBs have been described [30]. The USP9x gene is a member of the peptidase C19 family and encodes for an ubiquitin-specific protease. Ubiquitinating and deubiquitinating enzymes have emerged as key players in the regulation of membrane trafficking in organisms ranging from yeasts to mammals [31] (Figure 1).

6. The Selective Autophagic Pathway

During acute pancreatitis, the acinar cell activates VMP1-mediated autophagy. The immunomagnetic isolation of VMP1-autophagosomes containing zymogen granules from the EGFP-VMP1 transgenic mouse pancreas with acute pancreatitis allows the discovery of a new type of selective autophagy named zymophagy, which functions as an inducible cellular process that recognizes and degrades activated zymogen granules [7].

Zymophagy is characterized by the formation of autophagosomes containing zymogen granules. These organelles mediate the sequestration and degradation of pancreatitis-activated zymogen granules and are induced by secretagogues and probably other stimuli in acinar cells. Electron microscopy and immunofluorescence assays in human, mouse, and cultured pancreatic acinar cells show autophagic organelles at different maturation levels, suggesting that the autophagic flow progresses to acquire autolysosomal features and degrades the disease-altered secretory granules. CCK-R hyperstimulation in wild-type animals induced a markedly altered distribution pattern of the secretory granules such as fusion among zymogen granules as well as their fusion with condensing vacuoles. In addition, acinar cells lose their polarity, which results in the relocation of zymogen granules to the basolateral membrane. All these alterations in vesicular traffic are known to occur in acinar cells during acute pancreatitis and upon hyperstimulation of their CCK-R with cerulein. Surprisingly, ElaI-VMP1 mice subjected to CCK-R hyperstimulation reveal that acinar cells preserve their structure and polarity with negligible or no alteration in vesicular transport. Instead, pancreases from cerulein-treated ElaI-VMP1 mice present autophagosomes containing zymogen granules displaying a distinct localization to the apical area of

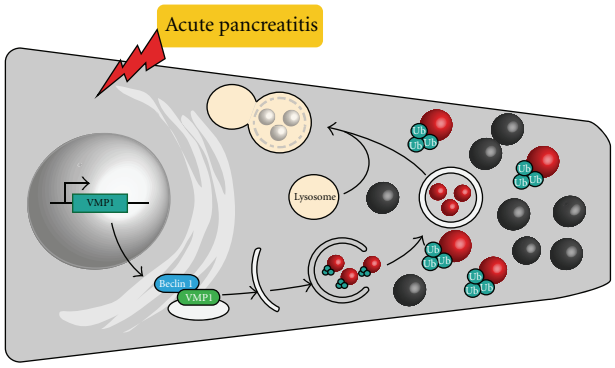

Figure 1: Upon acute pancreatitis, the acinar cell activates Vmp1 gene expression and triggers the autophagic degradation of those zymogen granules activated during the disease (in red) avowing the intracellular spreading of activated digestive enzymes. Altered zymogen granules are recognized by ubiquitination. Zymophagy is a novel inducible and selective form of autophagy, mediated by VMP1, which functionally links the autophagy pathway with the ubiquitin machinery to trigger a protective response to disease.

the acinar cell. These autophagosomes, containing secretory granules, were easily identifiable in apical regions as round high-density structures within double-membrane vesicles. After a systematic observation, we did not find evidence of other subcellular structures, such as ribosomes or mitochondria, within these autophagosomes. The observation of different maturation levels of selective autophagic vesicles as well as the degradation of p62 provides evidence that autophagic flow remains primarily unchanged under CCK-R hyperstimulation. Interestingly, similar to the case of *in vivo* autophagosomes, *in vitro* CCK-R hyperstimulation of ElaI-VMP1 mouse isolated acini induces a subcellular change in VMP1-containing autophagosomes to the zymogen granule-rich area (apical pole), therefore, in acinar cells from the ElaI-VMP1 mice, CCK-R hyperstimulation induces a progressive flow of autophagic vesicles containing zymogen granules that accumulate at the apical pole of acinar cells [7].

The distinctive characteristic of VMP1 as an autophagosome transmembrane protein allows the isolation of autophagosomes from pancreas tissue of treated and untreated ElaI-VMP1 mice. Autophagosomes containing zymogen granules are magnetically immunopurified from the pancreas of ElaI-VMP1 mice treated with cerulein, whereas in untreated animals, either empty or cytoplasm-containing autophagosomes are purified. In untreated animals, less than 20% of the autophagosomes contains zymogen granules, whereas in the cerulein treated ones, this percentage increases up to 70%. LC3-II is present in autophagosomal fractions from both cerulein-treated and untreated specimens. Notably, strong signals of p62 and trypsinogen are found only in magnetically immunopurified autophagosomes from cerulein-treated ElaI-VMP1 mice. This finding suggests that p62, which is an ubiquitin-binding protein that interacts with LC3 [4], may function as a cargo receptor during the selective autophagic pathway. Therefore, a nonselective autophagy pathway, which involves LC3-II but not p62, is triggered by VMP1 expression in untreated mouse pancreases. On the other hand, upon CCK-R hyperstimulation, p62 is involved in VMP1-mediated selective autophagy of zymogen granules. The description of zymophagy positions VMP1 and p62 in the same selective autophagy pathway. Immunofluorescence assays and western blot analyses of proteins from isolated selective autophagosomes demonstrate that these proteins colocalize and coexist in this autophagic organelle suggesting that p62 may act as cargo receptor during VMP1-selective autophagic pathway [7].

Zymophagy selectively degrades activated zymogen granules. The intracellular activation of trypsinogen induced by hyperstimulation of CCK-R in isolated murine pancreatic acini can be detected using rhodamine 110 bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride (BZiPAR), a cell permeable substrate that becomes fluorescent after the cleavage by the protease [32]. Upon CCK-R hyperstimulation, acinar cells from wild-type mice show early cytoplasmic trypsinogen activation, which is a hallmark of pancreatitis pathophysiology. Surprisingly, in acinar cells from ElaI-VMP1 mice, CCK hyperstimulation causes almost no activation of trypsinogen. Microscopic examinations reveal only few activated granules that highly colocalize with the VMP1-EGFP fluorescent signal showing that zymophagy selectively sequester the activated zymogen granules. Zymogen activation is an enzymatic chain reaction where initial zymogen granule alterations trigger rapid spread of active trypsin within the acinar cell. We think that the degradation of early-activated zymogen granules by zymophagy prevents this deleterious event. Interestingly, the inhibition of autophagic flow markedly increased trypsin activity within acinar cells in ElaI-VMP1 mouse pancreases under CCK-R hyperstimulation, confirming that zymophagy specifically degrades those zymogen granules that are initially activated by acute pancreatitis. This function of zymophagy is evident in the *in vivo* animal model of acute pancreatitis, where the hability of the ElaI-VMP1 mouse developing zymophagy clearly prevents the increment of enzymatic markers of pancreatic damage and pancreas morphological changes characteristic of acute pancreatitis.

Zymophagy specifically recognizes the pathologically activated zymogen granules. During zymophagy, the ubiquitin system serves as a targeting signal for zymogen granules. Proteins involved in the recognition of target for autophagy

are ubiquitin-binding proteins, such as p62 that binds directly to ubiquitin and LC3. Magnetically purified VMP1-mediated autophagosomes upon CCK-R hyperstimulation contain p62 and LC3 proteins and high colocalization of ubiquitin with amylase and with trypsinogen occurs early after CCK-R hyperstimulation. Moreover, the recruitment of ubiquitin with LC3, and the ubiquitin signal within LC3 decorated vesicles along with the colocalization between VMP1 and ubiquitinated granules, and the engulfment of ubiquitinated granules by VMP1-vesicles, demonstrate the selective sequestering of ubiquitinated zymogen granules by zymophagy [7]. More important, the selectively sequestered ubiquitinated granules are those activated by CCK-R hyperstimulation. GFP-Ub-transfected acinar cells subjected to CCK-R hyperstimulation show colocalization between activated granules and ubiquitin aggregates but do not show colocalization with unaffected or normal zymogen granules, indicating that the ubiquitin system serves as a targeting signal for activated zymogen granules during zymophagy. Therefore, activated zymogen granules are directly or indirectly ubiquitinated for their recognition by autophagic membranes, in which ubiquitin acts as a label for selective engulfment. This label might be subsequently removed for completing the formation of the autophagosome or even before this engulfment step. Nevertheless, activated zymogen granules are ubiquitinated upon acute pancreatitis and the VMP1-mediated selective autophagic pathway sequesters these ubiquitinated granules [7].

Notably, the ubiquitin-specific protease USP9x is showed to be an essential component of the machinery required to selectively degraded zymogen granules during zymophagy [7]. USP9x expression is highly induced in pancreatic acinar cells under CCK-R hyperstimulation. Also, USP9x is required to promote the selective degradation of altered zymogen granules. Interestingly, VMP1 interacts with USP9x early during zymophagy, supporting a direct functional role for this ubiquitin-specific protease in this selective autophagic pathway. Furthermore, downregulation of USP9x abolished activated zymogen granule degradation in acinar cells under CCK-R hyperstimulation, confirming the essential role of VMP1-USP9x interaction for zymophagy. USP9x is likely to modulate zymogen granule selective engulfment during acute pancreatitis by modulating VMP1 or providing a preferential recognition signal for altered zymogen granules. This data demonstrates for the first time that ubiquitin modifications may possess an additional function in acinar cells by promoting the degradation of highly harmful activated zymogen granules and strongly support the idea that there is a close cooperation between the autophagy pathway and the ubiquitin machinery required for selective autophagy. Alternatively, both ubiquitination and deubiquitination of distinct critical molecules might be required for selective autophagy. Thus, due to the potential importance of this type of regulation, these findings may fuel future investigations aimed to identify the potential E3 ligases and ubiquitinated substrate(s) required for zymophagy.

Zymophagy prevents pancreatic acinar cell death induced by CCK-R hyperstimulation [7]. Autophagosome formation inhibition with 3-methyl adenine as well as autophagy flux interruption with vinblastine significantly reduces acinar cell survival in a cell model of acute pancreatitis. Moreover, VMP1 downregulation (shVMP1) also significantly decreased acinar cell survival under CCK hyperstimulation showing that VMP1 expression is required to prevent acinar cell death in acute pancreatitis. These results indicate that zymophagy prevents pancreatic cell death induced by the activation of zymogen granules [33] and confirm that endogenous VMP1 expression is activated in acinar cells to mediate zymophagy as a protective cellular response against cell death.

Furthermore, VMP1 expression and zymophagy are present in human pancreas affected by acute pancreatitis [7]. VMP1 is not detectable in human normal pancreas tissue, but its expression is activated in human pancreatitis pancreas specimens and highly colocalizes with LC3 in autophagosomes. Moreover, autophagosomes markedly colocalize with zymogen granules. Remarkable, the finding of large autolysosomes without trypsin signal in human pancreatitis pancreas supports the experimental data and suggests that affected zymogen granules are eventually degraded by zymophagy during human pancreatitis. Results collectively demonstrate a previously unrecognized function for VMP1, mediating zymophagy, a novel selective form of autophagy, which functionally links the autophagy pathway with the ubiquitin machinery to trigger a protective cell response to disease.

7. The Function

The description of zymophagy provides further understanding of the autophagy molecular mechanisms relevant to human diseases, particularly acute pancreatitis. This condition has classically been considered an autodigestive disorder where the inappropriate activation of trypsinogen within the pancreatic acinar cell leads to the progression of the disease. The exact mechanism for the initiation of zymogen granule activation remains a subject of intensive investigation and debate [11]. Similarly, the role of autophagy in pancreatitis is seemingly incomplete, sometimes contradictory and requires further investigations. For instance, Hashimoto et al. [34] propose that autophagy is responsible for the zymogen activation within the acinar cell in acute pancreatitis. This suggestion is mainly based on the finding that deletion of the Atg5 gene in mouse pancreatic cells seems to prevent morphological alterations induced by CCK-R hyperstimulation. However, cells lacking Atg5 and Atg7 can still perform autophagy-mediated protein degradation [35], showing that deletion of these genes does not completely abolish autophagy. Moreover, no selective autophagic features were reported in the LC3 transgenic mouse pancreas after 48 hours starvation [36]. However, the induction of autophagy by starvation did not promote any morphological evidence of acute pancreatitis [36]. Interestingly, VMP1 constitutive expression induces the formation of autophagosomes in acinar cells but does not trigger pancreatitis [7, 17]. On the other hand, Mareninova et al. [37] have proposed that lysosomal proteases cathepsin

L and B are reduced, suggesting a defect in the autophagic flow during acute pancreatitis. These data also introduce the question whether the role of different types of autophagy has an impact on pancreatitis. In this regard, zymophagy is an inducible form of selective autophagy activated in response to disease. Zymophagy is not induced by starvation; it is triggered by CCK-R hyperstimulation and mediated by VMP1-USP9x-p62 autophagy-ubiquitin pathway [17]. This selective form of autophagy sequesters and degrades activated zymogen granules and prevents acinar cell death. Therefore, the outcome of pancreatitis differs when different types of autophagy pathways are considered, including selective and nonselective ones.

8. The Meaning

For the first time, it was demonstrated that there is activation of the VMP1-autophagic pathway and a selective autophagy of secretory granules during human pancreatitis [7]. These findings lead us to discuss how this knowledge fits the current theoretical framework regarding the occurrence and progression of this disease. Acute pancreatitis is a frequent, painful, and often deadly disease that ranges from a mild, edematous, and autolimited process, to a severe necrotizing and eventually lethal condition. Additionally, the etiology of this disease is diverse, and different stimuli can initiate the same autodigestion cascade via different mechanisms. VMP1 expression is activated during acute pancreatitis in humans, and there is formation of autophagosomes where p62, LC3, and zymogen granules colocalize [7]. The selective autophagic degradation of zymogen granules (Zymophagy) is a protective cell response that could explain, at least in part, the autolimited form of this disease seen in many patients. Hence, it is tempting to speculate that the more efficient zymophagic response by the pancreatic acinar cell, the less severity of the disease. In contrast, severe forms of pancreatitis offering an excess of cargo and an accelerated rate of degradation might overcome or disrupt the protective capacity of this selective autophagic process. The latter possibility is suggested by our studies since the inhibition of the autophagic flow impairs the protective role of zymophagy [17]. Interestingly, Fortunato and Kroemer [38] findings, regarding the reduction of the autophagosomelysosome fusion in human alcoholic pancreatitis, further support our hypothesis.

Finally, this novel autophagic pathway that selectively degrades altered secretory granules might be involved in other pathological processes affecting secretory cells, such as pancreatic beta cells in diabetes mellitus [26] or Paneth cells in Chron's disease [39]. Therefore, more studies on selective autophagy as a programmed cell response to pathological processes that affect protein secretion are important to significantly expand our knowledge of the role of autophagy in both cell biology and human disease.

9. Conclusions

Zymophagy revels a critical function of autophagy in secretory granule homeostasis and cell response to injury. It consists in the selective degradation of disease-induced activated secretory granules. This process can be reconstructed by the hyperstimulation of Gq-coupled receptors with CCK in a transgenic mouse model for studying VMP1-induced autophagy in pancreatic acinar cells (ElaI-VMP1 mice). A VMP1-USP9x-p62 molecular pathway is involved in this selective autophagic process. Zymophagy degrades the activated granules avoiding the spreading of their contents into the cytoplasm, thus preventing further trypsinogen activation and cell death.

Acknowledgments

Our work is supported by the Agencia Nacional de Promocion Científica y Tecnologica: Grant ANPCyT-PICT01627, Consejo Nacional de Investigaciones Cientificas y Tecnicas: Grant CONICET-PIP2527, and the University of Buenos Aires: Grant UBA-UBACyTM072.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 431684, 18 pages
doi:10.1155/2012/431684

Review Article

The Many Faces of Mitochondrial Autophagy: Making Sense of Contrasting Observations in Recent Research

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Received 14 November 2011; Accepted 21 December 2011

Academic Editor: Fulvio Reggiori

Research into the selective autophagic degradation of mitochondria—mitophagy—has intensified in recent years, yielding significant insights into the function, mechanism, and regulation of this process in the eukaryotic cell. However, while some molecular players in budding yeast, such as Atg32p, Uth1p, and Aup1p, have been identified, studies further interrogating the mechanistic and regulatory features of mitophagy have yielded inconsistent and sometimes conflicting results. In this review, we focus on the current understanding of mitophagy mechanism, induction, and regulation in yeast, and suggest that differences in experimental conditions used in the various studies of mitophagy may contribute to the observed discrepancies. Consideration and understanding of these differences may help place the mechanism and regulation of mitophagy in context, and further indicate the intricate role that this essential process plays in the life and death of eukaryotic cells.

1. Introduction

Even within large multicellular organisms, cells are not guaranteed a life within completely stable tissue environments. The ability of cells to adapt to stressful conditions, such as nutrient limitation, is a fundamental homeostatic requirement in order to survive and proliferate. The highly conserved process of autophagy is an important adaptation to the diverse challenges presented by environments in which unicellular and multicellular eukaryotic cells exist. Essentially, this process involves the transport of cellular components to the lysosome (in mammals) or vacuole (yeast) for degradation to fundamental components that are then recycled by the cell. In recent years, both nonselective and selective forms of autophagy, the uptake of bulk, random portions of the cytosol, or of specific targets, respectively, have been described.

Targets of the selective autophagic machinery include organelles, protein aggregates, and even invading microorganisms. Mitochondria, which accrue damage as they age, can present a challenge to the cell through uncontrolled generation of reactive oxygen species (ROS) and become increasingly inefficient in their generation of ATP. The selective removal of mitochondria by autophagy, known as mitophagy, is an important cellular adaptation to the challenge presented by this important organelle and the potential hazard it represents. Recently, studies have revealed key proteins involved in mitophagy, providing insights into the mechanism of this process. As the focus of research increasingly falls upon the physiological role of mitophagy within the cell, it is important to consider the wider meaning of results obtained to date in order to better understand the physiological role of mitophagy.

In this review, we briefly overview the current understanding of the mechanism of mitophagy, focussing on the model organism of the field, the budding yeast *Saccharomyces cerevisiae*. Stressors known to induce mitophagy in yeast will then be discussed, before recent research interrogating the regulation of mitochondrial turnover is addressed. Finally, discrepancies apparent in research undertaken to date will be addressed with reference to the experimental conditions employed in these studies and their relationship to our current understanding of mitophagy.

2. Autophagy and Mitophagy as Unique Forms of Intracellular Degradation

Macroautophagy (usually referred to as *autophagy*) involves the sequestration of cytoplasmic components (ranging from protein aggregates to whole organelles) into double-membrane structures known as autophagosomes (APs) (Figure 1(a)) [1]. Autophagosomes are delivered to the cell's degradative compartment, the vacuole (lysosomes in mammals), their contents degraded and subsequently returned to the cytoplasm for reuse. This important ability to "recycle" dangerous or unnecessary parts of the cell, described in numerous reviews [2–4], provides components during times of stress, allowing the cell to fulfil essential metabolic requirements [5]. However, autophagy also plays an important role in cellular homoeostasis, and a basal level of autophagy is evident in eukaryotic cells as a fundamental degradation pathway [6].

In the yeast *Saccharomyces cerevisiae*, the autophagy-related (*ATG*) genes encode proteins involved in autophagy, with 31 of these identified so far. Proteins implicated in all autophagic processes, encoded by the "core" *ATG* genes, constitute the basic autophagy machinery [7]. Homologues of many of these proteins have been identified in mammalian cells, demonstrating the highly conserved nature of autophagy throughout eukaryotic organisms [4]. While the process in yeast is described in detail elsewhere [3, 7, 8], it is useful to briefly consider key features of autophagy.

In yeast, a collection of 16 core Atg proteins are involved in the formation of the preautophagosomal structure (PAS), a transiently formed nucleation site [7]. From the PAS, membranes are recruited from a source that remains controversial; studies in yeast and mammalian cells variously suggest the plasma membrane [9], Golgi apparatus [10], endoplasmic reticulum [11], and the mitochondrion [12] as the source of membranes for AP expansion. As the AP expands, forming a double-membrane structure, it captures a portion of the cytosol destined for degradation [13]. The completed AP then traffics to the vacuole, where its outer membrane fuses with the vacuolar membrane, releasing the inner membrane and its contents (the autophagic body) into the vacuolar lumen. Cargo degradation is carried out by resident acid hydrolases, before membrane-bound effluxers such as Atg22p return components to the cytosol [14]. In order to monitor autophagy, Atg8p, a core Atg protein of ubiquitin-like function, is often used as a marker. This protein (the yeast homologue of mammalian LC3) is crucial to the formation of the AP through its conjugation to phosphatidylethanolamine and mediation of membrane-tethering events [15]. Atg8p serves as a useful marker of macroautophagy because this protein remains associated with the AP membrane, while other elements of the core Atg machinery only interact transiently [15].

Uptake of material into the vacuole lumen has also been shown to occur directly at the vacuolar membrane in a process known as microautophagy (Figure 1(b)) [16]. This process, observed in yeast, is characterised by the formation of an invagination at the vacuolar membrane, where cytosolic contents can be captured. The invagination grows into a tube-like structure within the vacuole that eventually pinches off from the vacuolar membrane, encapsulating the cytoplasmic contents within a single-membrane structure now located within the vacuolar lumen [16]. This microautophagic vesicle, along with its cargo, is subsequently degraded, apparently in the same manner as the autophagic body arising from the delivery of an AP to the vacuole. The molecular details of microautophagy and its physiological role largely remain unclear. Further work is required to characterise this phenomenon and whether observations made in yeast are relevant to mammalian cells [17].

As the relevance of autophagy as a fundamental cellular process has become increasingly evident in the literature, much research has focussed on selective manifestations of autophagy. The cytoplasm-to-vacuole targeting (Cvt) pathway, a selective application of the autophagy machinery uncovered early in yeast autophagy research, delivers at least two hydrolases (aminopeptidase I and α-mannosidase) to the vacuole, where they are processed to a mature, enzymatically active form [18]. This demonstrates that components of the autophagy machinery can be applied to biosynthetic pathways in addition to the canonical catabolic processes of intracellular turnover. In addition to the vacuolar delivery of aggregated molecules such as hydrolases, the formation of an AP allows the sequestration of a range of cellular materials, from soluble proteins to whole organelles. For example, the selective autophagic removal of peroxisomes (pexophagy, [19]), endoplasmic reticulum (reticulophagy, [20]), ribosomes (ribophagy, [21]), and mitochondria (mitophagy, [22–24]) has been described to date, while parts of the nucleus are also degraded by piecemeal microautophagy of the nucleus (PMN) at an early stage, and late nucleophagy (LN) following prolonged nutrient stress [25–27]. Even invading pathogens such as viruses [28] and bacteria [29] can be eliminated in higher eukaryotes through autophagic processes, collectively termed xenophagy. In *S. cerevisiae*, all types of selective autophagy identified to date (with the exception of LN) require the function of Atg11p, a protein that is thought to act as a scaffold or adaptor protein that brings the core *ATG* machinery into contact with targets selected for degradation [30, 31].

Mitophagy has recently become the subject of much scientific interest. This is due in part to the central role of this organelle in various cellular processes, as well as the association of mitochondrial dysfunction with pathological conditions in humans such as the neurodegenerative Alzheimer's and Parkinson's diseases [32–34]. The inherently dynamic mitochondrial network, which continuously undergoes fission and fusion events, is essential for eukaryotic life as the site for the provision of vast amounts of ATP [35]. However, as they age and accrue damage, mitochondria also present a potential challenge to cells through the leaking of excess reactive oxygen species (ROS) and other molecules, such as the proapoptotic protein cytochrome *c*, causing diverse cellular pathologies [36]. Mitophagy, working in concert with other degradative systems [37], serves as the primary means of eliminating those mitochondria that are damaged or surplus to requirements. As a selective manifestation of autophagy, mitophagy employs the core autophagy

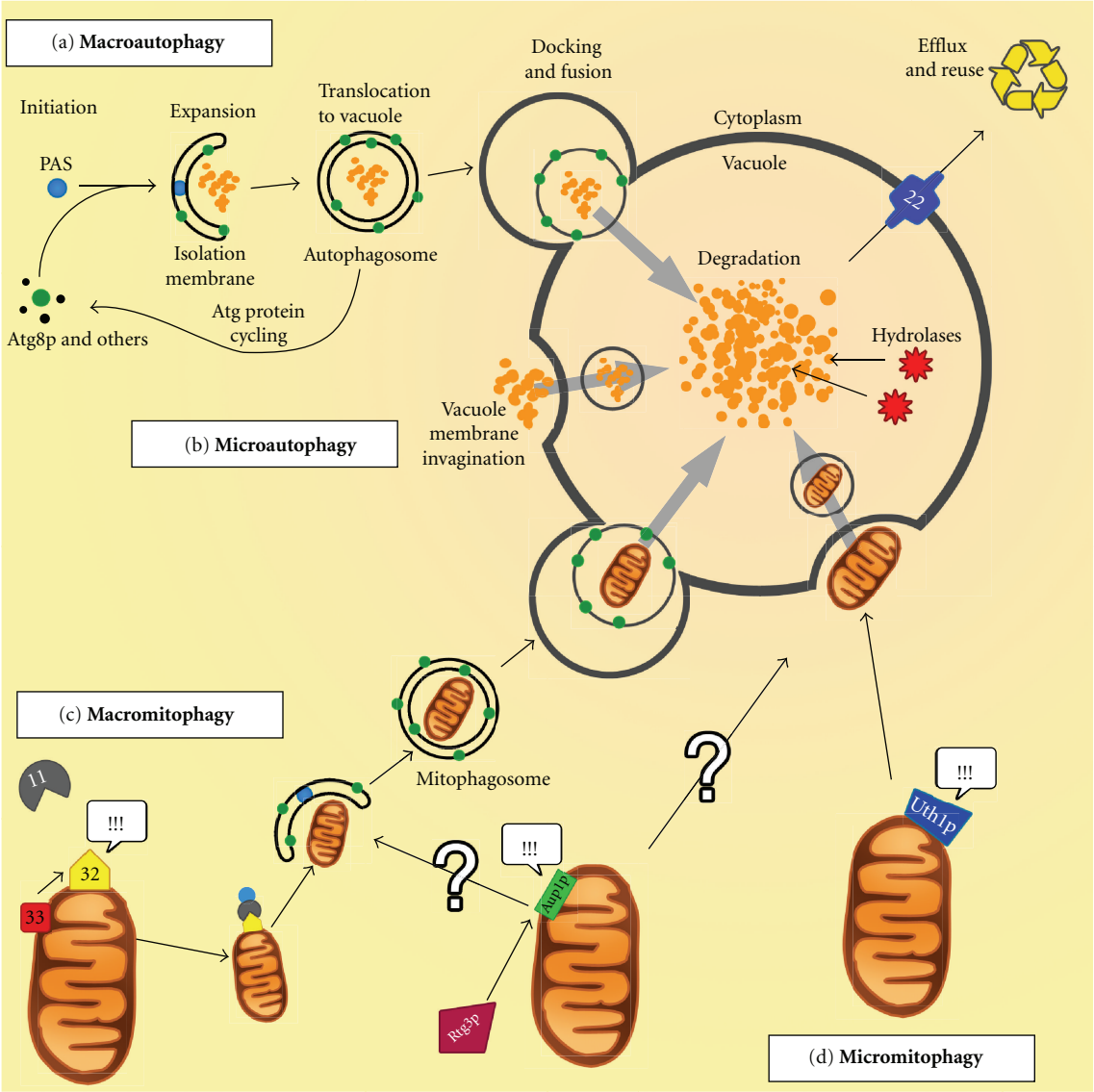

Figure 1: Overview of autophagy and mitophagy in yeast. (a) Macroautophagy, through the Atg proteins (including Atg8p, green dots), sequesters cytoplasmic components into autophagosomes for delivery to the vacuole for degradation. (b) Microautophagy involves invagination of the vacuolar membrane in order to take up cytoplasmic contents for degradation. (c) Mitochondria can be selectively degraded through a microautophagic mechanism. This requires the activity of Atg32p, Atg33p and Atg11p to bring the selected mitochondria into contact with the core autophagy machinery. (d) Mitochondria can also be removed by selective microautophagy, or micromitophagy, the mechanism of which remains unclear. While Atg32p, and Atg11p may be involved in micromitophagy, there is no definitive evidence to support this and the mechanism of Aup1p and Rtg3p function remains undetermined. See text for details. !!! = Inducing signal, ? = Uncertain mechanism.

machinery together with Atg11p [38] and several other gene products identified in recent research (Figures 1(c) and 1(d)) [39]. While proteins involved in mitophagy are diverse in structure and function, all cooperate to bring mitochondria destined for degradation into contact with the core autophagic machinery, thereby playing an important role in linking mitochondrial stress signals to autophagy. As we are still not able to completely describe the mechanism and regulation of mitophagy using the evidence collected so far, it is highly likely that further molecular components are yet to be identified.

In yeast, several genes have been associated with mitophagy. A summary of the key findings in yeast mitophagy research since the first study describing mitochondria within autophagic bodies [40] is provided in Table 1. Through investigation of deletion strain phenotypes, the *UTH1*, *AUP1*, *ATG32*, and *ATG33* genes have been directly implicated in mitophagy. *UTH1* encodes a 37 kDa SUN-family protein that localises to the outer mitochondrial membrane (OMM) and the cell wall [41]. This protein, which has previously been implicated in the maintenance of cell wall integrity, was shown to confer increased life span during nitrogen

Table 1: Key findings in yeast mitophagy research.

Author	Year	Primary finding	Notes	Assay	Carbon source*	Mitophagy induction*	Reference
Takeshige et al.	1992	Mitochondria within autophagic bodies	First observation of mitochondrial autophagy	Light microscopy, EM	Glucose, glycerol	Shift to N-starvation medium (glucose or glycerol)	[40]
Campbell and Thorsness	1998	Observation of damage-induced mitophagy	Further early evidence of mitophagy	EM	Various (respiratory)	Mitochondrial damage through disruption of *YME1*	[50]
Kiššová et al.	2004	*UTH1*	First mitophagy-specific gene identified	pGAL-CLbGFP (fluorescence microscopy)	Lactate & glucose	Shift to N-starvation medium (lactate and glucose) Rapamycin (0.2 μg/mL)	[41]
Priault et al.	2005	Mitochondrial damage triggers mitophagy	Impairing $\Delta\Psi_m$ results in preferential mitophagy of impaired mitochondria.	EM Pho8Δ60 (biochemical) Western (protein degradation)	Glucose (aerobic and anaerobic)	Used heat-sensitive Δ*fmc1* strain to precipitate mitochondrial damage.	[51]
Nowikovsky et al.	2007	*MDM38*	Found osmotic swelling triggers, and fission and is required for mitophagy	pCS-G/RFP ("Rosella", microscopy)	Galactose	Doxycyclin (5 μg/mL, induced *MDM38* depletion and mitophagy)	[52]
Kiššová et al.	2007	*UTH1*	Description of selective mitophagy and "micromitophagy."	EM	Lactate throughout	Shift to N-starvation medium (lactate)	[42]
Tal et al.	2007	*AUP1*	*AUP1* role in post-log phase mitophagy described	Western (aconitase degradation)	Glucose, lactate	Culture to post-log (glucose, lactate, up to 5 d)	[43]
Kanki and Klionsky	2008	*ATG11*	Further demonstration of selective mitophagy.	OM45-GFP, IDH1-GFP, ALP (biochemical)	Lactate	Shift to N-starvation medium (glucose)	[38]
Deffieu et al.	2009	Glutathione involvement	Indicates role of Redox in mitophagy induction	pGAL-CLbGFP (microscopy) EM	Lactate	Shift to N-starvation medium (glucose)	[53]
Kanki et al.	2009	*ATG33* (and 31 others)	Did not report *UTH1*, *MDM38*, *AUP1*, *RTG3* or *WHI2*. 8 reported genes overlap with Okamoto et al.	OM45-GFP (microscopy and western)	Lactate	Culture to post-log (lactate, 3 d) Shift to N-starvation medium (up to 6 h glucose)	[48]
Kanki et al.	2009	*ATG32*	Identified at same time as Okamoto et al.	OM45-GFP (microscopy & western)	Lactate	Culture to post-log (lactate, 3 d) Shift to N-starvation media (up to 6 h glucose)	[46]

Table 1: Continued.

Author	Year	Primary finding	Notes	Assay	Carbon source*	Mitophagy induction*	Reference
Okamoto et al.	2009	*ATG32* (& 52 others, including some known autophagy genes)	Did not report *UTH1*, *MDM38*, *AUP1*, *RTG3*, *WHI2* or *ATG33*. 8 reported genes overlap with Kanki et al.	p416GPD-mtDHFR-GFP (microscopy)	Glycerol	Culture to post-log (glycerol, 5 d)	[45]
Journo et al.	2009	*RTG3*	Also found *RTG3* regulates *AUP1*.	Fluorescence microscopy & Western analyses IDP1-GFP (microscopy)	Lactate	Culture to post-log (lactate, 3 d)	[44]
Mao et al.	2011	*HOG1*, *SLT2*	Shows MAPK signalling is involved in mitophagy in yeast	OM45-GFP(microscopy & western)	Lactate	Shift to N-starvation media (6 h, glucose) Culture to post-log (lactate)	[54]
Mendl et al.	2011	*WHI2*	Found fission is not essential for mitophagy.	pRS313-mtDsRed.T4 (microscopy)	Glycerol	Rapamycin (1 μM, 24 h, in DMSO)	[55]

* Where "*carbon source*" and "*mitophagy induction*" refer to conditions used to detect the primary finding.
GFP = green fluorescent protein, $\Delta\Psi_m$ = mitochondrial membrane potential, MOM = mitochondrial outer membrane, EM = electron microscopy, MAPK = mitogen activated protein kinase, N-starvation = nitrogen-starvation.

starvation (N-starvation) in a Δ*uth1* deletion strain and was not essential for macroautophagy. In a subsequent study, the same group demonstrated an early phase of mitophagy induced by N-starvation, which involves the sequestration of mitochondria directly by the vacuole, as observed by electron microscopy (EM). This suggests mitophagy can occur by a microautophagic mechanism, termed micromitophagy (Figure 1(d)) [42]. "Normal" macromitophagy, for which Uth1p is not required, follows this at a later stage.

In contrast, cells deleted for *AUP1*, which encodes a 49 kDa mitochondrial protein phosphatase, show perturbed mitophagy in long-term stationary-phase cultures and are characterised by decreased cell life span under these conditions [43]. A subsequent study linked *AUP1* function to the retrograde signalling (RTG) pathway, perturbation of which by deletion of the *RTG3* gene resulted in a defective mitophagy phenotype [44].

Two recently reported whole-genome screens for genes involved in mitophagy both identified the gene encoding Atg32p, a single-pass mitochondrial outer membrane protein with a predicted molecular mass of 59 kDa [45, 46], as being required for mitophagy. This protein is able to interact with both Atg8p (a core autophagy protein essential for the biosynthesis of APs [47]) and Atg11p (essential for all forms of selective autophagy described to date), linking mitochondria marked for degradation with the core autophagic machinery (Figure 1(c)). While the mechanism by which mitochondria are selected for autophagy remains poorly understood, it is hypothesised that Atg33p, which is believed to be a mitochondrial outer membrane protein, is able to report mitochondrial stress to Atg32p, especially during post-log (stationary) phase of growth [48]. What triggers Atg33p to relay this mitophagy-inducing signal remains unclear.

Proteins specific to mitophagy function in a sequential and controlled process of mitochondrial degradation. This tight control reflects the two-fold role of mitophagy in cells: it is involved in maintenance of mitochondrial homoeostasis (i.e., the dynamic maintenance of the functional stability of mitochondria), and as a response to stress (the physical and chemical demands of a particular environment) [49]. This review focuses on mitophagy as a response to stresses both intrinsic and extrinsic to the mitochondrion. Following a brief overview of the signalling pathways known to be involved in the regulation of this process, we identify and discuss discrepancies in the literature with reference to the diversity of mitochondrial stresses, and how the cell coordinates its response to bring about mitophagy. We conclude that these discrepancies are indicative of a complex integration of the basic mechanism of mitophagy into the cellular milieu, and that experimental conditions employed in studies of mitophagy must be considered to fully grasp the role of this process within the cell.

3. Mitophagy as a Response to Stress

Mitochondria play a fundamental role in cellular metabolism through the supply of energy as ATP. For the cell, the maintenance of a "balance" between healthy mitochondria and those that are damaged or dangerous is essential in order to ensure the most efficient production of energy. This is

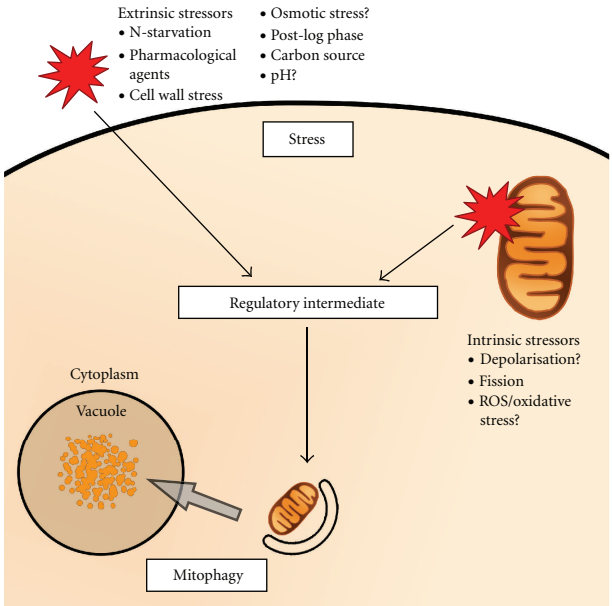

Figure 2: Mitophagy as a response to stress. Stress signals (red), arising from outside (extrinsic) or within (intrinsic) the mitochondrion, interact with regulatory intermediates in the cell. These intermediates coordinate the cell's response to these stresses, in this case promoting the removal of excess or dangerous mitochondria. As a consequence, mitochondria are then removed by an autophagic mechanism, mitophagy. Hypothesised, but as yet unconfirmed, stressors are indicated by "?".

a highly dynamic process requiring the cell's constant adaptation to changes in conditions within and outside of the cell. While any definition is necessarily problematic, for the purposes of this discussion, we define conditions that shift mitochondrial homeostasis in a direction favouring mitochondrial removal as stressors. Accordingly, cells that are subjected to such conditions are described as being in a state of, or exposed to, stress. Stress and stressors constitute the first step of mitophagy induction in which a stress signal is directed to the mitophagic response through a regulatory or signalling intermediate (Figure 2). It is important to recognise that stress, signalling/regulation, and mitophagy are overlapping terms in a continuum of controlled mitochondrial degradation. For the purposes of this discussion, we categorise stress as being either *intrinsic* (i.e., originating from within the mitochondrion itself) or *extrinsic* (arising anywhere outside of mitochondria, including within other parts of the cell) see Figure 2.

3.1. Intrinsic Stress. Much research has focussed on the role of the mitochondrion in triggering its own removal by mitophagy. Stresses originating from within mitochondria are often associated with mitochondrial damage, which affects the organelle's ability to produce energy efficiently without the release of excess ROS. In most physiologically relevant cases, mitochondrial damage is accompanied by the depolarisation of this organelle, or loss of the mitochondrial membrane potential ($\Delta\Psi_m$), which is essential for generation of ATP. The interest in mitochondrial damage as a trigger of mitophagy has been promoted by the finding that in mammalian cells, mitochondrial damage is a precursor to mitophagic degradation by proteins implicated in Parkinson's disease [56].

3.2. Depolarisation, Damage, and Dynamics of Mitochondria. While the importance of mitochondrial depolarisation and fragmentation to mitophagy are well established in mammalian cells, studies in yeast have yielded conflicting results. An early report suggests a role in mitophagy for *MDM38*, which encodes a membrane protein involved in K^+/H^+ exchange and protein export. This study indicates that yeast cells deleted for this gene are characterised by swollen and fragmented mitochondria that are targeted for removal by mitophagy [52]. Deletion of *MDM38* also results in abnormal mitochondrial morphology, with a collapse of the organelle cristae. These observations are consistent with the results of another study indicating that deletion of *FMC1*, which is required for ATP synthase assembly at high temperature, results in cells showing aggregation of ATP synthase F_1 catalytic subunits in the mitochondrial matrix, and evidence of mitophagy [51]. In contrast, a recent study assessing various fission-related yeast genes concluded that fission is not required for mitophagy and that fission is neither a precursor to, nor an inducer of, mitophagy [55]. Defective mitophagy in a Δ*fis1* mutant was attributed not to the role of Fis1p in fission, but an indirect disruption of the gene *WHI2*, which encodes a protein involved in the general stress response. Furthermore, while uncoupling agents such as carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) are able to induce mitophagy in mammalian cells, this is not the case in yeast [41, 46, 55]. The two recent, genome-wide screens for genes involved in mitophagy also

yielded conflicting results in terms of fission- and fusion-related proteins. According to data provided by Kanki et al., deletion of *DNM1*, encoding the important mitochondrial fission protein Dnm1p, significantly perturbs mitophagy [48], whereas Okamoto et al. did not detect the perturbation of mitophagy in strains deleted for any of the fission-related genes [45]. Clearly, further work is required to clarify these discrepancies.

3.3. Oxidative Stress and ROS. Oxidative stress often arises from within the mitochondrion, most commonly in the form of ROS. By definition, ROS are highly reactive molecules comprising oxygen, with reactivity attributable to the oxidising ability of unpaired valence electrons [57]. The healthy cell takes advantage of this useful oxidising property: controlled amounts of ROS play an important role in cell signalling and other redox-dependent processes [58]. However, ROS can also be hazardous to cells as they rapidly oxidise cellular components including amino acids, nucleic acids, and lipids. If ROS are allowed to accumulate, the consequences for the cell are dire and can result in death. A major source of ROS in cells is the mitochondrion, where ROS are a by-product of oxidative phosphorylation [59]. Under normal conditions, cells have adapted to cope with the production of ROS, eliminating these dangerous molecules by a range of antioxidant defences, such as superoxide dismutase, catalase, and glutathione [57].

The role of ROS as a regulator or inducer of mitophagy is not obvious given the available data, but work has provided several clues that allow preliminary speculation. As mitochondria produce the majority of intracellular ROS, imbalance of ROS levels and the resulting oxidative stress within these organelles is an attractive candidate for an inducer of mitophagy. ROS have been associated with nonselective macroautophagy in many recent studies in mammals [60, 61], as well as yeast nonselective autophagy [62]. However, in yeast, there is little evidence to suggest that ROS are able to induce mitophagy through any direct interaction with a component of the mitophagy machinery. A recent study by Suzuki et al. investigating cell death in autophagy-deficient yeast cells provided evidence that ROS accumulates in mutant strains lacking expression of certain *ATG* genes during nitrogen-starvation [63]. The implication here is that *ATG* genes play a role in the elimination of ROS-producing organelles, potentially by mitophagy. However, this is apparently due to the inability of these strains to upregulate expression ROS scavengers and respiratory chain components, rather than a direct inability of excess ROS-producing organelles to be removed by autophagy.

In a separate study, rapamycin (an established inducer of autophagy and mitophagy) was reported to reduce the cellular load of ROS in yeast cells, an effect attributed to an increase in mitophagy through target of rapamycin (TOR) signalling [64]. As removal of mitochondria by mitophagy reduces ROS load, these observations suggest that mitophagy is able to target ROS-producing organelles, although a direct relationship between ROS and mitophagy is not found. The fact that mitophagy could be further induced by rapamycin indicates that ROS alone in this case were not sufficient to induce complete mitophagy of these damaged organelles. At present, therefore, it seems that there is little evidence to support a direct role for ROS in mitophagy induction in yeast, although as important redox signalling molecules they are most likely indirectly involved.

3.4. Extrinsic Stress. In addition to the intrinsic factors dictating mitochondrial fate, a number of extramitochondrial stresses must be considered when considering mitophagy. Examples of such extrinsic stresses are pharmacological agents and the environmental conditions experienced by cells. Much research has investigated the link between extrinsic stresses and mitophagy in *S. cerevisiae*. Indeed, while mammalian cells generally exist within relatively stable tissue environments, unicellular organisms such as yeasts are often exposed to stressful environmental conditions. Such conditions can also be encountered by mammalian cells in unusual but clinically relevant circumstances, an example of which is the environment within tumours, where uncontrolled growth restricts the normal supply of nutrients to these extremely metabolically active cells [65]. The ease with which yeast cells can be exposed to environmental stress in the laboratory facilitates studies in this model organism. Yeast require a source of both nitrogen and carbon to survive and proliferate [66], and omission of either of these from the culture medium constitutes a starvation condition.

3.5. Nitrogen-Starvation. Nitrogen-starvation (N-starvation), in particular, is a well-established means of inducing both autophagy and mitophagy in yeast [40, 42, 48, 54, 63, 67]. This is achieved by transferring cells from a nitrogen-rich preculture medium to a medium omitting all sources of nitrogen, including amino acids. Such media can be supplemented with any source of carbon. Yeasts subjected to this form of stress cease proliferation and immediately activate autophagy in order to supply nitrogen for essential cellular processes [5]. Mitophagy is also induced by N-starvation, although the extent to which mitophagy is induced appears to depend on the particular carbon source available for cellular metabolism. For example, when yeast grown in rich media requiring mitochondrial function are subjected to N-starvation in media supplemented with sources of carbon that yeast can ferment by glycolysis (providing ATP independently of mitochondria, such as glucose), N-starvation results in mitochondrial turnover that is rapid and extensive [38, 41, 48, 53, 54]. Little mitophagy appears to occur, however, when yeast cells are subjected to N-starvation in medium supplemented with respiratory sources of carbon (requiring mitochondrial function to generate ATP, such as lactate) [38]. However, evidence discussed below suggests that exceptions to these rules are apparent—even when comparing carbon sources utilised by the same metabolic pathway, the extent and rate of mitophagy are not consistent. In any event, induction of mitophagy in this case can be attributed to TOR signalling (discussed below), although the mechanism by which mitophagy is suppressed in media containing a respiratory carbon source remains to be clarified.

3.6. Post-Log (Stationary) Phase of Growth. A form of stress observed to induce both mitophagy and autophagy is arguably the most natural condition of post-log (also referred to as "stationary") phase of growth. In their natural environment, yeasts, being immotile microorganisms, rapidly utilise any available nutrients to proliferate. After these nutrients have been exhausted, yeasts enter a quiescent state of low metabolic activity and may undergo sporulation in order to survive until more favourable conditions for growth are encountered once again. Mimicking these conditions in the laboratory by culturing yeast in nutrient-rich medium for extended periods induces both autophagy and mitophagy. Conditions ranging from 3 days [38] to 5 days [43, 68] of growth in nutrient-rich medium supplemented with respiratory or fermentative carbon source have been reported as strageies to induce and study mitophagy. While the level of mitophagy observed in stationary phase cultures is extensive and represents a physiologically relevant and natural response, it is difficult to determine the exact source of stress; whether mitophagy is induced by depletion of nutrients, the buildup of waste products or a combination of factors has not been determined.

Culture to stationary phase was employed in studies identifying the role of Aup1p in mitophagy [43, 44], while Okamoto et al. and Kanki et al. both performed genome-wide screens for mitophagy-related genes under stationary phase conditions, identifying the mitophagy-specific protein Atg32p [45, 48]. Indeed, in the genome-wide screens carried out and reported by Kanki et al., Atg33p was shown to be involved in stationary phase mitophagy, but not in mitophagy triggered by N-starvation [48]. It would, therefore, seem that, while it is difficult to attribute mitophagy to any particular stress during stationary phase, other factors apart from the exhaustion of nitrogen supply are at play under these conditions. In spite of these difficulties, the relevance of stationary phase as a naturally encountered stress is clearly important.

3.7. pH. pH-stress has been linked with autophagy, although its role in mitophagy is not as clear. A long-standing question of why *ATG* mutants die prematurely in comparison to wild-type strains when cultured in starvation media was recently addressed in a study finding that certain *ATG* mutants are extremely sensitive to low pH (around pH 3) in unbuffered starvation culture medium [63]. Perhaps unexpectedly, this effect is apparently due to defective mitochondrial respiration. While this might suggest the disruption of mitophagy-mediated quality control over the mitochondrion, comparison of respiratory function in a Δ*atg32* (mitophagy-deficient) strain and numerous autophagy-deficient strains indicates that perturbation of nonselective autophagy and not mitophagy is responsible for this phenomenon. However, methods of inducing mitophagy may be accompanied by changes in pH, and it is important to keep an open mind to the possible role of pH in mitophagy.

3.8. Pharmacological Agents. A number of pharmacological agents are able to induce autophagy and mitophagy, thereby acting as mitophagy-inducing stressors. These pharmacological agents have diverse effects on cells, but usually act through the manipulation of cellular signalling and regulatory pathways that control autophagy. A key pharmacological agent commonly used in the field is rapamycin, which induces autophagy and mitophagy through the inhibition of TOR signalling [69, 70], and other agents such as CCCP (discussed above) and oligomycin (an inhibitor of ATP synthase and oxidative phosphorylation) have also been used in studies of mitophagy [41, 55]. While these treatments are useful in studies that interrogate specific mechanistic and regulatory questions, they are artificial in action and do not generally represent naturally occurring conditions. For this reason, pharmacological agents do not necessarily replicate natural changes in the regulatory networks of cells, and, therefore, may not elicit natural autophagic responses of physiological relevance.

3.9. Osmotic Stress. Osmotic stress has been associated with autophagy relatively recently, and the role of osmolarity in mitophagy requires further investigation. The osmoregulatory protein Hog1p, which functions in the MAPK pathway (discussed below), has been implicated in macroautophagy [71]. Deletion of *HOG1* resulted in reduced autophagy under conditions of hypo- or hyperosmotic stress, indicating that *HOG1* is important in the coordination of autophagy in response to osmotic stress. While Hog1p and other MAPK proteins have been implicated in mitophagy (discussed below), the deletion of the *HOG1* gene was found to result in the most severe perturbation of mitophagy. While MAPK is a key signalling pathway in the cell that responds to a range of stresses, these phenotypes may suggest that the osmotic status of the cell has some bearing on mitochondrial turnover.

4. Regulation of Mitophagy

As the mechanistic details of mitophagy and types of stress that induce the process become better understood, attention has turned to the cellular regulatory pathways that control mitophagy. It is important to note at the outset that our understanding of how autophagy and mitophagy are regulated is very much in its infancy. Fully characterising the regulation of autophagy and mitophagy, and indeed the question of how autophagy and mitophagy are integrated into the complex systems of cellular signalling, remain important challenges in the field. These questions are particularly important as the clinical implications of our knowledge become increasingly relevant.

The regulation of autophagy, reviewed elsewhere in great detail [72–74], is beyond the scope of this article. Recent research, informed by advances in our understanding of autophagy regulation, has implicated several key regulatory pathways in the regulation of mitophagy. The most relevant of these are discussed below.

4.1. TOR Signalling. The TOR signalling pathway has long been known to play a role in the regulation of autophagy. TOR signalling is conserved in some form throughout all

eukaryotes and is intricately involved in cell proliferation and metabolism through its regulation of many cellular responses to nutrient status [66, 75]. TOR is sensitive to rapamycin treatment, and a vast body of literature supports the role this signalling pathway plays in the sensing of nitrogen supply [76–78]. Accordingly, TOR signalling is particularly relevant to regimes inducing mitophagy through N-starvation. However, due to the central role, it plays in the cell, TOR signalling is implicated in many stress responses, both related and unrelated to mitophagy.

The central components of TOR signalling in yeast are the two TOR complexes (TORCs), TORC1 and TORC2 [75]. Both TORCs are a collection of proteins that include Tor, a PI3-like protein kinase, but only TORC1 is sensitive to rapamycin and coordinates cell growth in response to nutrient availability [69, 79]. Under conditions of nutrient availability, TORC1 is active, allowing transcription and biosynthesis of genes and proteins required for cellular growth. Under such conditions, autophagy is repressed through the hyperphosphorylation of Atg13p, a core Atg protein required for autophagy [80]. In nutrient-poor conditions, however, TORC1 is inactivated and Atg13p is able to participate in the induction of autophagy. It is this inhibition of TORC1 that makes rapamycin such a potent and commonly used inducer of autophagy ([81, 82]).

While it is well established that TOR signalling is important for nonselective autophagy [83], the relationship between TOR and mitophagy remains unclear. In an early study demonstrating *UTH1* involvement in mitophagy, it was demonstrated that treatment of cells cultured in respiratory medium with rapamycin induced mitochondrial turnover, eventually causing cell death [41]. Another early report in mammalian cells provided evidence that mitophagy is suppressed by TOR activity (i.e., was induced following rapamycin treatment) [84]. A more recent study in yeast has reported that treatment with rapamycin is able to reduce ROS production in cells deficient in frataxin (a mitochondrial iron chaperone), possibly by stimulating the removal of damaged mitochondria by autophagy [64]. While there is currently little direct evidence of TOR involvement in mitophagy, the role that this pathway plays in nonselective autophagy and nitrogen sensing, in particular, suggests the need for further investigation.

4.2. Mitogen-Activated Protein Kinase (MAPK) Signalling. Results of recent studies in yeast illustrate the role in mitophagy of two MAPK proteins, including Hog1p, Slt2p, and additional proteins associated with Hog1p and Slt2p function, including Wsc1p, Ssk1p, Bck1p, Mkk1p, Mkk2p, Pbs2p, and Pck1p [54, 85]. The MAPK pathway is a highly conserved, broad-ranging signalling cascade involved in a variety of cellular processes. MAPK signalling is involved in a range of pathways [86] but can be separated into two categories according to their role in cell proliferation or the transduction of stress signals [87]. Hog1p and Slt2p, core components of two pathways comprising the MAPK proteins listed above, are both involved in the MAPK response to stress. While Hog1p has been implicated in the response of yeast cells to osmotic stress [88], Slt2p is important in responding to stress at the cell wall [89]. According to Mao et al. [54], the inhibition of mitophagy in both Δ*slt2* and Δ*hog1* deletion strains (and strains deleted for associated genes listed above) is marked, but not complete, suggesting that other as yet unidentified regulatory pathways are involved in the control of mitophagy. Temporally distinct regulation of mitophagy by Hog1p and Slt2p pathways is observed following the onset of N-starvation when monitored by Western blot analysis, echoing a trend described in the analysis of *UTH1*-dependent mitophagy [42]. Interestingly, Uth1p is also known to be involved in cell wall biogenesis [90], which suggests another link between this protein and Slt2p, and Wsc1p, which Mao et al. identify as having an effect on mitophagy, is also involved in the maintenance of cell wall integrity. The authors further find that autophagic role of Hog1p pathway proteins appear to be limited to mitophagy, whereas Slt2p associated proteins are also involved in the regulation of pexophagy, raising the prospect of crosstalk between the regulatory systems of different selective autophagy pathways. Indeed, hyperosmotic stress alone is not able to induce mitophagy, suggesting complexity in MAPK regulation of mitophagy [85]. It would, therefore, seem that consideration of mitophagy regulation in isolation of other selective autophagy pathways is unlikely to provide a complete understanding of the process.

4.3. Reduction-Oxidation Chemistry (Redox). Redox chemistry is known to participate in a range of regulatory systems (reviewed in [59]), and an increasing body of evidence supports a role for redox chemistry in mitophagy. The ongoing question of ROS involvement in mitophagy, which has important implications for cellular redox balance, is considered separately above. The direct role of redox in yeast mitophagy was recently described by Deffieu et al., who showed that glutathione, a key cellular moderator of redox state and antioxidant [91], is linked to mitophagy regulation [53]. In this study, *N*-acetyl-L-cysteine (NAC) was shown to have an inhibitory affect on mitophagy, while it had no affect on nonselective autophagy. This inhibitory effect was attributed to NAC-associated increases in glutathione levels, altering the redox state of the cell. The changes in glutathione levels were also shown to be *UTH1*-dependent, suggesting that different regulatory regimes might promote different phases of mitophagy. Another study indicates that treatment of cells with NAC suppresses the expression of Atg32p, which accordingly inhibits mitophagy, suggesting a direct link between the redox state of the cell and the mitophagy machinery [45]. The perturbation of redox homeostasis is inexorably linked to the health of mitochondria and thus should allow us to investigate the relationship between mitochondrial damage and mitophagy further.

4.4. The Retrograde Signalling Pathway. Mitochondria are able to elicit transcriptional responses from the nuclear genome through the retrograde signalling (RTG) pathway. The RTG pathway, which partially overlaps with TOR signalling, provides the mitochondrion with a means for reporting stresses and metabolic challenges to the nucleus. As a key player in mitochondrial homeostasis, mitophagy is also

regulated by the RTG pathway. Aup1p, a mitochondria-localised protein phosphatase required for stationary-phase mitophagy [43, 44], regulates the phosphorylation of the RTG transcription factor Rtg3p, as well as its localisation to the nucleus, leading to the activation of RTG genes [44]. Like Aup1p, Rtg3p was then shown to be required for stationary-phase mitophagy, but not nonselective autophagy or the Cvt pathway, although the redundancy of these proteins' actions was not determined. It is interesting to note, however, that TOR signalling regulated the localisation and activity of Rtg3p (and Rtg1p, another RTG transcription factor), suggesting a link between nitrogen sensing and mitophagy [92]. These findings suggest that the mitochondrion is not simply a passive subject of mitophagy; rather, mitochondria appear to play an active role in the regulation of their own removal by mitophagy. This is particularly interesting considering the increasing recognition of the mitochondrion as an active participant in cellular signalling on a number of different levels [93].

The regulation of mitophagy in yeast cells remains unclear, and further research in this area will provide further clues about the role of mitophagy and indeed mitochondria in the cell. The potential role of the TOR and MAPK signalling pathways as regulators of mitophagy suggests its integration into cellular responses to nutrient and other important stress signals. The implications of other potential regulators of mitophagy, including redox and RTG signalling, are not so obvious and warrant further research.

5. Mitophagy Mechanism and Regulation: Contrasting Observations

While significant progress has been made in our understanding of mitophagy, results emerging from a number of different studies remain to be reconciled. It is likely that the contrasting observations apparent amongst different studies, most clearly evident in the different outputs of the recent genome-wide screens for genes related to mitophagy, reflect the complex cellular integration of a variety of signal inputs in response to diverse conditions.

A variety of assays to monitor mitophagy have been employed, as well as a range of growth conditions and means of inducing mitophagy. We now examine the effect that these different experimental approaches might have on the mitophagy phenotypes observed with reference to contrasting observations that have emerged in yeast mitophagy research.

5.1. Inducing and Monitoring Mitophagy. Before discussing individual results, it is important to consider several practical aspects of yeast cell culture and mitophagy induction in the context of experimental design. The selection of relevant experimental conditions is paramount in order to best test a hypothesis. In terms of mitophagy, it is difficult to know what conditions are most relevant for the identification of mitophagy-related genes, even without considering the physiological relevance of experimental conditions. Several features, in particular, have to be considered when designing experiments. Firstly, the source of carbon in the medium has important implications for yeast cell phenotypes. Supplementation with a carbon source favouring fermentative growth, such as glucose, suppresses respiration in yeast (known as glucose repression) and the mitochondria fail to fully mature into an extensive reticular network [94]. In contrast, supplementation with sources of carbon utilised by respiration promotes the maturation of mitochondria into a filamentous reticular network that can be visualised under appropriate conditions. As mentioned above, the source of carbon has also been shown to affect the extent of mitophagy in cells. Transcriptional profiles of yeast cells differ significantly when cultured in carbon sources that must be utilised by the same means of catabolism [66]. The source of carbon in the culture medium is, therefore, an important consideration when interpreting results.

The means of inducing mitophagy is another important consideration when investigating mitophagy mechanisms and regulation. There are four different strategies adopted by researchers to experimentally induce mitophagy: N-starvation, treatment with pharmacological agents, causing mitochondrial dysfunction, or culturing cells to post-log phase. We do not currently have a complete understanding of the mechanisms by which each of these conditions trigger mitophagy, apart from understanding, for example, that TOR signalling is involved in both N-starvation and in response to treatment with rapamycin. This is of particular import in the case of post-log phase mitophagy induction; while this condition is most likely to replicate conditions experienced by yeasts in the wild, it is also likely the most problematic condition in terms of the isolation of variables inducing mitophagy. There is, therefore, a tension apparent between "natural" conditions, which result in more general induction, and "artificial" conditions, which manipulate particular variables. The former has the advantage of physiological relevance, while the latter can address more specific biochemical questions.

The assay used to detect mitophagy is an additional experimental feature that requires consideration (reviewed in [70, 95, 96]). Assays are characterised by different sensitivities (detection thresholds) and, in general, are only as useful as the biological mechanism upon which they depend. The range of different assays employed to monitor mitophagy in yeast, which are represented in Figure 3, use different molecular strategies to detect mitophagy and produce different outputs. Fluorescent proteins (FPs) are often used to monitor mitophagy, either by directly observing changes in fluorescence signal (e.g., delivery to the vacuole) at the microscope, or through biochemical techniques such as enzymatic activity. Access to such assays represents an important means of verifying results but is also a potential source of variability. An example of this is found in the fluorescent protein-(FP-) based analyses. Probes currently in use are targeted to different compartments of the mitochondrion, expressed from either a chromosomal location or a plasmid, under the transcriptional control of different promoters and report mitophagy in different ways (Figure 3). These variables can all have an impact on the nature of the information reported by assays, as well as how the data they yield are interpreted. However, the range of data generated in studies employing

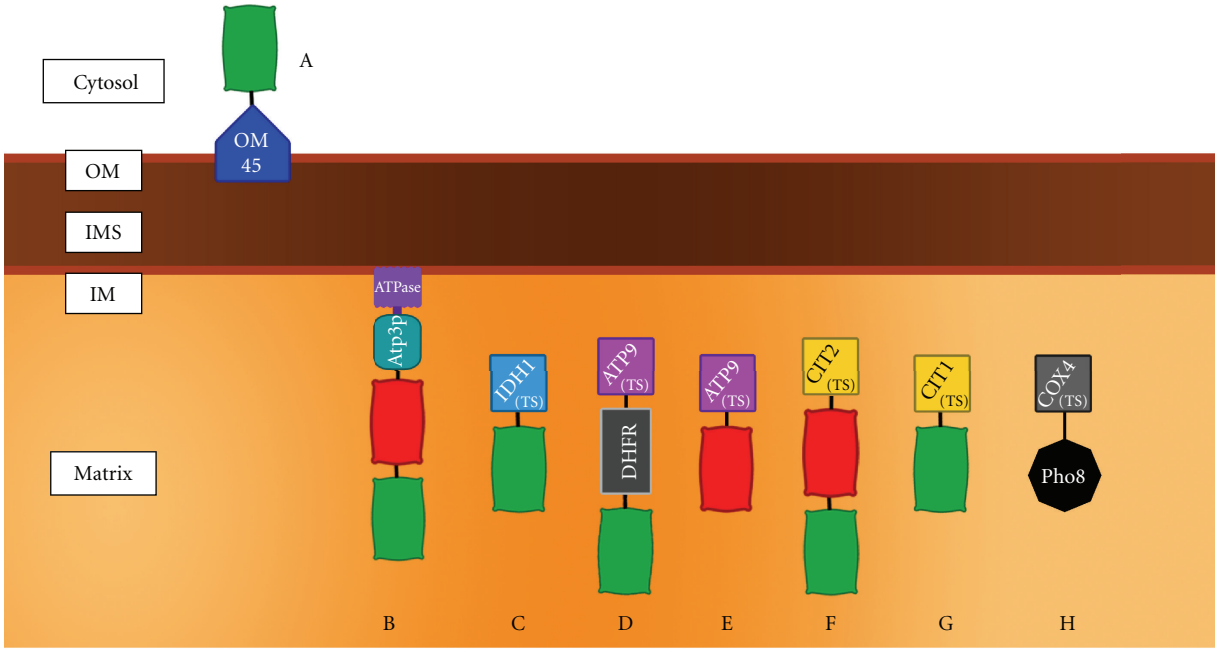

Figure 3: Biochemical probes for monitoring mitophagy in yeast. The localisation of probes within the different compartments of the mitochondrion is shown. A–G are fluorescence-based probes, while H is an enzymatic approach. (A) OM45-GFP is expressed from a chromosomal location in fusion with the endogenous OM protein OM45. GFP is exposed to the cytosol. (B) mt-Rosella II is an nonoligomerising biosensor comprising a red FP and pH-sensitive GFP expressed as a fusion to ATP3 from a genomic location (Lucarelli, May, Devenish and Prescott; unpublished). (C–H) Plasmid-derived combinations of FPs are targeted to the matrix space using different targeting sequences (TS) as follows: (C) isocitrate dehydrogenase, (D–E) F_0 ATP synthase subunit c and (F–G) citrate synthase. (H) mito-Pho8 is a an acid phosphatase that is only active at vacuolar pH. When targeted to the matrix by a *COXIV* TS, the enzymatic activity provides a measure of mitophagy in strains disrupted for the endogenous *PHO8* and *PHO13* genes. Alternative targeting sequences allow targeting of probes to different compartments. OM = mitochondrial outer membrane, IMS = intermembrane space, IM = mitochondrial inner membrane, TS = targeting sequence.

different assays can be important to confirm experimental outcomes.

5.2. Differences Evident in the Literature and Experimental Conditions. There are several contrasting suggestions documented in Table 2. While separate studies have identified different proteins, these inconsistencies are not irreconcilable and may be attributed to differences in technical strategy. *UTH1* was implicated in mitophagy by examining cells cultured first in lactate-supplemented medium that were shifted to N-starvation medium supplemented with either glucose or lactate [41, 42]. Under these conditions, mitophagy is observed from 2 hr after shifting to N-starvation. In contrast, the role of *AUP1* was determined in cells cultured to post-log phase of growth to induce mitophagy [43]. These genes are most likely involved predominantly in the mechanism of mitophagy induced in response to the particular experimental conditions employed in these studies. These findings exemplify the complexity of mitophagy in response to environmental cues. Indeed, such diversity in mechanism might not have been revealed without the use of different conditions to induce mitophagy.

As stated above, the physiological role of mitophagy in yeast is still not clearly understood. In 2005, Priault et al. analysed a temperature-sensitive Δ*fmc1* deletion strain, which is characterised by perturbation of both inner-membrane fusion and fission of the mitochondrial network at non-permissive temperatures [51]. Microscopy and biochemical analyses indicated that mitochondrial morphology was severely perturbed under these conditions and that mitochondria lost $\Delta\Psi_m$ before they were removed by mitophagy. Indeed, $\Delta\Psi_m$ alone was found to be capable of mitophagy induction as recapitulation of respiratory incompetence in a wildtype strain was sufficient to induce mitophagy. This is in contrast to data provided by Nowikovsky et al. indicating that conditional deletion of *MDM38*, causing perturbation of mitochondrial morphology (in the form of extensive fission), osmotic swelling of the organelle, and loss of $\Delta\Psi_m$, ultimately resulted in mitophagy [52]. These authors suggested that osmotic swelling and not alteration in $\Delta\Psi_m$ was important for the induction of mitophagy. A subsequent study [55] employed a temperature-sensitive strain, *mgm1-5,* that shows defective inner membrane fusion, causing mitochondrial fragmentation at elevated temperature. Growth at elevated temperature was not sufficient to induce mitophagy, suggesting that mitochondrial depolarisation and fission are not linked to mitophagy. Indeed, CCCP treatment of yeast cells was not able to induce mitophagy, and blocking of mitochondrial fission did not induce mitochondrial degradation.

Interestingly, cells used in the study by Mendl et al. [55] were cultured in respiratory medium containing glycerol as carbon source, whereas Priault et al. [51] and Nowikovsky

Table 2: Contrasting observations in yeast mitophagy research.

Observation	Supporting studies	Contradicting studies
Depolarisation triggers mitophagy	[51, 52]	[55]
Fission precedes mitophagy	[52]	[55]
Mitophagy by microautophagy (micromitophagy)	[42]	
Stress and regulation of mitophagy		
-*TOR*	[41, 55]	
-*MAPK*	[54]	
-*Redox*	[53]	
-*RTG*	[44]	[45, 48]*
-*General stress*	[55]	
-*pH*		[63]
Proteins required for mitophagy		
-*Uth1p*	[41, 42]	[45, 48]*
-*Aup1p*	[43]	[45, 48]*
-*Atg32p*	[45, 48]	
-*Atg33p*	[48]	
Requirement of nonrespiratory medium to induce mitophagy under N-starvation	[38, 48]†	[42, 52, 97]

*These studies reported no evidence of involvement, but did not directly contradict the observation.
†In this study, limited mitophagy is demonstrated during lactate-supplemented N-starvation.

et al. [52] assess cells grown in fermentative medium containing glucose or galactose, respectively. This might suggest that as long as mitochondria are required to utilise the available carbon source, mitophagy is inhibited, even following a significant mitochondrial insult such as mitochondrial fragmentation. This is in line with observations made by Kanki et al. [38, 98] that a shift to N-starvation medium supplemented with a respiratory source of carbon is not sufficient to induce mitophagy. However, galactose (as used by Nowikovsky et al.) is known to not completely suppress mitochondrial function [99], and Kiššová et al. found in their 2007 study that mitophagy does occur in cells subjected to N-starvation supplemented with lactate, which is utilised by respiration [42]. Yeasts subjected to N-starvation in medium supplemented with ethanol as a respiratory carbon source undergo extensive mitophagy (May, Devenish and Prescott, unpublished results and [97]).

These observations provide evidence suggesting that the source of carbon is an important factor influencing mitophagy phenotypes, even when comparing carbon sources utilised by the same metabolic pathway. A more comprehensive analysis of the influence of culture conditions on mitophagy phenotype should provide an interesting perspective on the place of mitophagy in metabolic homeostasis.

5.3. Two Genome-Wide Screens for Genes Involved in Mitophagy. There are some intriguing differences in the results generated by the two yeast genome-wide screens for mitophagy genes performed in 2009 by Kanki et al. and Okamoto et al. [45, 48], who analysed 4667 and 5150 deletion strains, respectively, for mitophagy defects. Kanki et al. conducted the screen in several phases, initially screening all deletion strains grown to post-log phase (3 days) in medium supplemented with lactate. This phase detected 290 deletion strains that were not deleted for *ATG* genes and grew normally. In the second phase, deletion strains were cultured in nutrient-rich lactate medium before being shifted to glucose-supplemented N-starvation medium to induce mitophagy. In total, 65 deletion strains were characterised by abnormal mitophagy, of which 32 had a clear defect in mitophagy. Ultimately, 23 of these were identified as novel mutants not otherwise linked to mitophagy.

In contrast, Okamoto et al. cultured cells to post-log phase in medium supplemented with glycerol for 5 days before determining mitophagy in deletion strains. This method detected 53 genes that when deleted conferred a defective mitophagy phenotype. Of these, 35 non-*ATG* genes were reported, of which 23 are novel candidates for involvement in mitophagy. Interestingly, only eight novel genes were characterised as having an unequivocal mitophagy defect in both screens—many genes reported were not detected in the alternate screen.

There are several possible reasons why the outputs of the two screens were different. In both screens, deletion strains were cultured in respiratory medium, although Okamoto et al. cultured cells further into post-log phase before analysing them for evidence of mitophagy. The use of N-starvation in the second phase of the Kanki et al. screen is a potential point of contrast between the two screens that may account for the different outcomes. However, there is little correlation in the identity of genes detected in the first phase of the Kanki et al. screen and the Okamoto et al. screen. Accordingly, the likely explanations are that either the probes used to detect mitophagy do not report the process with the same efficiency, the differences in culture length into post-log phase affect the type of mitophagy executed, or that the type of respiratory carbon source has an influence over mitophagy regulation or mechanism.

The fluorescent probes used in these studies differ in two ways: their mode of expression and their targeting to the mitochondrion (Figure 3 and discussed in [70]). Kanki et al. adopted an OM45-GFP probe that is encoded by a gene cassette integrated into the nuclear genome under expression control of the native OM45 promoter. OM45 localises to the mitochondrial outer membrane and is exposed to the cytosol. Okamoto et al. used a plasmid-borne gene cassette encoding GFP fused to dihydrofolate reductase (DHFR) and an ATP synthase subunit 9 targeting sequence, which delivers the probe to the mitochondrial matrix. Studies in mammalian cells have shown that the mitochondrial outer membrane can be delivered to other cellular compartments, such as the peroxisome [100], and that mitochondria can supply membranes during the membrane expansion step of AP formation [12]. The outer membrane may, therefore,

be processed differently to the matrix during mitophagy. OM45-GFP has previously proven to be a reliable indicator of mitophagy in yeast [38, 98], although the behaviour and targeting of the probe may change under different conditions. However, it seems most likely that culture conditions employed by the two screens are responsible for the observed differences in mitophagy phenotype. Thus, the additional time spent in post-log phase by cells assessed by Okamoto et al., or the carbon source itself, should be considered as factors potentially influencing the course of mitophagy.

It is noteworthy that both genome-wide screens failed to retrieve a number of genes, including *AUP1*, *UTH1*, *MDM38*, *RTG3*, or *WHI2*, that are reported to play a role in mitophagy in other studies. For most genes, this can be attributed to the differences in growth medium (carbon source) and the means by which mitophagy was induced in these studies in comparison to the genome-wide screens. *AUP1* and *RTG3* are the exceptions here, as strains deleted for these genes were demonstrated by Journo et al. [44] to be defective for mitophagy under virtually the same conditions as those employed by Kanki et al. [48]. In addition, the screen performed by Kanki et al. found a slight mitophagy defect in a strain deleted for *FMC1*, whereas Priault et al. [51] found that deletion of this gene and the associated mitochondrial damage incurred induced mitophagy. This may be due to the use of a different background strain of yeast—both Kanki et al. and Okamoto et al. used the same strain of yeast (although different mating types), while Journo et al. used a number of other strains of different genetic backgrounds.

In light of these themes, it is interesting that the two signalling pathways implicated in the regulation of mitophagy in yeast thus far, MAPK and redox (by glutathione), were detected by separate groups culturing cells under similar conditions [53, 54]. This suggests that even amongst cells exposed to similar stresses, regulation is complex and requires the coordination of different signalling pathways. Indeed, the genes detected in the genome-wide screens are involved in a very broad range of processes in the cell, suggesting that mitophagy is a well-integrated and fundamental process of cellular life. Untangling the complexity of mitophagy through comprehensive analyses of different conditions promises to enhance our understanding of this intriguing process.

5.4. Differences between Mitophagy in Mammalian and Yeast Cells. While mammalian cells are not the focus of this review, it is important to consider some of the apparent differences when comparing mitophagy in yeast and mammalian cells. Yeast is considered to be an important model for studying fundamental biological cellular processes including autophagy. Ultimately, such a discussion also helps us to understand the place and appropriateness of yeast as a model of mammalian cells.

In contrast to nonselective autophagy, it appears that the mitophagy mechanism in mammalian cells is different to that in yeast. Research carried out in mammalian cells has uncovered two mechanisms of mitophagy. The first, which is thought to be involved in mitochondrial quality control, requires the OM-localised Ser/Thr kinase PINK1, which detects a stress signal [101]. PINK1 then binds Parkin, a cytosolic ubiquitin ligase, which then ubiquitinates target proteins on the mitochondrion [102]. The target for ubiquitination and the implications of this process are not understood, but mitochondria marked in this way are subsequently degraded by mitophagy. Importantly, the PINK1-Parkin system is strongly linked to Parkinson's disease: a loss-of-function mutation in PARKIN is the most common mutation associated with the early onset form of the disease [103]. The second form of mitophagy encountered in mammalian cells, NIX-dependent mitophagy, is associated with reticulocyte maturation [104]. The Bcl-2 family protein NIX (NIP3-like protein X) interacts directly with LC3, the mammalian equivalent of Atg8p, facilitating mitophagy. NIX has been associated exclusively with the elimination of mitochondria from maturing reticulocytes and is dramatically upregulated in these cells immediately before the entire mitochondrial population is degraded by mitophagy, although a recent study has questioned whether NIX is essential for removal of mitochondria from reticulocytes [105]. It is also important to note that a mammalian homologue of Atg32p has not yet been identified in mammalian cells. Further research, however, is likely to uncover more molecular components in mammalian cells.

Beyond the mechanism of mitophagy, there also appear to be differences in the stressors that can induce mitophagy in yeast and mammalian cells. As discussed above, studies assessing whether membrane depolarisation acts as a precursor to mitophagy in yeast cells have provided inconsistent conclusions. In mammalian cells, however, depolarisation is closely associated with mitophagy. The first indications that depolarisation of mitochondria is linked to mitophagy were made by Elmore et al. [106] who illustrated that the mitochondrial permeability transition, a pathological state characterised by an increased permeability of mitochondria to small molecules, precedes mitochondrial autophagy in mammalian cells. Subsequent research has further characterised this phenomenon in mammalian cells with regard to the role of PINK1 and Parkin. More recently, it has been demonstrated that mitochondria characterised by reduced $\Delta\Psi_m$ are more likely to be separated from the intracellular population by fission events and that these depolarised organelles are unlikely to re-fuse [107]. These isolated mitochondria are more likely to be removed by mitophagy, supporting the hypothesis that mitochondrial dynamics and mitophagy coordinate to ensure the quality of a cell's mitochondrial population. Narendra et al. [56] subsequently demonstrated that Parkin is recruited in a selective manner to depolarised mitochondria and that Parkin localisation is essential for turnover by mitophagy. Interestingly, Amo et al. [108] found that swollen mitochondria and loss of $\Delta\Psi_m$ evident in PINK1$^{-/-}$ MEFs, which results in fragmentation and increased mitophagy, are due to disturbances in respiratory chain function. This result, which echoes the Suzuki et al. study of pH-effects on yeast mitophagy [63], suggests that permeabilisation may be a consequence rather than a cause of damage in this case. Interestingly, Dagda et al. recently

demonstrated in mammalian cells that localisation of PKA, an upstream modulator of TORC, to the mitochondrial outer membrane prevents mitophagy in PINK1-deficient mammalian cells [109]. In summary, unlike mitophagy in yeast, depolarisation is a well-established precursor to mitophagy in mammalian cells.

While the influence of fission and fusion events on mitophagy is contentious in the yeast literature, the role of mitochondrial dynamics in mitophagy is well-established in mammalian cells (reviewed in [110]). In addition to the important contribution made by Twig et al. [107], other studies have supported the importance of fission and fusion as a means of mitochondrial quality control. Evidence suggests that knockdown of PINK1 results in mitochondrial fission and mitophagy [101], while another study demonstrates that PINK1 and Parkin ubiquitinate and subsequently cause the degradation of mitofusins (proteins involved in fusion events) on damaged mitochondria, promoting their isolation from the healthy mitochondria network [111]. Müller and Reichert [112] have speculated that fission and fusion may still play a role in basal mitophagy in yeast but that the level of such mitophagy may be too low to detect. This clear distinction between the effect of mitochondrial dynamics in yeast and mammalian cells may reflect a shift in the emphasis of mammalian mitophagy from the yeast-like adaptation to starvation to basal, maintenance mitophagy.

Although still in its early stages, preliminary work interrogating the relationship between ROS and mitophagy in mammalian cells suggests that the two are linked. Assessment of PINK1 knockdown by Dagda et al. revealed that ROS and H_2O_2 in particular are important upstream preconditions for effective mitochondrial fission and mitophagy [101]. Schertz-Schouval et al. have also shown that ROS oxidise the mammalian Atg4 protein at a cysteine residue, promoting AP formation and autophagy, as well as perturbing $\Delta\Psi_m$ and causing mitochondrial permeability [113]. These data imply that ROS also play a role in redox regulation of autophagy and potentially mitophagy in mammalian cells. It will be interesting to determine whether ROS-induced APs are also involved in the removal of excess ROS-producing mitochondria. While the limited data available suggest that ROS are more relevant to mitophagy in mammalian cells, more evidence is required before we can begin to speculate on the meaning of these results. The optimisation of techniques used to monitor ROS should allow us to more confidently state the role in mitophagy of these molecules, which are notoriously difficult to follow due to their short-lived and reactive nature.

Differences in the implications of perturbed MAPK signalling for mitophagy are also observed between yeast and mammalian cells. As discussed above, there is strong evidence that specific stress-related MAPK proteins participate in mitophagy regulation in yeast [54]. Interestingly, the MAPK proteins most clearly implicated in mitophagy, the extracellular signal-related kinases (ERKs), are involved in cell proliferation rather than stress response [114]. Other MAPKs variously implicated in mammalian mitophagy and autophagy are known to coordinate stress responses (e.g., c-jun N-terminal kinase (JNK) and p38) [115]. However, the centrality of ERKs in mammalian mitophagy might also support the apparent emphasis on developmental and basal mitophagy in mammalian cells.

In light of such differences between mammalian and yeast mitophagy, it is important to reflect on the role of yeast in mitophagy research. As far as we can infer from the available data, there appear to be fundamental differences between mitophagy in yeast and mammalian cells, even at the level of basic mechanism. This is a reason to question the utility of yeast as a model of mammalian mitophagy. However, even though individual stressors, regulatory pathways or proteins involved in yeast mitophagy may differ from those in mammalian cells, yeasts still offer an opportunity to characterise an independent and highly responsive system of mitochondrial homeostasis. As discussed above, the differences evident in yeast cells and mammalian cells may be a reflection of the more complex role of mitophagy in multicellular organisms. Although mammalian cells exist within less stressful tissue environments, they are faced with greater developmental demands and must maintain their mitochondrial populations for a much longer lifespan. However, mammalian cells must still respond to mitophagy-inducing stress, especially under pathological conditions such as tumour growth and microbial invasion, which are of great clinical importance. The identification of such themes of physiological role and cellular context through yeast research offers a valuable base for studies in more complex mammalian cells. Understanding the relative importance of mitophagy in diverse aspects of cellular life, therefore, offers further depth in our understanding of fundamental cell biology.

6. Conclusion

Considerable advances in the basic mechanism of mitophagy have been described in both yeast and mammalian cells. However, our understanding of mitophagy is not complete, and accumulating evidence indicates that mitophagy is a complex and intricately regulated process within the cell. Even within the yeast literature, there is a significant number of contrasting observations concerning the mechanism and regulation of mitophagy. These differences, not yet fully reconciled, offer to provide researchers with a greater appreciation of the physiological relevance of mitophagy. The excistance of different mitophagy phenotypes observed under various conditions is itself evidence of an elaborate integration of mitophagy into the regulatory networks of the cell and strongly suggests that mitophagy plays an important role in the maintenance of cellular homeostasis. In order to deepen our understanding of this intriguing process, we contend that it is important to comprehensively assess, using a benchmark assay, the effect that individual changes in conditions such as carbon source and means of mitophagy induction have on mitophagy. With a greater understanding of how experimental variables affect mitophagy proteins and regulation, insights from yeast research promise to provide important information about the broader cellular context of this complex process, allowing us to better understand the significance of mitophagy.

Acknowledgment

The authors would like to thank Dr. Dalibor Mijaljica for helpful comments and discussions on the content of this review.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 872091, 11 pages
doi:10.1155/2012/872091

Review Article

Implications of Therapy-Induced Selective Autophagy on Tumor Metabolism and Survival

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Received 16 November 2011; Accepted 14 January 2012

Academic Editor: Masaaki Komatsu

Accumulating evidence indicates that therapies designed to trigger apoptosis in tumor cells cause mitochondrial depolarization, nuclear damage, and the accumulation of misfolded protein aggregates, resulting in the activation of selective forms of autophagy. These selective forms of autophagy, including mitophagy, nucleophagy, and ubiquitin-mediated autophagy, counteract apoptotic signals by removing damaged cellular structures and by reprogramming cellular energy metabolism to cope with therapeutic stress. As a result, the efficacies of numerous current cancer therapies may be improved by combining them with adjuvant treatments that exploit or disrupt key metabolic processes induced by selective forms of autophagy. Targeting these metabolic irregularities represents a promising approach to improve clinical responsiveness to cancer treatments given the inherently elevated metabolic demands of many tumor types. To what extent anticancer treatments promote selective forms of autophagy and the degree to which they influence metabolism are currently under intense scrutiny. Understanding how the activation of selective forms of autophagy influences cellular metabolism and survival provides an opportunity to target metabolic irregularities induced by these pathways as a means of augmenting current approaches for treating cancer.

1. Introduction

In order to evade barriers against cancer progression and treatment resistance, tumor cells undergo metabolic adaptations and develop mechanisms to resist apoptosis [1]. Apoptosis resistance in tumor cells can occur through multiple changes, none of which are mutually exclusive. For example, tumor cells enhance antiapoptotic signaling pathways and upregulate the removal or repair of damaged DNA as well as denatured proteins. Overcoming stressors that activate apoptosis requires higher rates of energy production and necessitates that tumor cells make metabolic changes to sustain antiapoptotic signaling, DNA repair mechanisms, and elevated protein turnover. While anticancer therapies that target these essential processes have been proven effective [2–4], improved outcomes may be achieved by combining them with metabolic inhibition.

Metabolic inhibitors have been shown to improve the efficacy of standard therapies in various cancer types [5–8]. Furthermore, the increase in toxicity that is achieved when metabolic inhibitors are combined with standard therapies is often well tolerated clinically, supporting the feasibility of this approach for treating cancer [9, 10]. As a result, there is a need to increase the development of therapeutic strategies that exploit key metabolic processes in tumors, while having minimal impact on normal cells. Anticancer drugs designed to activate apoptosis by causing mitochondrial depolarization, DNA damage, and misfolded protein aggregates restructure cellular metabolism in ways that could be targeted to enhance the selective killing of tumor cells. Stress induced by these drugs activates selective forms of autophagy that could play a central role in reprogramming cellular metabolism in tumor cells following exposure to anticancer therapy.

During autophagy, double-membraned vacuoles sequester bulk cytoplasm and whole organelles (so-called macroautophagy), or engulf selective cargo for degradation. In recent years, it has been discovered that autophagy selectively degrades damaged cellular constituents, such as the mitochondria (mitophagy) and portions of the nucleus

(nucleophagy), as well as misfolded protein aggregates (ubiquitin-mediated autophagy), during specific types of cellular stress [11–13]. In the following sections, we review general features of autophagy as well as unique characteristics of mitophagy, nucleophagy, and ubiquitin-mediated autophagy and consider how mitochondrial depolarization, nuclear damage, and the accumulation of misfolded protein aggregates induced by anticancer agents may impact tumor cell metabolism and viability.

2. General Features of Autophagy

Selective forms of autophagy share many common features with macroautophagy. It should be noted that the precise localization signals and protein-protein mediators of selective autophagy have not been fully defined; however evidence suggests that structures within the cell are degraded using components of the general autophagy machinery. An elongating phagophore encapsulates cellular cargo in a double-membraned vacuole called an autophagosome and fuses with lysosomes, resulting in the hydrolytic digestion of the autophagosome contents. Permeases efflux the digested cargo from the degradative compartment into the cytosol where molecules serve as either metabolic or biosynthetic precursors. To date, over 30 autophagy-related proteins have been reported downstream of the mammalian target of rapamycin (mTOR), a serine/threonine kinase and master regulator of autophagy [14]. When mTOR is inhibited, it ceases to negatively regulate autophagy [15, 16]. Central to the autophagy pathway is the Beclin1/Vps34 (phosphatidylinositol-3 kinase (PI3K) class III) complex, the ULK complex, and two ubiquitin-like systems: the Atg12-Atg5 conjugation system and the Atg8/microtubule-associated protein 1 light chain 3 (LC3) conjugation system [17–21]. In addition, other factors, such as Atg9L1, appear to be indispensable for autophagy to occur [22]. There are likely other converging pathways required for induction of autophagy and these may be context dependent. Collectively, these components appear to play integral roles in mediating autophagosome formation, elongation, and closure [17–21]. For a detailed discussion of the autophagy signal transduction cascade, the reader is directed to several recent reviews [23, 24]. Here, we focus on key components of the general autophagic machinery and consider how they interact with unique factors associated with various forms of selective autophagy.

3. Mitophagy

3.1. The Mitophagy Pathway. While studying the process of organelle turnover, it was assumed that autophagic degradation of mitochondria was a random process because autophagosomes were observed to contain a variety of cytoplasmic components including proteins, endoplasmic reticulum, peroxisomes, and mitochondria [25]. However, recent evidence suggests that autophagic digestion of mitochondria is a selective process [26] (Figure 1). One way that mitophagy can be induced is by the opening of mitochondrial membrane permeability transition pores (mPTP) and the depolarization of the electrochemical proton gradient across the inner mitochondrial membrane [11, 27, 28]. Following mitochondrial membrane potential ($\Delta\Psi m$) depolarization, PTEN-induced putative kinase 1 (PINK1) localizes to the mitochondria and recruits Parkin, an E3 ubiquitin ligase that forms polyubiquitin chains linked through K27 and K63 on voltage-dependent anion channel (VDAC) proteins on the outer membrane of mitochondria [28]. These polyubiquitin chains appear to serve two purposes: first, to tether clusters of dysfunctional mitochondria together and second, to target these structures for autophagic degradation [28]. Interestingly, both K27 and K63 polyubiquitin linkages have been correlated with lysosomal localization and/or autophagic degradation of proteins [28–30]. These linkages differ from the canonical G76–K48 ubiquitin linkages characteristic of proteins destined for proteasomal degradation [31, 32], supporting the hypothesis that site-specific ubiquitination targets mitochondria for selective autophagic degradation.

The mechanisms responsible for ushering ubiquitinated mitochondria to the nascent phagophore for autophagic degradation are controversial. Initially, Geisler et al. proposed p62 to be the principle mediator of crosstalk between the selective and degradative machinery of mitophagy, as silencing of p62 was observed to inhibit the degradation of mitochondria, polyubiquitin, and Parkin but not the colocalization of these structures following $\Delta\Psi m$ depolarization [28]. This hypothesis is supported by evidence demonstrating that p62 binds K63-linked polyubiquitin [33] as well as the lipidated form of the autophagosome bound protein, LC3 [34], which plays an important role in autophagosome formation and closure [35]. However, contrary to the results of Geisler et al., two recent studies have independently demonstrated that p62 is essential for clustering but not degradation of depolarized mitochondria [36, 37]. These disparate results are difficult to reconcile given that the investigators used the same cell types and siRNAs in their respective studies [28, 36]. However, the existence of p62-independent mitophagy does not exclude the possibility that multiple adapter molecules capable of binding polyubiquitin and LC3 such as p62, Nrb1, and Nix function redundantly to bridge the selective and degradative machinery of mitophagy [36, 37].

In addition to the selective machinery described above, the process of mitophagy also employs conventional proteins associated with macroautophagy and thus can be blocked pharmacologically with general autophagy inhibiting drugs such as chloroquine, 3-methyladenine, and wortmannin [38, 39]. These drugs are commonly used inhibitors of lysosomal acidification and autophagy inducing signals generated by class III PI3Ks. To date, it remains unclear how the autophagic machinery is activated in concert with PINK1, Parkin, and p62 during $\Delta\Psi m$ depolarization. One possibility is that Parkin stimulates the generation of autophagy inducing signals from the Beclin1/Vps34 class III PI3K complex by interacting with the autophagy promoting protein, Ambra1 [40]. In addition, given that mitochondria are responsible for maintaining the majority of cellular

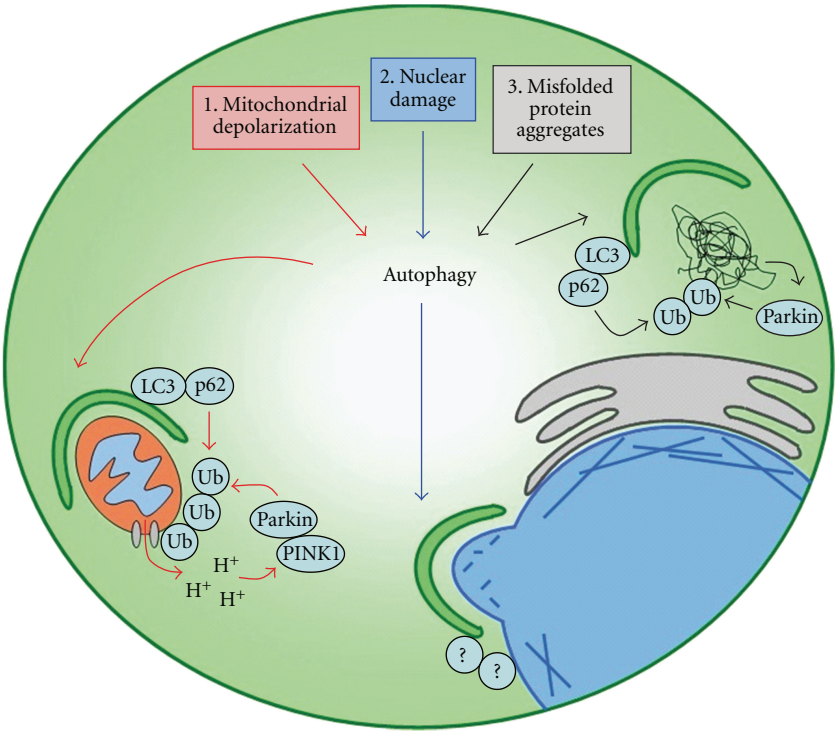

Figure 1: Anticancer agents may activate selective forms of autophagy by causing ΔΨm depolarization, nuclear damage, and misfolded protein aggregates. (1) Drugs that open mPTPs are known to cause ΔΨm depolarization, which may result in the recruitment of PINK1 and Parkin. It is hypothesized that this would promote mitochondrial polyubiquitination and selective targeting to the autophagosome through the LC3:ubiquitin adapter proteins, such as p62. (2) DNA damaging agents may promote the selective autophagy of structurally damaged portions of nuclei in mammals in a process dependent on the cleavage of lamin and emerin intermediate filaments in the nuclear periplasm. To date, the mammalian adapter proteins that target the autophagosome to the nucleus have not been identified. (3) Drugs that inhibit the proteosome are known to cause an accumulation of misfolded protein aggregates in tumor cells, which results in Parkin mediated polyubiquitination and targeting to the autophagosome through p62.

adenosine triphosphate (ATP) pools, it is likely that energy sensors detecting increases in the intracellular ratio of adenosine monophosphate (AMP): ATP, such as 5′ AMP-activated protein kinase (AMPK), activate autophagy during ΔΨm depolarization.

3.2. Mitophagy Inducing Signals Are Generated by Anticancer Agents. Many ionophores including carbonyl cyanide m-chlorophenylhydrazone (CCCP), p-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP), 2,4-dinitrophenol, and fluoride curcumin derivatives have been demonstrated to induce mitophagy by causing ΔΨm depolarization [28, 41, 42]. In addition to activating mitophagy, these uncoupling agents and numerous other drugs that open mPTPs cause mitochondrial swelling and depolarization, signaling for the induction of apoptosis [43]. Among these mPTP targeting drugs are several clinically used anticancer agents, including 1-β-D-arabinofuranosylcytosine, butyrate, doxorubicin, etoposide, lonidamine, paclitaxel, and vinorelbine (Table 1). Drugs targeting mPTPs are attractive for cancer therapy because they mediate cytochrome c release, a potent apoptotic trigger [43]. While some of these drugs have been reported to activate autophagy, their ability to induce mitophagy specifically has not been investigated. Given that mitochondrial depolarization is a powerful inducer of mitophagy as well as apoptosis, further work must be done to determine whether mPTP targeting drugs do in fact activate mitophagy and how this impacts cellular viability.

3.3. Implications of Mitophagy on Tumor Cell Metabolism and Survival during Therapeutic Assault. The fate of cells that undergo ΔΨm depolarization is dependent on a variety of factors including the level of apoptotic signaling from the mitochondria and cellular metabolism. The degradation of dysfunctional mitochondria by mitophagy promotes cell survival by preventing the production and release of toxic byproducts such as reactive oxygen species and cytochrome c that signal for apoptosis [43, 89]. However, the bioenergetic consequences of mitophagy on cellular viability are more complex. On one hand, homeostatic levels of mitophagy may promote cell survival by liberating metabolites that can be oxidized in functional mitochondria for energy. Conversely, hyperactivation of mitophagy renders cells either incapable of meeting energetic demands or solely dependent upon glycolytic substrates for survival [41]. Given that many tumors are inherently dependent on aerobic glycolysis for bioenergetics (so-called Warburg effect) [90, 91], hyperactivation of mitophagy would solidify their glycolytic addiction

Table 1: Clinically used anticancer agents that may induce mitophagy, nucleophagy, and ubiquitin-mediated autophagy in tumor cells.

Drug	Mechanism of action	Cancer type	Confirmed autophagy inducer
Mitophagy			
1-β-D-arabinofuranosylcytosine	DNA synthesis inhibitor [44, 45], mPTP opener [46]	Leukemia [47], lymphoma [48]	no
Butyrate	mPTP opener [49, 50]	Leukemia [51]	yes [50]
Doxorubicin	mPTP opener [46]	Breast [52], lung, melanoma, sarcoma [53]	yes [54]
Etoposide	Topoisomerase inhibitor [55], mPTP opener [46]	Gastric [56], Kaposi's sarcoma [57], lung [58]	yes [59]
Lonidamine	Hexokinase inhibitor [60], mPTP opener [61]	Brain, lung, ovarian [60]	no
Paclitaxel	Microtubule stabilizer [62], mPTP opener [63]	Breast [64], head and neck [65], Kaposi's sarcoma [66], lung [67], ovarian [68]	yes [69]
Vinorelbine	Microtubule formation inhibitor, mPTP opener [70]	Breast [71], lung [72]	yes [73]
Nucleophagy			
1-β-D-arabinofuranosylcytosine	DNA synthesis inhibitor [44, 45], mPTP opener [46]	Leukemia [47], lymphoma [48]	no
Camptothecin	Topoisomerase inhibitor [74]	Gastric [75], lung [76], pancreatic [77]	yes [78]
Cisplatin	DNA intercalating agent [79]	Ovarian [68], lung [68]	yes [80]
Etoposide	Topoisomerase inhibitor [55], mPTP opener [46]	Lung [58], gastric [56], Kaposi's sarcoma [57]	yes [59]
Ubiquitin-mediated autophagy			
Bortezomib	Proteasome inhibitor [81–83]	Mantle cell lymphoma [84], multiple myeloma [85]	yes [81–83]
NPI-0052	Proteasome inhibitor [86]	Leukemia [87], multiple myeloma [88]	yes [86]

by diverting the flux of metabolites away from the mitochondria. Evidence in support of this hypothesis has been demonstrated in HeLa cells, a human tumor cell line that does not endogenously express Parkin, and thus cannot undergo $\Delta\Psi$m depolarization-induced mitophagy [41]. When HeLa cells are pretreated with the $\Delta\Psi$m depolarizing agent, CCCP, the cells survive, presumably because they are able to utilize amino acids and other metabolites in the mitochondria to generate energy [41]. However, HeLa cells pretreated with CCCP and transfected with Parkin do not survive glucose withdrawal because their mitochondria are degraded by mitophagy, preventing oxidative metabolic pathways from sustaining energy pools [41]. In this model, it appears that Parkin-dependent mitophagy may promote survival by coordinating a metabolic shift from oxidative phosphorylation to glycolysis when mitochondria become dysfunctional. However, when cells undergo excessive mitophagy, the nutrient environment of the cell dictates whether cells will survive or succumb to energy crisis followed by cell death (Figure 2).

This finding may have important implications for chemotherapeutic strategies for treating some cancers. For example, administration of glycolytic inhibitors in combination with mitophagy inducing chemotherapies may potentiate killing of tumor cells as a result of increased tumor cell dependency on glycolysis following excessive mitophagy. This may explain why the efficacies of several chemotherapies that result in mPTP opening, such as paclitaxel and doxorubicin, are enhanced significantly when administered with lonidamine, a combinatorial hexokinase inhibitor and mPTP opener [92, 93].

4. Nucleophagy

4.1. Nucleophagy Pathway in Saccharomyces cerevisiae and Mammals. Maintaining proper structure, organization, and dynamics of the nucleus is essential for the vitality of most cell types [94]. Emerging evidence suggests that the selective digestion of portions of the nucleus by autophagy plays a central role in upholding nuclear integrity when structural damage occurs [12]. While nucleophagy in mammalian cells has recently been reported [12, 95–97], this process has primarily been described in yeast [94]. In most yeast models, nutrient deprivation is the stressor of choice used to induce nucleophagy [98, 99]. Following nutrient deprivation, junctions between nuclei and vacuoles (the yeast lytic compartment) are seen to increase in surface

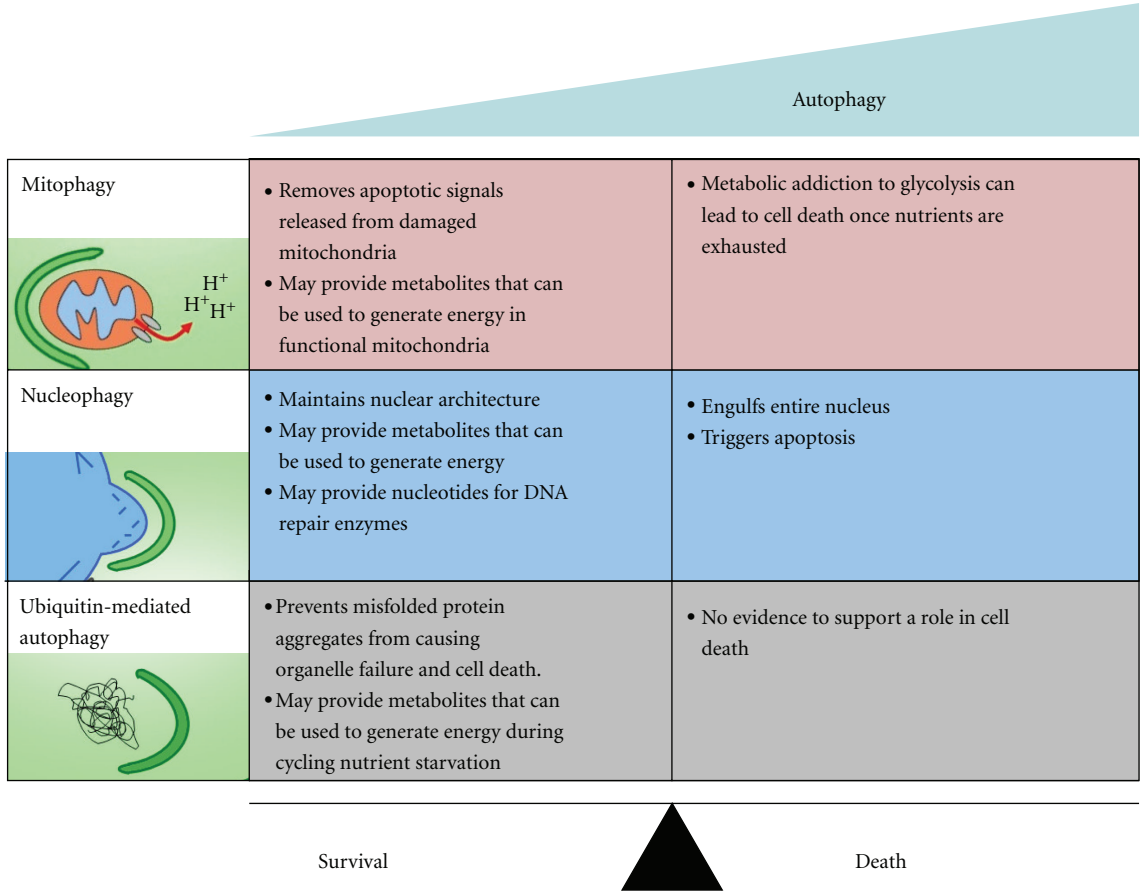

Figure 2: Mitophagy, nucleophagy, and ubiquitin-mediated autophagy are associated with cell survival or cell death depending on the level of activation. A homeostatic level of mitophagy promotes cell survival by liberating nutrients and by clearing dysfunctional mitochondria that signal for apoptosis. Conversely, hyperactivation of mitophagy can lead to a loss in the cell's ability to generate ATP, resulting in cell death. Similarly, a homeostatic level of nucleophagy protects cells against the accumulation of structural damage to the nucleus and may provide energetic and biosynthetic resources that aid in repair. Nucleophagy also appears to be associated with cell death in specialized cell types facing extreme stress. Ubiquitin-mediated autophagy appears to function solely as a survival pathway that clears misfolded protein aggregates and liberates metabolites that may be used for energy production.

area as a result of interactions between Nvj1p, an outer nuclear membrane protein, and Vac8p, a vacuolar membrane protein [98]. Within nucleus-vacuole junctions, the nuclear envelope begins to form bulges and blebs that pinch off and are sequestered in the vacuole for degradation [99]. This gradual degradation of nuclear content is referred to as piecemeal microautophagy of the nucleus (PMN) [99]. In contrast to macroautophagy, PMN does not involve the formation of autophagosomes to envelop content to be degraded [99]. However, it has been shown that nucleophagy in yeast requires macroautophagic machinery, including the two ubiquitin-like conjugation systems and the PI3K class III complex, to mediate terminal vacuole enclosure and fusion stages [100].

In contrast to PMN seen in yeast, mammalian cells undergoing nucleophagy are able to form large autophagosomes characteristic of macroautophagy [12, 95–97]. These large double-membraned macroautophagosomes are observed to colocalize with LC3 and have been seen to envelop large portions of structurally deformed nuclei as well as small nuclear blebs [12]. While mammalian orthologs of nucleophagy adapters such as Nvj1p and Vac8p have not yet been identified, it has been shown that mutations in A-type lamins and emerin in $Lmna^{H222P/H222P}$ mouse embryonic fibroblasts cause structural deformations in the nuclear envelope resulting in the induction of nucleophagy, possibly through similar selective adapter proteins to those described in yeast [12] (Figure 1).

4.2. Nucleophagy Inducing Signals Are Generated by Anticancer Agents. Cancer treatment regimens often include DNA-damaging agents in an attempt to target the nuclear content of rapidly dividing cells. One feature of many DNA-damaging agents is their activation of caspases that disassemble the nuclear lamina by cleaving lamin intermediate protein filaments. While lamin cleavage is known to increase nuclear envelope plasticity and contribute to nuclear blebbing during apoptosis [101], there is evidence that it also activates nucleophagy. Park et al. demonstrated

that mutated lamins lead to deformations in the nuclear envelope that induced nucleophagy [12]. Therefore, DNA-damaging agents that disrupt the nuclear envelope may be predicted to have a similar effect. Some clinically used anticancer agents that induce DNA damage and lamin cleavage include etoposide, camptothecin, cisplatin, and 1-β-d-arabinofuranosylcytosine [102] (Table 1). In addition to these drugs, the cation vanadyl(IV) has been confirmed to activate oxidative stress and DNA damage resulting in nucleophagy of whole chromosomes in mitotic cells [96, 97]. While the exact mechanism of nucleophagy induction following vanadyl(IV) exposure has not been elucidated, it is possible that it may involve a similar lamin-dependent mechanism.

4.3. Implications of Nucleophagy on Tumor Cell Metabolism and Survival during Therapeutic Assault. Activation of nucleophagy appears to be a double-edged sword. In some models, activation of nucleophagy in response to DNA damage has been demonstrated to promote cell death by degrading whole chromosomes in oxidatively stressed mitotic cells [96, 97]. Similarly, whole autophagic degradation of the nucleus has been seen in protozoans such as *Tetrahymena thermophila*, leading to programmed cell death, albeit through different autophagic machinery than what is observed in yeast or mammals [103]. In contrast, nucleophagy has also been demonstrated to promote survival in mammalian cells by maintaining nuclear structure, and possibly through the release of nutrients for energy production [12]. To date, nucleophagy has yet to be defined in tumor cells. However, several DNA damaging anticancer agents may induce nucleophagy in tumors cells as a result of their ability to cause cleavage of lamin filaments [102] (Table 1). Some of these drugs have also been shown to facilitate a cytoprotective, autophagy-dependent surge of ATP [59], raising the possibility that nucleophagy contributes in mediating this ATP surge. It may be that ATP pools are utilized to fuel the energy costly process of DNA repair, the perpetuation of nucleophagy, or both. In addition, liberation of nucleic acids through the nucleophagic degradation of damaged DNA may contribute to increased rates of DNA repair by providing substrate for DNA repair enzymes. To counteract the potential protective role of nucleophagy, anticancer agents that induce this process could be combined with inhibitors of amino acid or lipid catabolism (major macromolecules associated with the nuclear envelope) or inhibitors of nucleophagy itself. However, given the controversial role of nucleophagy in promoting cell survival and cell death (Figure 2), further consideration must be given to systemic inhibition of nucleophagy for cancer therapy.

Another caveat to the systemic inhibition of nucleophagy is that this may lead to off-target toxicity in normal tissue. By removing the potential survival advantage imparted by nucleophagy, normal cells with DNA damage caused by nonspecific therapeutics may succumb to normal apoptotic pathways. In addition, long-lived cells such as neuronal tissue or immunological memory cells may require nucleophagy for normal maintenance of nuclear structure. Dysregulation of this process may lead to unforeseen toxicities in these cell types.

5. Ubiquitin-Mediated Autophagy

5.1. Ubiquitin-Mediated Autophagy Pathway. Tumor cells inherently have high levels of misfolded proteins due to rapid proliferation and increased intracellular acidification caused by lactic acid production during glycolysis [104, 105]. In response to misfolded proteins, cells have been shown to upregulate molecular chaperones that promote refolding of denatured proteins, proteasomal degradation of soluble misfolded proteins, and ubiquitin-mediated autophagy of protein aggregates [106–108]. The first line of defense against an aggregation of misfolded proteins is the activation of molecular chaperones of the heat shock protein family, which shield hydrophobic surfaces of denatured proteins to aid in restoration of proper folding [106]. If denatured proteins persist, ubiquitin-mediated autophagy is activated [13] (Figure 1). This process requires an intact microtubule cytoskeleton and the cytoplasmic deacetylase, histone deacetylase 6 (HDAC6), presumably to coordinate the transport of protein aggregates, autophagic machinery, and lysosomes [109, 110]. Protein aggregates are subsequently polyubiquitinated through K63 linkages by E3 ubiquitin ligases, such as Parkin [111]. This promotes the recruitment of the autophagosome adapter protein, p62, resulting in selective autophagic degradation [111, 112]. Similar to mitophagy, it appears that K63 linked polyubiquitination selectively targets misfolded proteins to the autophagosome, while crosstalk with the degradative autophagic machinery is mediated through adapters such as p62.

The general autophagic machinery appears to be activated in concert with the selective apparatus of ubiquitin-mediated autophagy by a variety of mechanisms. Following an accumulation of unfolded proteins, activating transcription factor 4 (ATF4) is stabilized, which promotes the activation of autophagy by increasing the transcription of LC3 [81, 113]. In addition, signaling from the IRE1-c-Jun NH(2)-terminal kinase pathway has been shown to be necessary for the activation of autophagy in response to proteasome inhibition [82]. Therefore, it appears that the general autophagy pathway is activated through convergent mechanisms in response to unfolded proteins.

5.2. Ubiquitin-Mediated Autophagy Is Induced by Anticancer Agents. The observation that tumor cells have elevated levels of misfolded proteins, and thus protein turnover, has stimulated interest in targeting components of the proteasome in order to induce proteotoxic stress in tumor cells [114]. Proteotoxicity refers to molecular damage caused by misfolded protein aggregates that can lead to organelle dysfunction and cell death [115]. The most well-known inhibitor of the proteasome, bortezomib (or Velcade(TM)), has been tested in numerous recent clinical trials and is now commonly used for the treatment of multiple myeloma and mantle cell lymphoma [84, 85]. Bortezomib has also shown some promise in other cancers, such as prostate cancer and

non-small-cell lung cancer [116, 117]. Since bortezomib and other proteasome inhibitors, such as NPI-0052, compromise the cell's ability to dispose of misfolded proteins, proteasome inhibition can upregulate ubiquitin-mediated autophagy of misfolded protein aggregates as a compensatory strategy [109, 110, 118] (Table 1). Given that autophagy helps cells to degrade misfolded protein aggregates caused by proteasomal inhibition [86], it is not surprising that preclinical studies have reported increased efficacy of proteasomal inhibitors when coupled with autophagy inhibitors in colon, prostate, and breast cancer cell types [81–83]. The potential for increased therapeutic efficacy of proteasomal inhibition when combined with autophagy inhibition has even led to the initiation of a clinical trial combining bortezomib with the autophagy blocking drug, chloroquine, for the treatment of multiple myeloma (NCT01438177).

5.3. Implications for Ubiquitin-Mediated Autophagy on Tumor Cell Metabolism and Survival during Therapeutic Assault. In contrast to mitophagy and nucleophagy, it is unclear how the selective autophagy of misfolded proteins may restructure tumor cell metabolism. Unlike mitophagy, this process does not appear to skew nutrient utilization toward any particular pathway. Furthermore, there is no evidence that the ubiquitin-mediated autophagy of misfolded proteins promotes bioenergetics in ways similar to nucleophagy. On the contrary, the fact that tumor cells are capable of sustaining the energetically costly process of protein translation to the point where misfolded proteins aggregate and become toxic indicates that cells undergoing this type of stress are not lacking intracellular energetic resources. Collectively, these observations suggest that the ubiquitin-mediated autophagy of misfolded proteins is activated solely to remove harmful intracellular structures out of necessity. However, considering the dynamic metabolic milieu found in the tumor microenvironment, there may be a yet undefined metabolic advantage to this process over prolonged periods of time.

As a result of rapid tumor cell proliferation and fluctuations in local vasculature supplying nutrients to the tumor bed, cancer cells often undergo cycling periods of hypoxia, and presumably starvation [119]. In order to fuel essential cellular processes in the absence of exogenous metabolites, the selective autophagy of misfolded proteins may provide an internal reserve of nutrients that can be utilized during cycles of nutrient withdrawal. Therefore, blocking amino acid catabolism in tumors *in vivo* may prove to be an efficacious adjuvant to proteasome inhibition.

6. Conclusion

To date, anticancer agents that nonspecifically target rapidly proliferating cells remain the best treatment option for many forms of cancer. In order to ensure complete killing of tumor cells, patients are sometimes maintained on these drugs for years at a time, increasing the probability of harmful side effects. Consequently, there is a need to develop strategies to enhance the toxicity of these drugs with greater specificity towards tumor cells so that lower doses of cancer therapeutics can be administered for shorter periods of time with the same or better antitumor effect.

Irregular metabolism is a fundamental hallmark of nearly all cancerous cells [1]. Therefore, finding ways to exploit unique metabolic adaptations and irregularities induced by anticancer agents in tumors may prove to be an effective adjuvant to standard therapies. The activation of selective forms of autophagy that degrade metabolically significant structures such as mitochondria, nuclei, and proteins may be one feature of tumors that can be exploited to cripple tumor survival.

Abbreviations

AMP: Adenosine monophosphate
AMPK: 5′ AMP-activated protein kinase
ATF4: Activating transcription factor 4
ATP: Adenosine triphosphate
CCCP: Carbonyl cyanide m-chlorophenylhydrazone
FCCP: p-trifluoromethoxy carbonyl cyanide phenylhydrazone
HDAC6: Histone deacetylase 6
LC3: Microtubule-associated protein 1 light chain 3
mPTP: Mitochondrial permeability transition pore
mTOR: Mammalian target of rapamycin
PI3K: Phosphatidyl-inositol-3 kinase
PINK1: PTEN-induced putative kinase 1
PMN: Piecemeal microautophagy of the nucleus
VDAC: Voltage-dependent anion channel
$\Delta\Psi m$: Mitochondrial membrane potential.

Acknowledgments

The authors thank Katelin Townsend, Dr. Katrin Schlie, and Dr. Quinn Matthews for their helpful comments and discussion. They also wish to acknowledge support from the CIHR New Investigator Award (JJL), CIHR Operating Grant (JJL), CIHR Emerging Team Grant (JJL), British Columbia Cancer Foundation Studentship Award (VIP), Frederick Banting and Charles Best Canada Graduate Scholarship: Masters Award (JES), Ovarian Cancer Canada Teal Heart Scholarship (JES), University of Victoria Graduate Fellowship (JES), and University of Victoria Graduate Award (JES).

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 282041, 12 pages
doi:10.1155/2012/282041

Review Article

Lipophagy: Connecting Autophagy and Lipid Metabolism

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Received 5 December 2011; Accepted 17 January 2012

Academic Editor: Anne Simonsen

Lipid droplets (LDs), initially considered "inert" lipid deposits, have gained during the last decade the classification of cytosolic organelles due to their defined composition and the multiplicity of specific cellular functions in which they are involved. The classification of LD as organelles brings along the need for their regulated turnover and recent findings support the direct contribution of autophagy to this turnover through a process now described as lipophagy. This paper focuses on the characteristics of this new type of selective autophagy and the cellular consequences of the mobilization of intracellular lipids through this process. Lipophagy impacts the cellular energetic balance directly, through lipid breakdown and, indirectly, by regulating food intake. Defective lipophagy has been already linked to important metabolic disorders such as fatty liver, obesity and atherosclerosis, and the age-dependent decrease in autophagy could underline the basis for the metabolic syndrome of aging.

1. Introduction

Autophagy, or the process of degradation of intracellular components in lysosomes, has been traditionally linked to cellular energy balance and to the cellular nutritional status [1, 2]. In fact, although during the recent revival of the autophagic process, most of the emphasis has been placed on its role in other cellular functions such as cellular quality control, remodeling, or cell defense, the first descriptions of the autophagic process in the early 1960s already stated that conditions such as starvation lead to its activation [3–5]. These early studies proposed that autophagic activation during starvation was necessary to maintain the cellular energetic balance. Later studies in yeast, in fact confirmed that activation of autophagy was essential to preserve cellular viability during nutritional starvation (nitrogen depletion in yeast), and that mutants defective in autophagy were lethal [6, 7]. In most of these studies emphasis was placed on the ability of autophagy to supply through degradation of protein products the amino acids required to maintain protein synthesis under the extreme nutritional conditions.

However, the contribution of autophagy to the cellular energetic balance may not be solely dependent on this capacity to provide free amino acids, which in fact, are a relatively inefficient source of energy when oxidized to urea and carbon dioxide. Recent studies support that autophagy can also provide energetically more efficient essential components, such as free fatty acids (FFAs) and sugars. In this paper, we focus on the contribution of autophagy to lipid catabolism and the consequences of this novel autophagic function in the cellular energetic balance as well as in specific lipid-mediated regulatory functions. Lastly, we also discuss the possible implications of alterations in the autophagic breakdown of lipids in human health and disease, with emphasis on common metabolic disorders.

2. Mobilization of Lipid Droplets by Autophagy

2.1. The Upgrading of Lipid Droplets to the Organelle Category. Cells store fat in the form of lipid droplets (LDs)—intracellular deposits of lipid esters surrounded by a monolayer

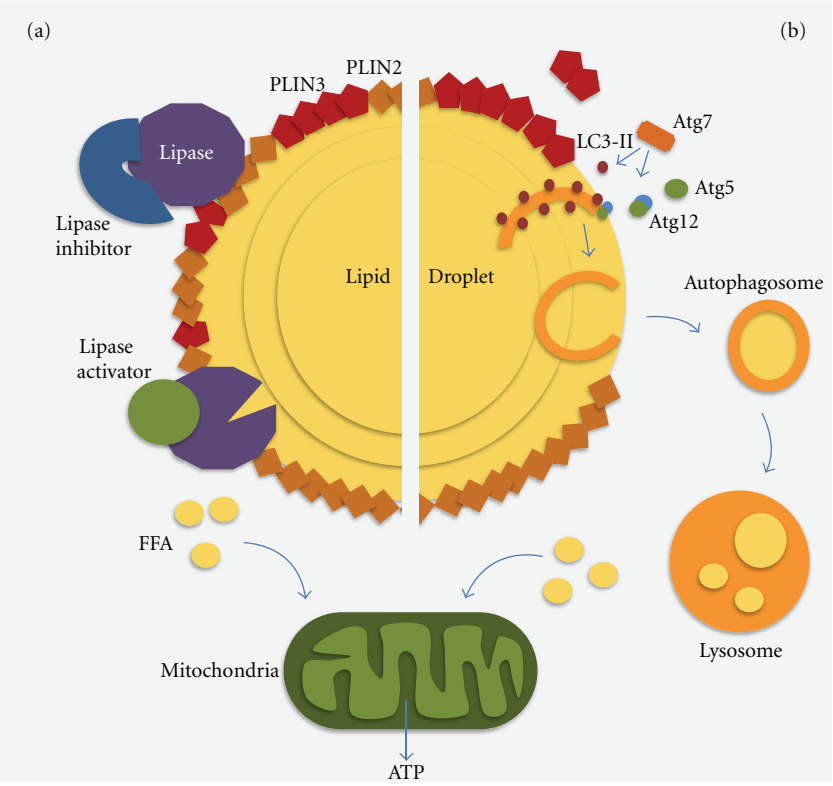

Figure 1: Lipolysis of lipid droplets. (a) Schematic representation of the main lipid and protein components of lipid droplets (LDs) and mechanisms of lipid mobilization (lipolysis) by cytosolic lipases. (b) Lipolysis by lipophagy. Schematic representation of the formation of autophagic vacuoles at the surface of an LD. PLIN: perilipin; Atg: autophagy-related protein; FFA: free fatty acids.

of phospholipids and separated from the hydrophilic cytosolic environment by a coat of structural proteins, known generically as perilipins (Figure 1) [8, 9]. Despite their misleading appearance of inert stores, studies during recent years have revealed that LD are sites of high activity and that their functions are not limited to passive store of lipids. In fact, their dynamic nature, multifunctionality, and defined identity have now conferred upon them the category of intracellular organelles [8, 9]. Furthermore, as many other organelles, LDs have been shown to interact in a regulated manner with other intracellular compartments (likely, to provide them with specific lipids for their membranes) and to adapt to changes in the cellular environment [10, 11]. A growing theme in the field of LD research is now the identification of functions for the LD beyond those related to lipid metabolism or supply of membrane lipids. Pioneering among those has been the finding that the hydrophobic matrix of the LD can become a sequestering surface for misfolded proteins that if left free in the cytosol could organize into oligomeric and aggregated products highly toxic for cells [12, 13]. LD sequestration of proteins does not only apply to pathogenic proteins destined for degradation, but also may have a regulatory role in the availability of some fully functional proteins. For example, certain histones elude nuclear translocation through dynamic and reversible interactions with LD [14]. Interestingly, pathogens such as some types of viruses, have found in the LD ideal platforms for assembly [15, 16].

2.2. Mobilization of Intracellular Lipids through Lipolysis: Novel Role of Autophagy. Although lipid droplets are particularly prominent in the adipose tissue—where they organize as a single large droplet (up to 100 μm diameter) that occupies almost the totality of the cytoplasm—all cells contain lipid droplets to variable extents that range from 0.1 to 10 μm. In addition to size differences, the adipose tissue LD has a core predominantly formed by triglycerides (TGs) whereas in most cells cholesterol and TG share the nuclear core of the LD [8, 9].

LD originate from the ER and maintain a close connection with this organelle, which facilitates exchange of lipids and proteins between both compartments to accommodate to the metabolic requirements of the cell [10]. Mobilization of the lipids inside the LD occurs through lipolysis. The interaction of lipases present at the surface of the LD with the structural proteins that surround LD and with inhibitory proteins in the cytosol contributes to modulate the rate of lipolysis [17]. Cells activate lipolysis not only when they need energy but also in response to a large affluence of lipids to prevent stores from becoming compromisingly enlarged for the cell.

Although, traditionally, mobilization of LD by lipolysis has been solely attributed to the LD-associated lipases, recent studies have revealed a role for autophagy in LD breakdown (Figure 1). The presence of lipases in the lysosomal lumen, along with a large variety of hydrolases such as proteases, glycases and nucleases, has been acknowledged since the early

days of the discovery of this organelle. However, lysosomal lipases, also known as "acid lipases" because of their optimal acidic pKa, were thought to serve mainly in the degradation of lipids contributed by the diet through endocytosis or those present in the membranes of the organelles digested during the autophagic process. The elevation of LD to the category of cytosolic organelles was in part a motivation to address their turnover by autophagy. This catabolic process is capable of sequestering whole cytosolic organelles inside double-membrane vesicles known as autophagosomes, which deliver this cargo to lysosomes upon heterotypic fusion with their membrane [18, 19]. Each of the events of the autophagic process is coordinated by a complex network of more than 32 genes and their protein products (autophagy-related genes (ATGs) and proteins (Atgs)). Atgs participate at the level of: (1) activation, (2) nucleation of the autophagosome membrane that forms *de novo* through conjugation of proteins and lipids from different cellular compartments, (3) elongation of the membrane and sealing to form the autophagosome, (4) trafficking toward the lysosomes, and (5) fusion of the two membranes [18].

The first hint that LD could become substrates of the autophagic process originated from studies in cultured hepatocytes knocked down for Atg5, one of the genes essential for the formation of autophagosomes [20]. Hepatocytes respond to an acute oleic challenge by increasing lipolysis, which would prevent massive enlargement of the LD compartment. Oleic challenge resulted in a marked increase in the number and size of LD in cells with compromised macroautophagy. The same was true *in vivo*, when knockout in liver of another essential autophagy gene (Atg7) led to an accelerated development of liver steatosis (fatty liver) in the autophagy compromised animals, when compared to control animals [20]. Detailed biochemical and functional analyses helped in establishing that the observed lipid accumulation did not result from increased formation of LD or reduced lipid secretion from hepatocytes, but that, instead, it could be explained almost exclusively on the basis of reduced lipolysis [20].

It is possible that, through mechanisms yet to be identified, changes in autophagic activity may modulate the LD-associated lipases and contribute to the observed changes in lipolysis. However, independent of this possibility, there is now evidence that the autophagic system contributes directly to the mobilization of lipids from LD to lysosomes, wherein luminal lipases mediate their lipolysis. In fact, neutralization of the lysosomal pH, that would have a marked effect on the lysosome-resident lipases but does not modify the activity of the cytosolic lipases, was enough to almost completely block the lipolysis activated in response to a lipid challenge [20].

2.3. Recognition of LD by the Autophagic System. Sequestration of cytosolic components inside the forming autophagosome was considered for a long time a nonselective in-bulk process by which cytosolic material was randomly delivered for lysosomal degradation. However, recent years have revealed the existence of a growing number of proteins dedicated to the tagging and recognition of cytosolic components for autophagic degradation [21]. In the case of intracellular protein aggregates, the presence of polyubiquitinated chains formed through specific types of linkage (the best characterized uses the lysine 63 in ubiquitin to link one ubiquitin moiety to another in the polyubiquitin chains) is the tag identified by the cargo-recognition machinery [22]. Similarly, ubiquitination of proteins on the surface of peroxisomes and of different pathogens contribute to their segregation towards the autophagic system [23, 24]. However, ubiquitin is not the only signal identified as marker for autophagic degradation. Selection of mitochondria for mitophagy (selective degradation of mitochondria by autophagy) has been shown to occur through different mechanisms, which likely coexist in most cells. In most cases, changes in structural components of the mitochondrial membrane are identified by partner cytosolic proteins (such as Parkin or Nix), that once bound at the surface of this organelle, tag it for degradation [25]. All cargo recognition molecules or autophagy receptors share their ability to bind to the tagging molecule in the organelle to be degraded as well as to specific components of the autophagic machinery (in almost all cases the light chain protein 3 or LC3) [21, 26]. This recruitment of autophagic components toward the cytosolic material to be degraded is proposed to initiate the *in situ* formation of the autophagosome around this material and to mediate selectivity.

Some levels of lipophagy may always occur even during the random sequestration of cytosolic material by "in-bulk" autophagy. In fact, analysis of the components inside autophagosomes in cells maintained in basal conditions revealed the presence of lipid material and LD structural proteins inside these vesicles, along with other cytosolic material [20]. However, as described in the previous section, when lipophagy is activated in response to a lipid challenge or prolonged starvation, there seems to be a switch toward the preferential sequestration of LD, supporting some level of selectivity in this process [20]. An intriguing observation in the studies of hepatic lipophagy was the fact that LD do not always seem to be sequestered as a "whole" by the autophagosomes, but, on the contrary, in many instances, only fractions of the LD underwent autophagy (Figure 1) [20]. Thus, membranous structures enriched in LC3, in support of their autophagic origin, appear to grow from the surface of the droplet towards the inner core. Often these membranes curve to finally seal, giving rise to double-membrane vesicles of a slightly smaller size than a conventional autophagosome (50–100 nm) and contain only components of the LD in their lumen. Interestingly, formation of the membranes seems to be polarized in only one site on the surface of the LD. Other components of the autophagic machinery, such as Atg5 and Atg7, also localize to these areas of the LD in further support that formation of the limiting membrane occurs at the surface of the LD (Figure 1) [20].

In the process of *de novo* formation of the autophagic membrane, Atg7 acts as the enzyme regulating conjugation of Atg12 to Atg5 (to serve as scaffold for assembling other components of the forming membrane), as well as the conjugation of LC3 to a lipid (phosphatidylethanolamine, PE) to generate LC3-II that is one of the best characterized structural components of the autophagosomes [18].

Interestingly, Atg7 is not required for the recruitment of LC3 to the LD, since this protein, although in its nonconjugated form, is still found associated to LD in cells defective in Atg7 [20]. PE, the only lipid known to conjugate to LC3, is among the phospholipids that contribute to form the delimiting phospholipid monolayer of LD [27]. The specific mechanism by which the limiting autophagosome membrane grows from the LD surface is still poorly characterized, but the presence of membrane-like structures in the LD core has been previously described [28]. In addition, although the core is predominantly composed of lipids, some proteins can also be detected in this region. Early studies have suggested that these proteins may form complexes with phospholipids to form structures compatible with the hydrophobic environment inside the LD [29]. In this respect, conjugation of LC3 to PE on the surface of the LD may provide the right conformation for the forming membrane to advance towards the inside of the LD.

The determinants for autophagic initiation on the surface of a LD remain unknown. Polyubiquitination has been detected in polarized areas of LD in part resulting from the accumulation of clusters of polyubiquitinated apolipoprotein B (ApoB) on their surface [13]. The fate of ApoB in this location seems to be to undergo lysosomal degradation. Whether or not the lysosomal degradation of ApoB occurs as a result of the activation of lipophagy and how the accumulation of this protein contributes to the initiation of the process requires future investigation. Recent studies have shown now the integration into the LD surface of the ancient ubiquitous protein 1 (AUP1), which bears a c-terminus able to bind enzymes involved in ubiquitination [30]. Whether or not the presence of AUP1 in LD is necessary or precedes the arrival of the autophagic machinery requires future investigation.

Of particular interest is the fact that LDs have been shown to dynamically interact with two of the organelles that have been proposed as sites of formation of the limiting membrane of the autophagosomes—the ER and the mitochondria (Figure 1) [11, 31]. Interactions with the ER may be related to LD biogenesis, as this is the compartment from where these organelles originate, but may also favor the distribution of lipids from the LD towards other organelles through the endosecretory pathway. In the case of mitochondria, the close interaction between LD and the outer-membrane of this organelle could facilitate delivery of the FFA released by lipolysis for mitochondrial β-oxidation. However, considering the described association of the autophagic initiation complex to punctual areas in the membrane of the ER and the mitochondria, and the formation of cup-like precursors of the limiting membrane of the autophagosomes from these regions [32, 33], it is tantalizing to at least propose that the previously described interactions of LD with these organelles, could also contribute to the initiation of their autophagic degradation.

3. Physiology of Lipophagy

3.1. Liver Lipophagy. As described in previous sections, mobilization of LD by autophagy was first observed both in cultured hepatocytes in response to fatty acid exposure, and in liver of mice maintained on a diet enriched in fat for prolonged periods of time (4 months) [20]. The liver responds to the massive influx of lipids from the blood by upregulating LD biogenesis, as a mechanism of defense against the toxicity of FA, which upon esterification get converted into TG and stored into LD [17]. However, in order to prevent uncontrolled expansion of LD, activation of lipolysis also occurs under these conditions and contributes to maintain LD size. Failure to regulate lipid accumulation in hepatocytes may be the basis of pathogenic conditions such as liver steatosis and steatohepatitis [8]. Autophagy has now been added to the mechanisms that control the growth of the hepatic LD under these conditions (Figure 2).

Besides lipid challenges, other stimuli such as starvation also engage the lipolytic contribution of the autophagic system. Classic measurement of protein catabolism in liver during starvation, revealed that most of the protein degradation in this organ occurs during the 4–6 h that follow starvation, and that protein breakdown, even of autophagic origin, decreases markedly once the 8–10 h of starvation are reached [4]. However, this decrease in autophagic degradation of proteins by autophagy is not equivalent to a decrease in overall autophagic activity. Formation and clearance of autophagosomes seems to be maintained throughout the starvation period, but there is a consistent change in the type of cytosolic components sequestered in these vesicles [20]. Whereas cytosolic proteins and some organelles are the main cargo of the autophagic process during the early hours of starvation, as lack of nutrients persists, there is a gradual change towards preferential sequestration of lipid droplets inside autophagosomes (Figure 2) [20]. This selective autophagy of lipid stores, known now as lipophagy, accounts for a high percentage of the lipolysis occurring during prolonged starvation in liver.

Interestingly, although lipophagy is markedly upregulated in response to lipid challenges and during prolonged starvation, it is possible that a certain percentage of degradation of lipid stores in lysosomes occurs continuously in many cell types. Thus, blockage of autophagy through knockdown of any of the essential Atg in hepatocytes in culture, leads to a significant increase in the number of lipid droplets in these cells even when maintained under normal nutritional conditions and in the absence of any additional challenge [20]. Similar basal lipophagy has also been observed in cell types not typically known to store fat such as fibroblasts, macrophages, T cells, dendritic cells, lymphoblasts, glia, striatal cell lines, and even primary neurons [20, 34–36], although the relative contribution of autophagy to basal lipolysis may vary depending on the cell type. Further studies are necessary to determine the reasons behind the coexistence of the two different mechanisms for lipolysis—the one mediated by the cytosolic lipases and the one occurring through the autophagic system. It is possible that activation of one or the other may mainly lead to quantitative differences (i.e., lipophagy may be able to provide large amounts of FFA in shorter time). However, because the lysosomal lipases have been poorly characterized, it is also possible that the quality and type of the resulting lipolytic

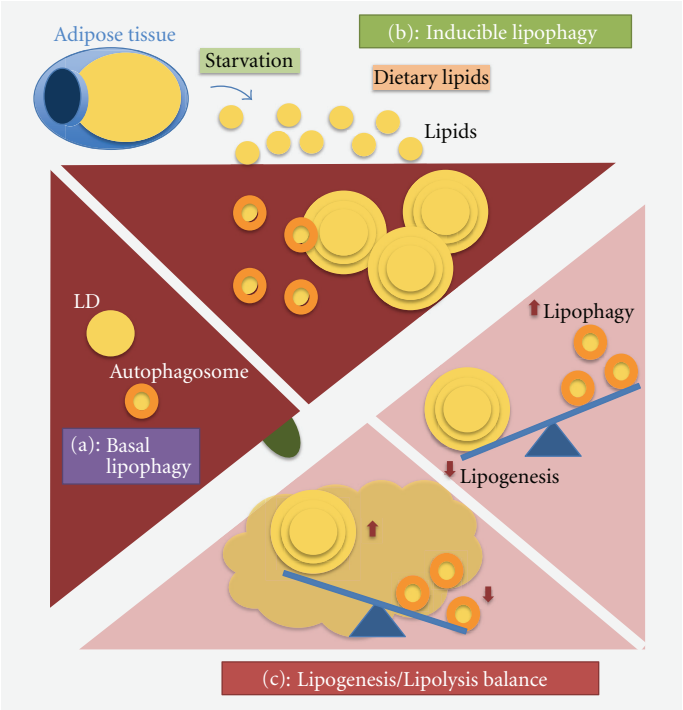

Figure 2: Autophagy and lipid metabolism in liver. (a) Basal lipophagy: some level of mobilization of LD by autophagy occurs continuously in all tissues including the liver. (b) Inducible lipophagy: stimuli such as prolonged starvation or maintained lipid challenges induce liver lipophagy to regulate LD growth. Failure to upregulate autophagy under these conditions could results in liver steatosis. (c) Lipogenesis/lipolysis balance: autophagy may also contribute to LD formation by mechanisms still unknown. A partial blockage of the autophagic process may modify the lipogenic-lipolytic balance in one direction or another depending on the cellular conditions.

products differs between cytosolic and lysosomal lipases. Lastly, in light of the growing evidence in support of the heterogeneity of the cellular LD, it is also plausible that the two lipolytic systems target different subpopulations of LD.

3.2. Specialized Lipophagy in the Hypothalamus. As detailed earlier, lipophagy appears to contribute significantly to the mobilization of cellular lipids for provision of energy [2]. However, the identification of lipophagy in cell types other than those involved in lipid storage, such as in immune or neuronal cells, suggests that autophagic turnover of lipids is perhaps a generic mechanism for the utilization of cellular fat stores in diverse cell types. In fact, recent reports have now included two additional cell types; hypothalamic neurons [35] and macrophage foam cells [37], to the increasing list of cells where lipophagy has been shown to be present and functionally important.

The neurons within the mediobasal hypothalamus (MBH) form part of a focal neural network that integrates nutritional and hormonal information from two main cellular kinases, the mammalian target of rapamycin (mTOR) [38] and the phosphoinositol-3-kinase (PI3K) [39] to control food intake and energy balance [40]. Hypothalamic fatty acid metabolism, amongst other neuronal mechanisms, has been linked to the regulation of appetite [41, 42]. Although recent work suggests that neuronal FFA availability and oxidation provide the energetic requirements for activation and firing of orexigenic agouti-related peptide (AgRP) neurons [41], the lipolytic mechanisms that generate neuron-intrinsic FFA have remained poorly elucidated. Since autophagy is activated by starvation in most cells [43], it was plausible that autophagic mobilization of lipids in the hypothalamus could contribute to the generation of neuronal FFA during starvation that, in turn, trigger mechanisms driving food intake. In fact, a recent study in mice knockout for Atg7 in AgRP neurons shows that the hypothalamus, indeed, needs autophagy to upregulate expression of AgRP in response to nutritional depletion [35]. This indicates the distinctive characteristic of hypothalamic neurons in their ability to activate autophagy, quite unlike other regions of the brain in which autophagy does not seem to be under this type of nutritional regulation [1]. The activation of autophagy occurred in parallel to starvation-induced increases in hypothalamic FFA uptake (Figure 3), suggesting that, as in the case of the liver [20], acute FFA stimulus might be a mechanism for activation of hypothalamic autophagy during starvation. Indeed, the exposure of hypothalamic cells to FFA or FFA-rich serum from starved rodents increased autophagy (Figure 3) [35].

The activation of autophagy in hypothalamic neurons upon acute lipid stimulus associated with increases in the levels of phosphorylated AMPK and ULK1 [35], a kinase involved in the regulation of the autophagic process and recently described to be an AMPK substrate [44]. These findings indicate that hypothalamic AMPK and ULK1 may contribute to a FFA sensing mechanism that modulates

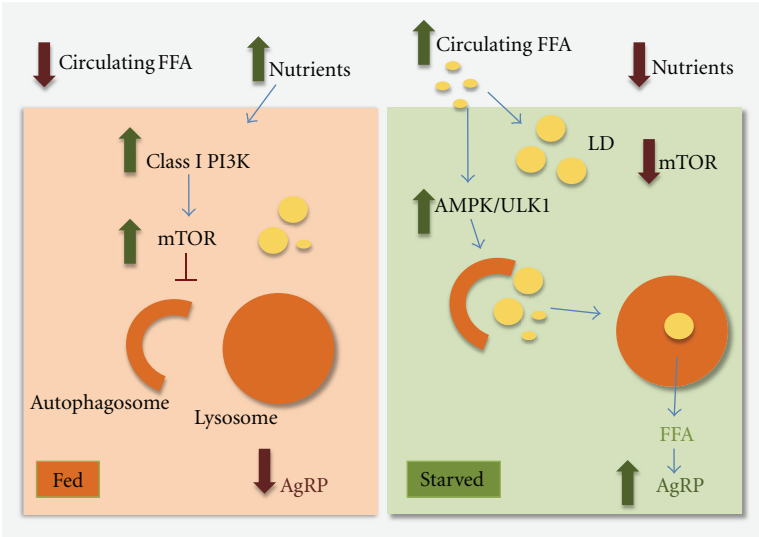

Figure 3: Conceptual model for hypothalamic lipophagy in control of food intake. In the fed state, active PI3K/mTOR signaling maintains autophagy at basal lower levels. Starvation increases circulating free fatty acids (FFAs), which activate hypothalamic autophagy by mechanisms that may in part require activation of AMPK/ULK1. These FFAs taken up by hypothalamic neurons are rapidly esterified into neutral lipids within lipid droplets. Activation of hypothalamic autophagy mobilizes neuronal lipids for the controlled availability of neuron-intrinsic FFAs that increase AgRP expression and food intake. AgRP: Agouti-related peptide, AMPK: AMP-activated protein kinase, FFA: free fatty acids, LD: lipid droplets, PI3K: phosphoinositide 3-kinase, mTOR: mammalian target of rapamycin, and ULK1: unc-51-like kinase 1.

autophagy in response to changes in nutrient signals. However, the mechanisms connecting FFA release and AgRP expression remain unknown for the most part. Although it is possible that FFA modulate some of the signaling cascades involved in AgRP regulation [45], a direct effect of the autophagic process on secretion of AgRP-containing vesicles cannot be ruled out.

The immediate fate of the FFA taken up by hypothalamic cells is esterification into neuronal LD, which underscored the requirement of a lipolytic mechanism to liberate neuronal FFA during starvation. Indeed, the activation of hypothalamic autophagy observed under these conditions, leads to increased mobilization of neuronal lipids to lysosomes [35]. The physiological consequence of these interactions is the generation of neuronal FFA, since inhibiting lysosomal hydrolysis or interfering with the autophagic process significantly decreases hypothalamic FFA levels. Lipophagy-generated hypothalamic FFA directly regulate the increase in orexigenic AgRP expression that occurs in AgRP hypothalamic neurons in response to starvation or to exposure to extracellular FFA [35]. In fact, selective blockage of autophagy in AgRP neurons has been shown to reduce fasting-induced increases in hypothalamic AgRP levels, food intake, and body weights. Interestingly, mice deficient in autophagy in AgRP neurons also displayed higher levels of the anorexigenic peptide α-melanocyte stimulating hormone (MSH), which is produced within the adjacent proopiomelanocortin (POMC) neuronal population, which could contribute to the reduced adiposity in these mice.

In this respect, it is interesting to note that the effect of autophagy in lipid mobilization and the downstream consequences of neuronal lipophagy may be very neuronal type-specific. For example, a recent study using acute intrahypothalamic injection of siRNA against Atg7 revealed increased adiposity and glucose intolerance in the injected mice [46], in contrast to the lean phenotype observed when only AgRP autophagy is compromised. It is possible that adiposity in this model occurred from concurrent reduction of atg7 in both AgRP and POMC neuronal populations, or from autophagic deficiency in additional cell types, for instance hypothalamic glial cells, also shown to regulate glucose homeostasis [47]. However, as the actual impact of the siRNA injection in the autophagic flux in this model is not known, is not possible to discard that differences in efficiency of the autophagic compromise between POMC and AgRP neurons are the real reason behind the different phenotype, or even that autophagy was not affected in the AgRP neurons and the phenotype only resulted from reduced POMC autophagy.

Although future investigation is required to clarify the cell type differences and to identify additional stimuli that may also modulate hypothalamic autophagy, the current findings highlight an exciting new role for lipophagy in control of food intake and whole body energy balance by modulating the "controlled production" of neuronal FFA that regulate AgRP levels [35] (Figure 3). In this way, the contribution of autophagy to the cellular and organismal energetic balance is no longer merely limited to its role in active breakdown of macromolecules or cellular stores to obtain energetic products, but has been raised to a more global regulatory function that includes the modulation of food intake. Forthcoming studies should help in addressing the possible implications of these findings for metabolic diseases such as obesity and the impact that these metabolic changes could have on hypothalamic autophagy.

3.3. Autophagy in the Adipose Tissue. The presence of lipophagy in diverse cell types raises the question whether lipophagy might also serve to mobilize lipids within the principal fat storing organ in the body, the adipose tissue. Surprisingly, recent reports by two independent groups demonstrate a completely new and unexpected function of autophagy in regulating adipose physiology [48, 49], which is quite distinct from the role of autophagy in mobilizing lipids observed in other cell types [20, 34–36]. In fact, adipose-selective knockout of essential autophagy genes in mouse significantly reduced adipocyte lipid droplet content and fat tissue mass [48, 49]. Likewise, blocking autophagy in cultured preadipocytes decreased cellular triglyceride content and levels of key adipogenic transcription factors CEBP-α (CCAAT/enhancer-binding protein *alpha*), CEBP-β (CCAAT/enhancer-binding protein *beta*) and PPAR-γ [48]. Conceivably, reduced expression of adipocyte-specific genes led to the formation of an adipose tissue that predominantly consisted of immature fat-deficient preadipocytes judging by their reduced levels of terminal differentiation markers (fatty acid synthase, fatty acid-binding protein-4 (FABP-4/aP-2), glucose transporter 4 (GLUT4), or stearoyl CoA desaturase 1) [48].

Intriguingly, selective inhibition of autophagy in white adipose tissue (WAT) *in vivo* not only impaired WAT differentiation but also introduced brown adipose tissue-(BAT-) like features in autophagy-deficient WAT [48]. The autophagy-deficient white adipocytes exhibited a cellular morphology that resembled closely that of brown adipocytes. For instance, atg7-deficient white adipocytes displayed increased number of smaller multiloculated lipid droplets and mitochondria, rounded nuclei, and larger cytoplasmic size [48, 49]. The molecular characteristics of the adipose tissue in the autophagy-deficient mice also mimicked those of BAT as reflected by increased levels of brown adipogenic factors, PPAR-γ transcriptional coactivator (PGC-1 α), and uncoupling protein-1 (UCP-1) [48]. Acquisition of BAT-like properties resulted in higher adipose tissue β-oxidation rates in knock-out animals than in controls [48, 49].

The physiological consequences of this shift in WAT phenotype were a decrease in body weight, reduced adiposity and resistance against high fat diet-induced alterations in glucose homeostasis [48, 49]. Although, the mechanism by which autophagy controls adipocyte differentiation or modulates the phenotypic switch from WAT to BAT-like is unclear, a likely possibility is that autophagy modulates levels of key regulatory proteins to control adipocyte cell fate, differentiation and fat storage. However, it is still plausible that specific autophagy-related components may be directly involved in the process of adipogenesis and that this function is not only limited to the adipose tissue. This concept is supported by studies performed at ages before adulthood in the same mouse model null for autophagy in liver used in the discovery of hepatic lipophagy [50]. In contrast to the massive accumulation of lipids observed in the adult animals, very young animals, at least when unchallenged, had consistently lower hepatocyte content of LD [50]. The previously described association of LC3-II to LD [20], also confirmed in this latter work, was proposed to be required for LD formation. Since expression of the enzyme used to flox out the autophagic gene does not start in this mouse model until 3-4 months after birth, it is possible that if autophagy is involved in both formation and mobilization of LD, the partial blockage of autophagy was initially sufficient to cope with the lipophagic requirements of the young animals and prevent the accumulation of LD. However, as the animals reached adulthood, the complete and persistent blockage of the autophagic system tilted the balance between lipogenesis and lipolysis toward the former one, leading to LD accumulation.

This dual involvement of autophagy in lipogenesis (LD formation) and lipolysis proposed in the liver now raises the question of whether a similar dual role could also occur in the adipose tissue. In fact, recent studies with a mouse model deficient in caveolin 1 have shown lipoatrophy of the adipose tissue mediated by massive upregulation of autophagy in this tissue [51]. Future studies with inducible autophagy knockouts in the adipose tissue of adult mice are needed to determine if autophagy does contribute to lipid mobilization also from fully formed adipose tissue.

4. Pathology of Lipophagy

4.1. Dual-Effect of Lipids on Autophagy. After the first observations demonstrating the existence of lipophagy and the upregulation of this process in response to a lipid challenge [20], numerous studies have confirmed the stimulatory effect of dietary lipids on the autophagic process. Upregulation of autophagy in response to increased FFA has been demonstrated in neurons, muscle, pancreas, mammary epithelial cells, liver-derived cells, and even in colon cancer cells [52–56]. Although the mechanisms that modulate the activation of the autophagic process under these conditions are still poorly elucidated, at least in the case of the pancreatic beta cell, autophagic activation has been proposed to occur through activation of the c-Jun N-terminal kinase 1 pathway in a manner independent of ER or oxidative stress [53].

In contrast to this stimulatory effect of a lipid challenge on the autophagic system, an equal number of studies have started to report inhibition of autophagy in response to exposure to high concentrations or particular type of lipids (Figure 4). For example unsaturated FFA such as oleic acid has a marked stimulatory effect on autophagy in many cells, at least up to some concentrations [20, 57, 58]. In contrast, saturated FFA such as palmitic acid—maybe due in part to its lower incorporation into LD—remains in the cytosol at higher concentrations and suppresses autophagy [58]. Likewise, in animals exposed to a high-fat diet for prolonged periods of time it is possible to detect an increase in autophagic activity during the first weeks of treatment, which is progressively followed by a gradual decrease in autophagy. This decrease further contributes to the expansion of the LD compartment, eventually leading to hepatotoxicity and steatosis [20, 57, 58]. Interestingly, the switch from activation to inhibition of autophagy in response to lipogenic stimuli can also be cell type dependent. Thus, same amounts of oxidized low-density lipoprotein that stimulate autophagic activity in schwannoma

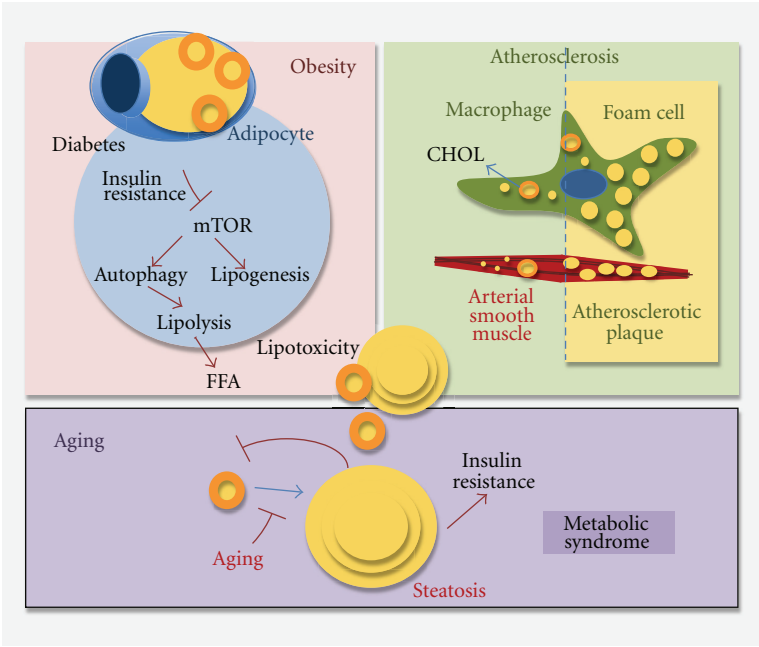

Figure 4: Lipophagy in pathology and aging. Alterations in the autophagic system and in its ability to mobilize intracellular lipids may underline the basis of important human disorders. The possible links of lipophagic malfunctioning with obesity, atherosclerosis, and the metabolic syndrome of aging are depicted.

cells have been shown to be toxic for neuroblastoma cells [54].

Although many mechanisms could contribute to the inhibitory effect of FFA on autophagy, a systematic analysis of the different steps of the autophagic process has revealed a primary defect in the fusion between autophagosomes and lysosomes in cells exposed to high concentrations of FFA or in animals subjected to prolonged high-fat diet [57]. Interestingly, this failure to deliver autophagosome cargo directly to lysosomes is initially compensated for by increasing fusion of autophagic compartments with late endosomes (to generate what is known as an amphisome) [57]. However, as the high levels of intracellular lipids persist, defective intracellular turnover becomes evident, either because of further compromise of the pathway or maybe because of an additional failure in the endocytic system as autophagic cargo builds up in these compartments. Analysis of autophagic vacuoles from animals exposed to a high-fat diet revealed that changes in the lipid composition of the membrane of these vesicles are behind their compromised fusogenicity [57].

This-dual effect of dietary lipids on autophagy and lipophagy should be taken into consideration when contemplating manipulations of the autophagic system as a therapeutic strategy for metabolic disorders.

4.2. Liver Lipophagy and Hepatic Diseases. The fast development of steatosis and fatty liver observed in mice defective for autophagy in this organ [20] strongly supported the contribution of altered autophagy to the pathogenesis of this common disease. In fact, a compromise in hepatic autophagy has been proposed to underline also the basis for the accumulation of LD upon exposure to toxic concentrations of ethanol [59]. Furthermore, recent studies have demonstrated that pharmacological upregulation of autophagy reduces hepatotoxicity and steatosis in an alcohol-induced model of fatty liver [59]. However, future studies are needed before autophagy activation can be used as a generalized treatment against this disease, because, for example, upregulation of the autophagic process in hepatic stellate cells has been shown to favor their activation and consequently initiate liver fibrosis [60]. The liver responds to some stressors through global activation of autophagy, including degradation of lipids, proteins, and organelles. However, in other instances upregulation of autophagy as a protective mechanism against liver injury can be specific for lipophagy. For example, the autophagy upregulated as a first line of defense against alcohol-induced toxicity in liver, selectively targets mitochondria, and lipid droplets, while excluding soluble cytosolic proteins and other organelles [59]. Future efforts should focus in understanding how selective forms of autophagy can be individually modulated for therapeutic purposes.

The recently discovered capability of the autophagic system to mobilize hepatic lipids is also utilized by viruses to favor their replication. Earlier studies have shown that although autophagy is usually an efficient mechanism in the defense against most viral infections, upregulation of autophagy could also favor replication of some viruses such as the dengue virus. Recent studies have demonstrated that dengue virus-dependent induction of autophagy mediates LD breakdown and release of FFA necessary to maintain the high levels of intracellular ATP required for dengue viral replication [61]. Future studies are needed to determine

which subset of hepatotropic virus makes use of lipophagy for their own replication and whether blockage of the autophagic system can be performed in this organ in a selective way to preferentially affect virogenesis but not normal liver metabolism.

4.3. Metabolic Disorders. The finding that autophagy is required for adipogenesis has elicited a considerable interest in the interplay between autophagy and metabolic disorders such as obesity.

Studies performed in human subjects with different types and degrees of obesity have revealed a direct correlation between autophagic activity and the sizes of various fat depots. Interestingly, autophagy was found to be inappropriately active in omental fat tissues extracted from obese individuals, and, in fact, autophagic activity was remarkably raised in insulin-resistant obese subjects [62]. This indicates that although functional autophagy may be a requirement for adipose differentiation during development, autophagy might also be involved in the maintenance of adipose tissue size and lipid storage in adults. The fact that autophagic upregulation occurs before obesity-associated morbidity becomes manifested, still leaves open the possibility that this system could be activated as a defensive mechanism against the increase in intracellular lipids. However, the final outcome and autophagy effect may be very different depending on the metabolic status. For example, in adipocytes from type 2 diabetes patients characterized by unresponsiveness to insulin, maintained attenuation of mTOR has been recently described and proposed as the main mechanism responsible for the upregulation of autophagy in these cells. The concomitant increase in LD formation under these conditions along with their enhanced autophagy favors cellular toxicity due to the excessive release of FFA from LD. It is anticipated that blockage of autophagy, or at least downregulation to a normal level, may be better under these conditions [63].

Lipophagy has been recently proposed as a possible defensive mechanism against atherosclerosis, or the thickening of the artery walls due to abnormal accumulation of lipid deposits in macrophage foam cells [37]. The recent finding that autophagy contributes to lipolytic mobilization of LD in macrophages also, provides now a new possible mechanism for the pathogenesis of atherosclerosis. This study has revealed that macrophage lipophagy is upregulated both *in vitro* and *in vivo* in response to lipid loading and that failure to upregulate the autophagic system, in mice defective for this pathway, results in inefficient clearance of cholesterol in macrophages [37]. In light of the inhibitory effect that high concentrations of intracellular lipids can have on autophagy [57], it is reasonable to propose that chronic exposure to high levels of circulating lipids may compromise the autophagic system of the artery wall macrophages and lead to their transformation into "foam cells" as lipids accumulate in their cytoplasm. This massive accumulation of lipids is the seeding for the subsequent formation of the atherosclerotic plaque. Current therapeutic strategies in this disease are aimed at promoting cholesterol efflux from these macrophages to reduce the size of the lipid-enriched plaque that they form beneath the endothelial cells. Consequently, manipulations aimed at enhancing macrophage autophagy and thus favoring cholesterol efflux from these cells, may have therapeutic potential in the atherosclerotic artery walls. Interestingly, the contribution of changes in autophagy to atherosclerotic plaque development may go beyond macrophages and involve also the smooth muscle cells of the arterial wall. Recent studies have shown compromised autophagy in these cells as a consequence of the inflammatory response associated to the plaques [64].

4.4. Aging. Autophagic activity decreases with age in most tissues and organisms [65]. The fact that a decline in autophagic activity, and in particular in lipophagy, would contribute to intracellular accumulation of LD and that, as described in previous sections, these abnormally expanded lipid stores would further reduce autophagic activity, makes this an attractive feedback loop for the perpetuation of the metabolic syndrome of aging (characterized by hypercholesterolemia, accumulation of lipid deposits in organs, and insulin resistance) (Figure 4). Interestingly, and in some way contra intuitively, treatment with antilipolytic agents has been shown to improve the age-related hypercholesterolemic phenotype and overall health-span in old mouse models [66]. However, recent studies support that most of the beneficial effect observed with these agents is dependent on their ability to induce autophagy, likely as a response to the increase in intracellular lipid stores [67].

Genetic connections among autophagy, lipid atherosclerotic, and longevity have also been recently highlighted in studies in C. elegans [68]. Functional autophagy is necessary in this model to maintain the activation of a cellular lipase (LIPL-4) and conversely, this lipase is required for induction of autophagy. Interestingly, both the activity of this lipase and autophagy are required to attain the extension in life span observed upon germline removal in worms. Although the specific lipid targets of this lipase and the way in which it participates in autophagy remain unknown, it is tempting to propose that part of the effect in life-span could be due to better intracellular lipid handling by lipophagy.

5. Concluding Remarks

The recent discovery of lipophagy has contributed to link two major intracellular catabolic pathways autophagy and lipolysis. This new function of autophagy in lipid metabolism expands the physiological relevance of the autophagic process by making its contribution to the energetic balance more relevant (when considering the higher energetic value of lipids versus proteins), but also including now under the list of autophagic functions the control of many of the regulatory activities that lipids exert inside cells.

There are however a large number of standing questions that deserve immediate attention. Does lipophagy regulation occur through similar signaling pathways to those described for other types of autophagy? How are LDs selectively targeted by the autophagic machinery? Is there preferential degradation of a subset of LD by lipophagy? Are there differences between the types of lipid byproducts generated by lipophagy when compared to cytosolic lipolysis? What determines the

threshold for the switch from a stimulatory to an inhibitory effect of FFA on lipophagy? And also, in a more general context, is upregulation of autophagy protective against all types of lipotoxicity *in vivo*? What are the possible effects of lipophagy on the combinations of metabolic defects that often coexist in our population such as obesity, diabetes, and hyperlipidemia? Does defective hypothalamic lipophagy with age contribute to the reduced food intake observed in advanced aging [69]?

Although these are still early days for lipophagy, there is now ample evidence that organ-specific targeting of this process may have implications for development of novel therapeutic interventions against common human metabolic disorders such as obesity and insulin resistance.

Acknowledgments

The authors thank Dr. Susmita Kaushik for critically reading the paper. Work in our groups is supported by NIH Grants AG021904, AG031782, DK041918, NS038370, a Hirsch/Weill-Caulier Career Scientist Award (A. M. Cuervo), and NIH Grant DK087776 and a pilot award project from the Einstein Nathan Shock on Basic Biology of Aging (AG038072) (R. Singh).

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 512721, 18 pages
doi:10.1155/2012/512721

Review Article

Pexophagy: The Selective Degradation of Peroxisomes

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Received 16 October 2011; Accepted 23 November 2011

Academic Editor: Masaaki Komatsu

Peroxisomes are single-membrane-bounded organelles present in the majority of eukaryotic cells. Despite the existence of great diversity among different species, cell types, and under different environmental conditions, peroxisomes contain enzymes involved in β-oxidation of fatty acids and the generation, as well as detoxification, of hydrogen peroxide. The exigency of all eukaryotic cells to quickly adapt to different environmental factors requires the ability to precisely and efficiently control peroxisome number and functionality. Peroxisome homeostasis is achieved by the counterbalance between organelle biogenesis and degradation. The selective degradation of superfluous or damaged peroxisomes is facilitated by several tightly regulated pathways. The most prominent peroxisome degradation system uses components of the general autophagy core machinery and is therefore referred to as "pexophagy." In this paper we focus on recent developments in pexophagy and provide an overview of current knowledge and future challenges in the field. We compare different modes of pexophagy and mention shared and distinct features of pexophagy in yeast model systems, mammalian cells, and other organisms.

1. Introduction to Peroxisome Biology

Peroxisomes were initially described as "microbodies" in a Ph.D. thesis on the cellular morphology of rodent kidneys [1] and were characterized as novel eukaryotic organelles by De Duve and Baudhuin in the 1960s [2]. Biochemical analysis of isolated peroxisomes from rat liver resulted in the identification of several enzymes involved in hydrogen peroxide generation and detoxification and thus led to the term "peroxisome" for this new organelle. Almost 50 years later, despite significant insights regarding peroxisome function, several aspects of peroxisome biology still remain unresolved. This is partly based on the fact that peroxisomes display an unusually high variability in function, morphology, and biochemical features. For example, the presence of enzymes involved in the glyoxylate cycle has resulted in the denotation "glyoxysomes" for some plant peroxisomes [3], while the same organelle is dubbed "glycosome" in trypanosomatids because it houses glycolytic enzymes [4, 5]. Exemplifying the remarkable specialization of peroxisomal enzymes is the protein luciferase and proteins required for synthesis of penicillin. Luciferase is responsible for the bioluminescent characteristic of the firefly *Photinus pyralis* [6, 7] and the enzymatic cascade involved in penicillin production derives from the fungus *Penicillium chrysogenum* and its relatives [8, 9]. In vertebrates, peroxisomes harbor the enzymatic pathways for synthesis of specialized ether phospholipids vital for integrity of the central nervous system [10].

In contrast to these specializations, most peroxisomes share the enzymatic components for the β-oxidation of fatty-acyl-CoA derivatives, as well as for the production and degradation of hydrogen peroxide and other reactive oxygen species (ROS). The common evolutionary origin of all peroxisome subtypes is best illustrated by the ubiquitous presence of orthologs of a specific set of *PEX* genes, encoding peroxins, involved in peroxisome biogenesis, maintenance, and division. Additional commonalities are that all peroxisomal proteins are encoded in the nucleus, translated in the cytosol and imported into the peroxisomes by a highly conserved set of localization signals (called peroxisomal targeting signals or PTSs) and corresponding receptors and transporters [11, 12]. Figure 1 summarizes shared and unique metabolic and enzymatic functions of peroxisomes.

The evolutionary origin of peroxisomes is still a matter of debate [13]. Their presence in all main eukaryotic taxa and the mentioned similarities argue for a singular evolutionary origin in a common ancestor of eukaryotic cells, most likely as a consequence of an increase in oxygen levels in

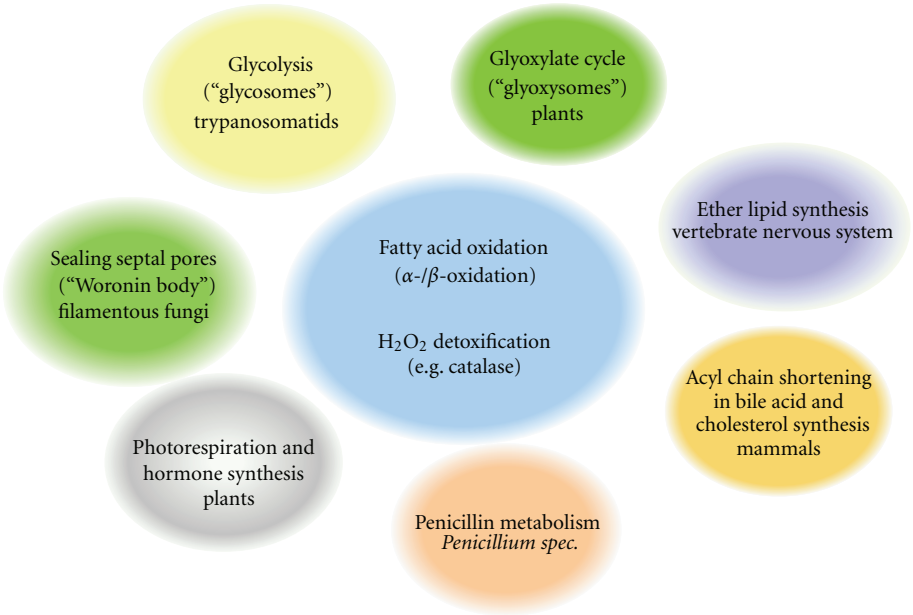

Figure 1: Overview of peroxisome functions in different organisms and tissues. Peroxisomes display a great variety in metabolic pathways as defined by their respective enzymatic content. Most eukaryotes share peroxisomal enzymes for fatty-acyl-CoA metabolism (α- and β-oxidation) and detoxification of hydrogen peroxide by catalase. In addition, several specialized metabolic pathways housed in the peroxisomal matrix of various organisms or tissues are shown.

the archaic atmosphere. While it was initially hypothesized that peroxisomes evolved in the course of events related to endosymbiosis, similar to mitochondria and plastids [14–16], research in the past decade has provided conclusive evidence that peroxisomes are not remnants of endosymbiotic microorganisms but have evolved from specialization of distinct parts of the endoplasmatic reticulum (ER) [17, 18]. Peroxisomes (unlike mitochondria and chloroplasts) have a single membrane, do not possess their own genome, and require several peroxisomal membrane proteins (PMPs) that transit via the ER before reaching their final destination in the peroxisomal membrane [19–22]. In the light of these findings it is generally believed that peroxisomes represent organelles originating from a specialization of the endomembrane system, rather than examples of endosymbiotic events.

The vital importance of peroxisomes in higher eukaryotes is documented by the dramatic effects of peroxisome dysfunction on human health. Peroxisomal disorders (PDs) are subdivided into two major groups: "single peroxisomal enzyme/transporter deficiencies" (PEDs) and "Peroxisomal Biogenesis Disorders" (PBDs). PEDs are caused by a functional defect in one peroxisomal pathway and include metabolic syndromes such as acatalasia, Acyl-CoA deficiency and X-linked adrenoleukodystrophy [23]. PBDs are caused by mutations affecting a set of at least 12 human genes, which function in peroxisome biogenesis and assembly (*PEX* genes), resulting in manifestation of numerous pathological conditions [24, 25]. PBDs involve autosomal recessive neurodevelopmental disorders that display numerous other symptoms including skeletal and craniofacial dysmorphism, liver dysfunction, and retinopathy. These diseases are caused by complete or partial loss of peroxisome functionality and include the Zellweger syndrome spectrum disorders (e.g., Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum's disease) as well as rhizomelic chondrodysplasia punctata [26, 27].

The severity of these defects emphasizes the pivotal role of peroxisomal metabolism for cellular integrity, especially in neuronal cells. In line with these observations, peroxisomes serve an important function in the central nervous system for the formation and maintenance of the myelin sheath and for the preservation of long-term axonal integrity [10, 28, 29]. In addition, recent reports point to a specific role of peroxisomal metabolism as a determinant of the cellular aging process, with peroxisome-derived ROS being triggers of antiaging pathways (at low concentrations), but also being decisive accelerators of aging by damage accumulation (at high concentrations) [30, 31]. This is further underscored by the finding that aging human fibroblasts accumulate peroxisomes with impaired protein import capacity, leading to ROS accumulation and exacerbation of the aging process [32]. Homeostasis in peroxisome number and functionality not only plays a role in the described disease settings, but also for the physiological aging process.

2. Peroxisome Homeostasis

Due to their importance for a variety of metabolic functions, peroxisome number is tightly controlled by environmental conditions. Yeasts (e.g., *Hansenula polymorpha, Pichia pastoris, and Saccharomyces cerevisiae*), are capable of utilizing different carbon sources and increasing peroxisome number and biomass when grown in these media requiring peroxisomal metabolism (Figure 2). Conversely, the shift of

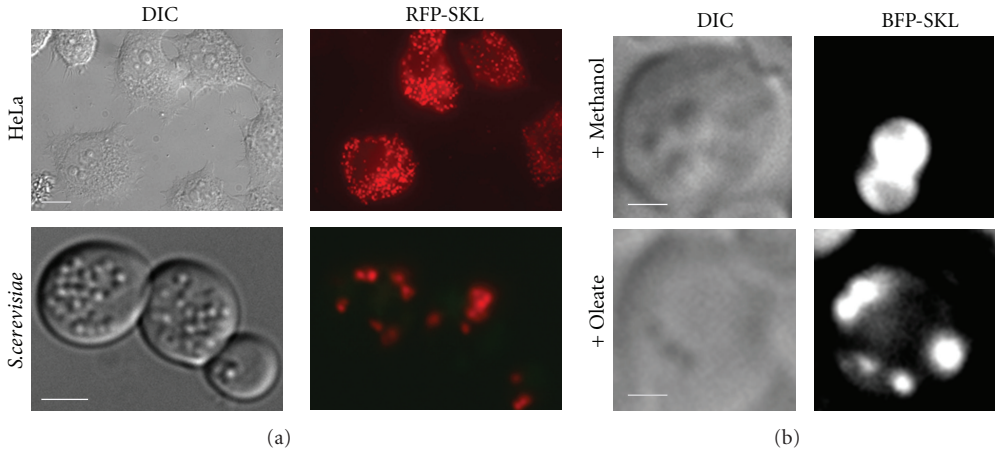

Figure 2: Comparison of peroxisome number and morphology in different eukaryotic cells and under different proliferation conditions. (a) Upper panel: Human HeLa cells expressing the peroxisomal marker, RFP-SKL, under basal growth conditions. Lower panel: *S. cerevisiae* cells expressing RFP-SKL after peroxisome induction in oleate medium. The relative number of peroxisomes per cell differs greatly between different eukaryotic cell types. Size marker = 2 μm. (b) Grayscale images of *P. pastoris* cells expressing BFP-SKL as peroxisomal marker. Upper panel: large, clustered methanol-induced peroxisomes; lower panel: small, unclustered oleate-induced peroxisomes. Note the difference in size and appearance of peroxisomes induced by different carbon sources. Size marker = 2 μm.

these cells from peroxisome induction conditions to carbon sources wherein peroxisomes are unnecessary triggers the degradation of superfluous peroxisomes by autophagy. These observations of peroxisome induction and removal have resulted in utilization of yeasts as model organisms to study peroxisome biogenesis, and turnover, which have led to the identification of several genes and mechanisms controlling peroxisome homeostasis [11, 33–47]. In rodents, the administration of phthalate esters or hypolipidemic drugs such as fibrates results in upregulation of peroxisomal proteins and a concomitant increase in peroxisome number [48]. This process is dependent on members of a special class of nuclear receptors, called "peroxisome proliferator-activated receptors (PPARs)" [49, 50]. However, this effect does not represent a general conserved mechanism since PPAR agonists fail to induce peroxisome proliferation in human cells [51, 52]. In contrast, it has been demonstrated that drugs, such as 4-phenylbutyrate (4-PBA), that act as chemical chelators and/or affect histone deacetylase (HDAC) activity can act as nonclassical peroxisome proliferators independent of PPAR activity in human cells [53, 54].

Here we present model systems to study peroxisome turnover and outline mechanisms that contribute to peroxisome homeostasis by regulating the selective degradation of peroxisomes. The main focus will be on selective degradation of peroxisomes in the vacuolar/lysosomal compartment, a process mediated by components of the general autophagy core machinery and usually referred to as pexophagy.

3. Methylotrophic Yeasts as Model Systems for Pexophagy

The large peroxisome clusters of methylotrophic yeasts (e.g., *P. pastoris* and *H. polymorpha*), as well as the experimental ease of manipulation of peroxisome number, volume, and content by media shifts in a genetically tractable organism, have facilitated studies on pexophagy. These yeasts, when grown in media containing methanol as the sole carbon source, rapidly proliferate their peroxisomes, which can occupy up to 40% of the cell volume. This makes fluorescence imaging of tagged proteins involved in pexophagy, as well as biochemical analysis of peroxisomal markers, much easier to monitor than in mammalian or other yeast systems. The autophagic degradation of peroxisomes was first noted by De Duve and Baudhuin [2] when they observed the appearance of peroxisomes within the lysosomes of mammalian cells, thus documenting the earliest description of pexophagy. Since then, much has been learnt from studies on pexophagy conducted in methylotrophic yeasts.

4. Modes of Pexophagy: Micropexophagy and Macropexophagy

All organisms from yeast to humans possess basal and inducible macroautophagy. During macroautophagy (referred to here as "autophagy"), a double membrane originates from a site known as the phagophore assembly site (PAS) to engulf cargo into a double-membrane vesicle known as the autophagosome, which upon fusion with a lysosome (or vacuole in yeast cells), releases into the lysosomal/vacuolar lumen an autophagic body comprised of a single membrane surrounding the cytosolic cargo. Once in the lysosomal lumen, the membrane and other macromolecular contents of the autophagic body are degraded by hydrolases to their constituent building blocks for reuse by the cell. This entire process, from the assembly of the PAS, to the engulfment of cargo into autophagosomes, fusion of autophagosomes with the lysosome/vacuole and subsequent degradation of the cargo, is orchestrated by the hierarchical recruitment of autophagy-related (Atg) proteins [80]. An alternative process called

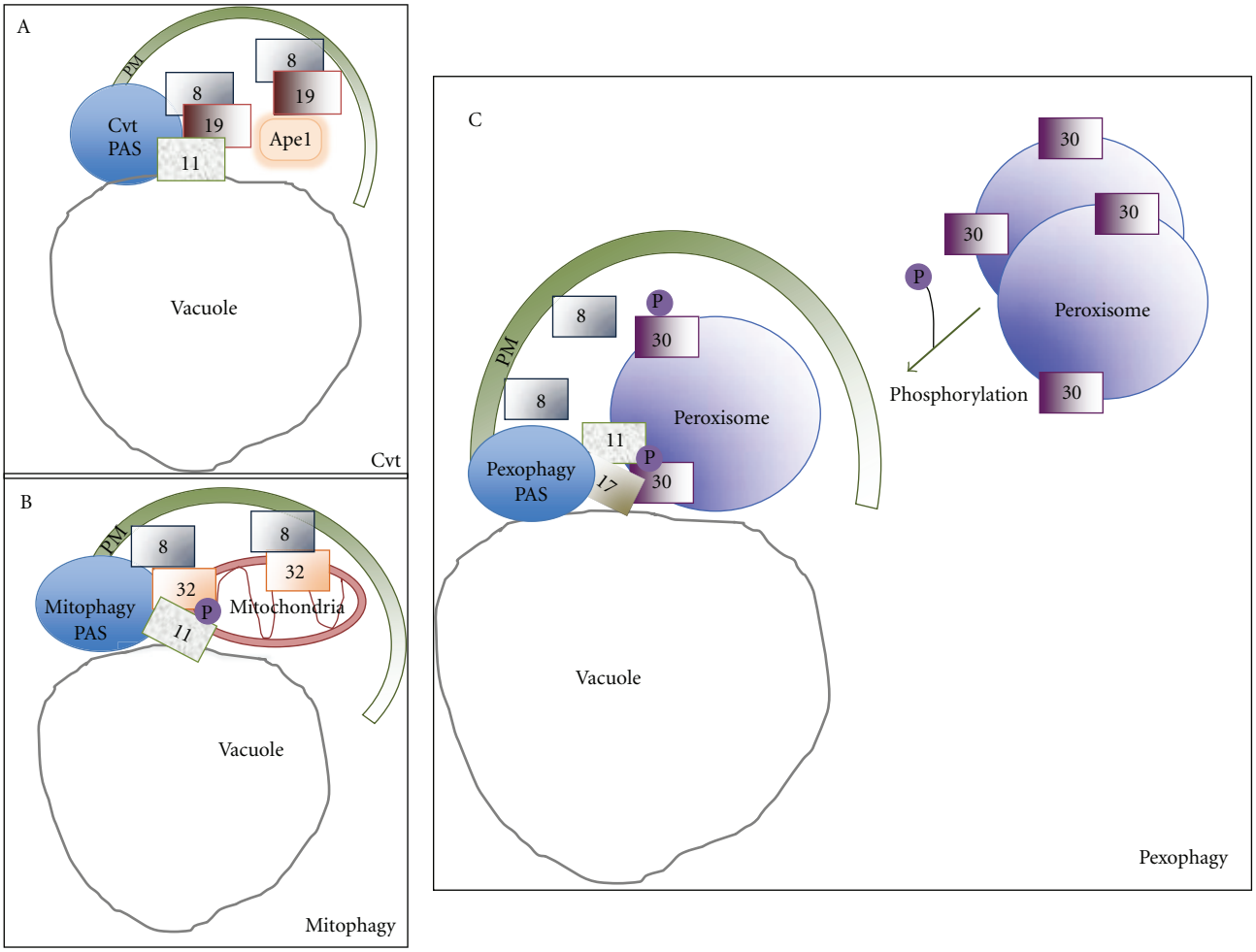

Figure 3: Similarities and differences between selective autophagy pathways. Various selective autophagy pathways share similar molecular mechanisms. They require a receptor that interacts with the cargo, recruits a scaffold protein (Atg11) that organizes the core autophagic machinery at the PAS, and mediates recruitment of Atg8, which initiates phagophore elongation from the PAS. In the Cvt pathway (a) Atg19 and Atg34 are the receptors for the cargo proteins aminopeptidase I (Ape1) and alpha-mannosidase, respectively. These receptors bind to Atg11 at the Cvt-specific PAS to initiate membrane expansion of the phagophore. (b) The mitophagy-specific phagophore membrane expansion from the PAS is initiated by Atg32, a mitochondrial outer membrane protein. Atg32 also interacts with Atg11 and Atg8. (c) The pexophagy receptor, Atg30, is localized at the peroxisome membrane, via interaction with the PMPs, Pex3, and Pex14. It is phosphorylated upon induction of pexophagy resulting in interaction of Atg30 with core autophagic machinery components, Atg11 and Atg17. In the case of pexophagy, the direct Atg8 interaction partner is still unknown.

microautophagy also exists, in which the lysosome/vacuole membrane invaginates to engulf cytosolic cargo directly to degrade and recycle it [81, 82].

In contrast to the nonselective nature of cargo engulfed by macroautophagy and microautophagy, other autophagy-related pathways capture cargo selectively from the cytosol. These include oligomeric proteins delivered to the vacuole by the cytosol-to-vacuole targeting (Cvt) pathway, ribosomes (ribophagy), and subcellular organelles such as peroxisomes (pexophagy), mitochondria (mitophagy), parts of the ER (ER-phagy), and segments of the nucleus (micronucleophagy) [38]. In most of these selective processes, the phagophore membrane, originating from specific PAS structures required for each form of selective autophagy (e.g., Cvt- or pexophagy-specific PAS, Figures 3(a) and 3(c)), engulfs the specific cargo and delivers it to the lysosome/vacuole for degradation. The source of the phagophore membrane is a widely debated topic within the field of autophagy, with the focus primarily on how Atg9 (ATG9L1 in mammals), which is the only transmembrane protein of the core Atg machinery, is recruited to the PAS [83]. Atg9 is thought to be involved directly or indirectly in trafficking membrane and/or lipid components during phagophore expansion from the PAS. However, this mechanistic concept remains a hypothesis [84–86].

To date, 35 autophagy-related (*ATG*) genes involved in several autophagy-related pathways have been discovered. Macropexophagy and micropexophagy (described next) are both used in *P. pastoris* for selective peroxisome degradation [87, 88]. As these represent specialized types of autophagy,

it should not be surprising that they require many of the core genes also used for autophagy as well as specific genes in addition (see Table 1) [38, 89]. Many yeast mutants with pexophagy defects provide insights into the mechanism of the two pexophagy modes (see Table 1).

Micropexophagy occurs when a cluster of peroxisomes is directly engulfed by vacuolar sequestering membranes (VSMs) that extend from a septated vacuole, and a double-membrane structure called the micropexophagy-specific membrane apparatus (MIPA) [60]. The MIPA extends from the PAS to form a cup-shaped lid over the VSM-engulfed peroxisomes and fuses with the VSMs to completely sequester the targeted peroxisomes from the cytosol and to ultimately deliver the pexophagic body into the vacuole lumen to be degraded by resident vacuolar enzymes (Figure 4(a)). During macropexophagy, an individual peroxisome is surrounded by the phagophore membrane originating from the pexophagy-specific PAS to form a double membrane-bounded pexophagosome (Figure 4(a)), before the outer membrane fuses with the vacuole membrane in a process resembling macroautophagy [41]. In *P. pastoris*, the choice between induction of either micro- or macropexophagy is determined by ATP levels in the cell [90]. High levels of ATP induce micropexophagy while lower levels activate macropexophagy. One explanation for this observation may be that the massive vacuolar rearrangement during micropexophagy and formation of the MIPA may be a more energy-intensive process than formation of the pexophagosome and thus may demand more energy in the form of ATP from the cell.

5. Nutrient Conditions That Induce Pexophagy

In *S. cerevisiae*, pexophagy is induced by transferring cells from growth media containing oleate as a carbon source to glucose-containing medium without a nitrogen source [91]. In *P. pastoris*, peroxisomes can be induced when cells are grown in media containing methanol, oleate, or amines. Transferring cells grown in methanol to ethanol or from oleate or methylamine to glucose without nitrogen induces macropexophagy (Figures 4(b) and 4(c)) [92]. Shifting cells from methanol medium to glucose induces micropexophagy (Figures 4(b) and 4(c)) [93]. Intriguingly, the two modes of pexophagy can be triggered by different experimental conditions in different yeasts. In *H. polymorpha*, macropexophagy, rather than micropexophagy, is induced when cells are shifted from methanol medium to glucose [47].

Interestingly, it was shown that simultaneous treatment of *H. polymorpha* with both nitrogen limitation and excess glucose conditions results in concomitant induction of both microautophagy and macropexophagy, thus exemplifying the fact that selective (i.e. pexophagy) and nonselective (autophagy) pathways can be initiated in parallel [94].

6. Regulation of Yeast Pexophagy

It has long been realized that not only surplus, but also damaged components or potentially toxic structures within the cytosol of eukaryotic cells can be selectively removed by autophagy. Using ectopic expression of a temperature-sensitive degron-Pex3 fusion, it was recently shown in *H. polymorpha* that damage to peroxisomes by abruptly removing the essential PMP, Pex3, causes pexophagy to occur [71]. This conditional selective degradation was apparent even when cells were placed in conditions that would normally require peroxisome biogenesis for cell growth. In methanol-excess conditions the authors saw a transient increase of ROS in wild-type cells that corresponds with the degradation of the peroxisome matrix protein, alcohol oxidase, as well as PMPs, Pex3, and Pex14, suggesting the possible physiological significance of pexophagy. However, it is unclear at present if there is a similar requirement of Pex3 removal from the peroxisome membrane for pexophagy in other methylotrophic yeasts such as *P. pastoris*, where Pex3 is actually essential to recruit the pexophagy receptor, Atg30, to the peroxisome, before the organelle is targeted for pexophagy.

The signaling events that regulate the specific removal of cellular components are still poorly understood. The emerging role of intracellular signaling pathways controlling pexophagy was shown by our group and has since been replicated and refined further. Using the degradation of a peroxisomal marker to investigate the role protein kinases play in pexophagy in *S. cerevisiae*, the Slt2 mitogen-activated protein kinase (MAPK) and several other upstream components of this signaling pathway were shown to be required for pexophagy, but not for pexophagosome formation, suggesting a block at the step of pexophagosome targeting or pexophagosome-vacuole fusion [37].

This theme of the involvement of yeast MAPK in selective autophagy has been extended by the recent discovery that Slt2 also plays a role in mitophagy [95], along with another MAPK (Hog1) (Figure 5) [95, 96]. Slt2 is crucial for recruiting mitochondria to the PAS, a step required for the specific packaging of cargo into autophagosomes. Interestingly, mitophagy in mammalian cells is activated by ERK2, another MAPK [97]. Thus, the differential involvement of MAPK pathways represents a central process in controlling diverse selective autophagy pathways [95].

Other signaling pathways have also been shown to have a direct role in pexophagy [35, 77, 98]. The phosphoinositide, phosphatidylinositol-3-phosphate (PtdIns3P), as well as the sole phosphatidylinositol 3-kinase, Vps34, that generates PtdIns3P in yeast, are required for all autophagy-related pathways, including pexophagy [36, 99]. In addition, phosphatidylinositol-4-phosphate (PtdIns4P), as well as the kinase that is responsible for PtdIns4P generation (Pik1) and Atg26, a sterol-glucosyltransferase that binds PtdIns4P via its GRAM domain, are necessary for micropexophagy in *P. pastoris* [46].

7. General Themes of Selective Autophagy Pathways

Since all autophagy-related pathways share common components required for PAS assembly, elongation of the phagophore membrane around cargo, vesicle formation, fusion and vacuolar degradation, the key decision point in any selective autophagy pathway is the mechanism by which the

Table 1: Genes involved in macro- and micropexophagy in methylotrophic yeasts. Involvement of the respective genes in the different modes of pexophagy is indicated by check marks. Genes denoted in bold font are (by current knowledge) exclusively involved in pexophagy, but not in other autophagy pathways. Genes denoted in regular font represent components of the core machinery involved in different autophagy pathways in the methylotrophic yeasts *Pichia pastoris (Pp)* and *Hansenula polymorpha (Hp)*. Empty spaces and parentheses depict the current lack of conclusive evidence. Table adapted from Sakai et al. [55].

Gene	Description of molecular events	Macropexophagy		Micropexophagy	Reference
		Pp	*Hp*	*Pp*	
ATG1	Serine/threonine kinase required for PAS formation	✓	✓	✓	[42, 56, 57]
ATG2	Peripheral membrane protein required for Atg9 recycling	✓		✓	[58]
ATG3	E2-like ubiquitin ligase that catalyzes lipidation of Atg8			✓	[59]
ATG4	Protease that processes Atg8 as prerequisite for conjugation with phosphatidylethanolamine (PE)			✓	[60, 61]
ATG6	Subunit of PI3K complexes I and II	✓			[35]
ATG7	E1-(ubiquitin activating enzyme)-like protein involved in conjugation of Atg12-Atg5 and Atg8-PE conjugates	✓		✓	[42, 62]
ATG8	Ubiquitin-like protein that is anchored to the expanded phagophore membrane in its processed and lipidated form, involved in phagophore membrane expansion	✓	✓	✓	[33, 60, 61]
ATG9	Transmembrane protein cycling between the PAS and a peripheral compartment	✓		✓	[57, 63]
ATG11	Coiled-coil adaptor protein that interacts with the core machinery and known receptors for selective autophagy	✓	✓	✓	[64]
ATG16	Essential component of the Atg12-Atg5-Atg16 complex	(✓)		✓	[42]
ATG17	Scaffold protein that is responsible for PAS organization	✓		✓	[34]
ATG18	PtdIns3P-binding protein whose localization is dependent Atg9 and PtdIns-3P; recruits Atg2 and needed for Atg9 recycling	✓		✓	[65, 66]
ATG21	WD40 protein with phosphoinositide binding domain that is involved in pexophagosome formation		✓		[67]
ATG24	Sorting nexin protein involved in fusion events with the vacuole	✓		✓	[33]
ATG25	Coiled-coil protein that co-localizes with Atg11 at the PAS, required for macropexophagy		✓		[39]
ATG26	Sterol glucosyltransferase that plays a role in phagophore membrane expansion	✓		✓	[44, 46, 68]
ATG28	Coiled-coil protein required for peroxisome sequestration during micropexophagy and vacuole fusion of pexophagosomes in macropexophagy	✓		✓	[69]
ATG30	Pexophagy receptor that interacts with peroxins, Pex3 and Pex14, and adaptor proteins, Atg11 and Atg17	✓		✓	[34]
ATG35	Localizes to the perinuclear structure; regulates MIPA formation and interacts with Atg28 and Atg17			✓	[43]
GCN1-4	Involved in general amino acid control			✓	[42]
Sar1	Sec protein required for MIPA and proper pexophagosome formation		✓	✓	[70]
PEP4	Vacuolar protease			✓	[60]
PEX3	PMP peroxin required for peroxisome biogenesis and for recruitment of pexophagy receptor	✓	✓	✓	[34, 71, 72]
PEX14	PMP peroxin required for peroxisome biogenesis and for recruitment of pexophagy receptor	✓	✓	✓	[34, 73]
PIK1	PtdIns-4-kinase required for MIPA formation			✓	[46]
PFK1	Subunit of phosphofructokinase complex			✓	[62]
TUP1	Transcriptional repressor	✓			[74]

Table 1: Continued.

Gene	Description of molecular events	Macropexophagy		Micropexophagy	Reference
		Pp	*Hp*	*Pp*	
VAC8	N-myristoylated armadillo-repeat protein of the vacuolar membrane, required for VSM formation			✓	[75, 76]
VAM7	SNARE protein that is involved in vacuolar fusion events with the phagophore membrane		✓		[45]
VPS15	Regulatory subunit of PI3K	✓	✓	✓	[42, 77]
VPS34	Phosphatidylinositol-3-kinase (PI3K)	✓	✓	✓	[36, 78]
YPT7	Rab GTPase involved in phagophore membrane fusion	✓	✓	✓	[71, 79]

core autophagy machinery is redirected to degrade primarily selective cargo. The study of these selectivity factors for pexophagy in yeast has revealed a set of key principles. Where applicable, we describe how these events are relevant to other selective autophagy pathways.

(1) Every selective autophagy pathway studied to date requires a specific cargo receptor. Examples of these include Atg30 for pexophagy [34], Atg19 and Atg34 for the Cvt pathway [100–102], and Atg32 for mitophagy [103, 104].

(2) These cargo receptors typically have a tripartite role in (a) cargo binding, (b) interaction with Atg11, a protein required by all selective yeast autophagy pathways to create the specific PAS structures from which the phagophore membrane will expand [100, 103, 104], and (c) interaction with Atg8, via an Atg8-interaction motif (AIM) [105], to allow phagophore expansion [100, 103, 104]. The receptors Atg19 and Atg32, required for the Cvt and mitophagy pathways in yeast, have all these properties, but as of now, only the first two roles have been attributed to Atg30 during pexophagy [34].

(3) The selective autophagy receptors are often synthesized even under conditions wherein the cargoes are not degraded, but receptor activation often relies on protein modifications, such as phosphorylation or ubiquitination [34, 96, 106].

(4) Some of the pexophagy-mediating factors, such as Atg11 and the sterol glucosyltransferase Atg26 that binds PtdIns4P [46], are required in an absolute fashion for the degradation of large cargoes, but are partially dispensable when the cargo size is small [107]. We predict that since the phagophore membrane has to engulf cargoes of varying sizes from individual cytosolic proteins to organelles, bacteria and viruses, analogous factors will be required for selective autophagy of other large cargoes.

(5) Specialized membrane structures, such as the MIPA, are needed for micropexophagy, and not for macropexophagy. Indeed, the protein Atg35 is needed for MIPA formation during micropexophagy, but not for pexophagosome formation during macropexophagy [43].

(6) Generally the receptors are degraded in the vacuole along with the cargo.

8. The Pexophagy-Specific PAS

Like autophagy, pexophagy is also initiated at a specific PAS (Figure 3(c)) that is distinct from other types of PAS for selective autophagy (Figures 3(a) and 3(b)). The autophagy-specific PAS is organized by Atg11, Atg17, Atg29, and Atg31, but Atg11 is dispensable [108]. The Cvt-specific PAS requires Atg11 and Atg19 for its organization [100–102] (Figure 3(a)), whereas the mitophagy-specific PAS uses Atg11 and Atg32 [103, 104] (Figure 3(b)). The pexophagy-specific PAS is organized by Atg11, Atg17, and Atg30 [34, 107].

For the onset of pexophagy in *P. pastoris*, Atg30 phosphorylation by a hitherto unknown kinase occurs and facilitates direct physical interaction with Atg11 [34]. The two proteins colocalize at the PAS, and Atg30 also directly interacts with Atg17. The roles of Atg11 and Atg17 are as scaffolds at the PAS that recruit other proteins, such as constituents of the core autophagy machinery described next. Surprisingly, there is a size requirement of the scaffolding proteins. For degradation of small peroxisomes, Atg11 and Atg17 are only partially required, but are essential for degradation of large peroxisomes in nitrogen-starvation conditions [107].

The assembly of a specific PAS is followed by the recruitment of core proteins of the autophagic machinery to the PAS including, but not limited to Atg1, Atg2, Atg5, Atg8, Atg9, Atg12, Atg13, Atg16, Atg18, Atg23, Atg24, Atg25, Atg27, Atg28, Atg35, and the PtdIns3-kinase (PI3K) complex. These proteins typically assemble in a complex hierarchy [109], such as our demonstration that the recruitment of PtdIns-3-Kinase to the PAS precedes Atg8 recruitment [78].

9. Elongation of the Phagophore Membrane

The protein Atg35 is a micropexophagy-specific protein recruited by Atg28 and is required for efficient MIPA formation but not for pexophagosome formation, giving the first evidence that the formation of the MIPA could be genetically distinct from the formation of the pexophagosome in macropexophagy [43].

Oku et al. [44] discovered that Atg26, a sterol glucosyltransferase that synthesizes sterol glucoside, is essential for

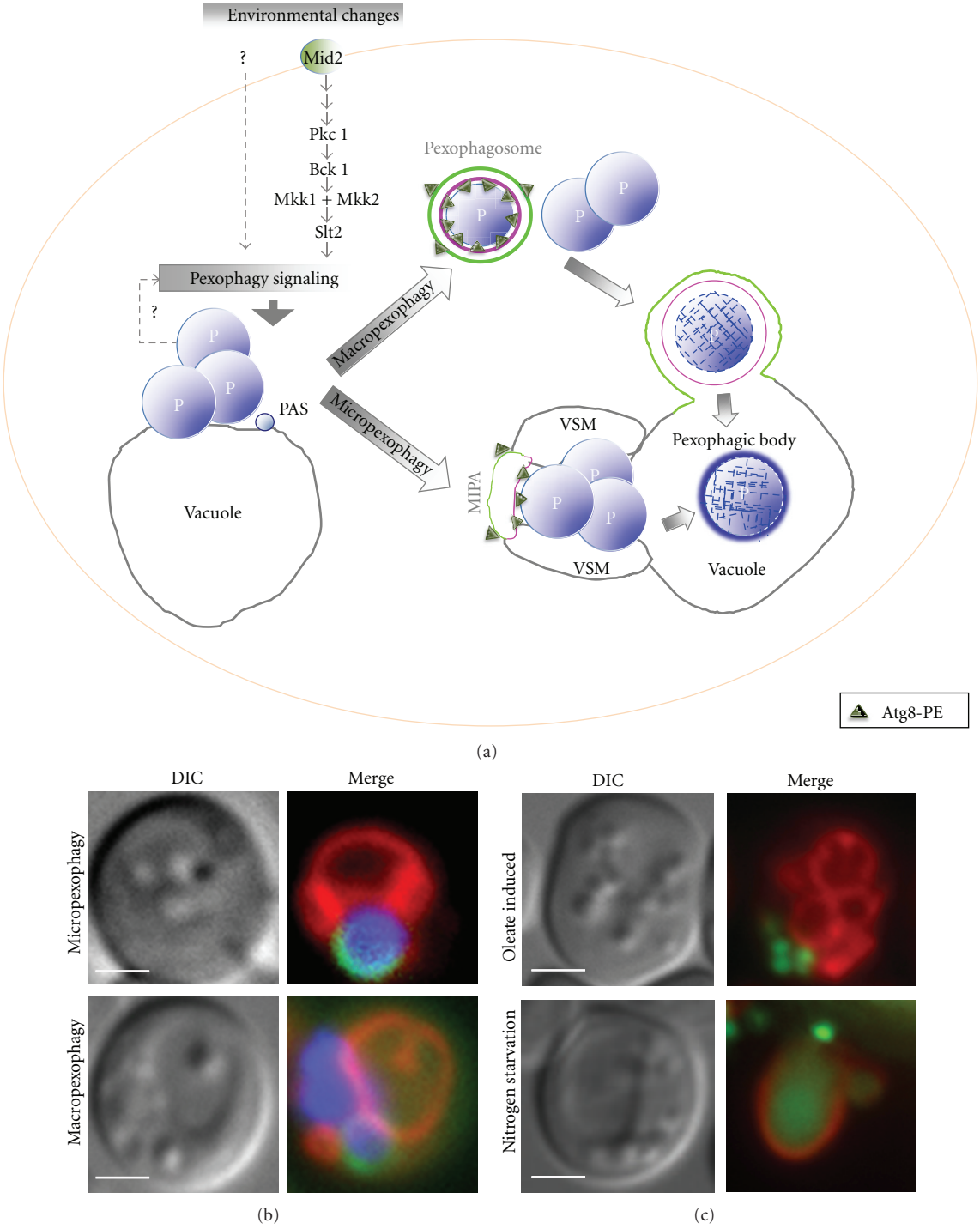

Figure 4: Micropexophagy and macropexophagy. (a) Micropexophagy differs from macropexophagy in vacuole dynamics and formation of the MIPA instead of the pexophagosome. A pexophagy-specific PAS, required for both forms of pexophagy, is characterized by its localization near the peroxisome and also touching the vacuolar membrane. Micropexophagy can target a peroxisome cluster for degradation by vacuole remodeling to form cup-like vacuolar sequestration membranes (VSMs) and a lid-like cover called the MIPA (micropexophagy-specific membrane apparatus). Macropexophagy is characterized by individual sequestration of targeted peroxisomes into a pexophagosome, followed by its fusion with the vacuole for degradation and recycling. Pexophagy signaling is dependent on Mitogen-activated protein kinase (MAPK) pathways (Mid2-Slt2 cascade), but may also be triggered by internal (unknown) factors, including signals related to the status of, or metabolic need for, (e.g., damaged or superfluous) peroxisomes. (b) The upper panel depicts a single *P. pastoris* cell that has undergone peroxisome induction (in methanol) and has then been switched to micropexophagy conditions (glucose). The vacuole (red, FM 4–64) is shown surrounding the targeted peroxisome cluster (blue, BFP-SKL). The MIPA (green, GFP-Atg8) forms a lid over the cup-like VSMs. The lower panel illustrates pexophagosome formation around a single peroxisome under macropexophagy conditions (ethanol). (c) *S. cerevisiae* cell labeled with GFP-tagged thiolase (a peroxisome matrix marker) and vacuole marker (FM 4–64, red) shows proliferated peroxisomes under nutrient-rich conditions (in oleate, top panel). When the cells are switched to glucose without nitrogen, peroxisomes are targeted to the vacuole by macropexophagy and GFP accumulates in the vacuole (lower panel).

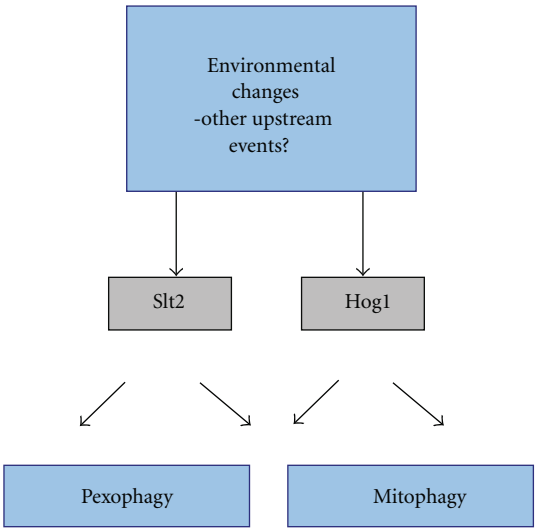

Figure 5: Signal transduction cascades regulating selective autophagy in yeast. Mitogen-activated protein kinase (MAPK) cascades contribute to differential regulation of selective autophagy pathways. As recently shown, the Slt2 and Hog1 signal transduction pathways regulate both mitophagy and pexophagy [37, 96]. Besides the obvious role of environmental factors such as nutritional conditions, details of other upstream events are poorly understood.

pexophagy, but not autophagy in *P. pastoris*. They showed that the protein is associated with the MIPA during micropexophagy, and that a single amino acid substitution within the GRAM domain (domain found in glycosyltransferases, Rab-like GTPase activators, and myotubularins) of the protein abolished this association [44]. However, it was found that although Atg26 is required for utilization of decane in *Y. lipolytica*, it was unnecessary for pexophagy in this yeast, showing that sterol glucosyltransferase play different functional roles in the two yeasts [68].

In *P. pastoris*, phosphatidylinositol-4-phosphate (PtdIns4P) initiates *de novo* membrane synthesis that is required for pexophagy. PtdIns4P, generated primarily by the PtdIns-4-kinase, Pik1, recruits Atg26 via its GRAM domain [46], and the sterol glucosyltransferase activity of Atg26 at the nucleation complex is necessary for the elongation of the membrane.

In both *S. cerevisiae* and *P. pastoris*, the only integral membrane protein of the autophagy machinery, Atg9, cycles between a peripheral compartment comprising a reservoir of Atg9 and the PAS, or PAS-like structures. The shuttling mechanism has been studied in both organisms but the process is better understood in *S. cerevisiae* and is therefore described next, before the role of this protein in pexophagy is described.

In *S. cerevisiae*, Atg9 colocalizes at the PAS but is not present on completed autophagosomes, suggesting it must be recycled during autophagosome formation. It cycles between a peripheral compartment and the PAS [85]. The anterograde trafficking of Atg9 from the peripheral compartment to the PAS requires Atg11, Atg23, and Atg27 [86]. Atg9 retrieval from the PAS is regulated by the Atg1-Atg13 signaling complex and requires Atg2, Atg18, and the PtdIns3P generated by the Atg14-containing PtdIns-3-kinase complex [110]. However, only Atg2, Atg18, and PtdIns3P are necessary for Atg9 recycling, while the Atg1-Atg13 complex and Atg1 kinase activity, but not Atg2, Atg18, and PtdIns3P, are necessary for Atg23 cycling to and from the PAS [110].

The subcellular movement of Atg9 in *S. cerevisiae* requires interaction with the actin cytoskeleton as has been shown by the sensitivity of relocation of Atg9 to the inhibitor Latrunculin A, as well as by the phenotype displayed by conditional mutants of actin and the actin-related protein Arp2 [111, 112].

The proteins Atg11 and/or Atg17 are necessary for Atg9 recruitment to the PAS [84, 113]. Also required at the PAS is PtdIns3P, generated by the Vps34 (PtdIns-3-kinase) complex, to recruit PtdIns3P-binding proteins (e.g., Atg18 and Atg24), which then recruit yet other proteins, such as Atg2, to the PAS [65].

P. pastoris Atg9 (*Pp*Atg9) is necessary for the formation of the VSM, assembly of the MIPA, and for pexophagosome formation. As in *S. cerevisiae*, the *P. pastoris* Atg9 also shuttles to the PAS from a peripheral compartment, perhaps supplying the membrane to the PAS and elongating the phagophore membrane to form the VSM, MIPA, and pexophagosome [63]. *Pp*Atg9 shuttles from a peripheral compartment near the ER/mitochondria to unique perivacuolar structures (PVS; PAS-like structures) that contain Atg11, but not Atg2 or Atg8. Atg9 then traffics from the vacuole surface to the VSMs that engulf peroxisomes for degradation [63]. Movement of the PpAtg9 from the peripheral compartment to the PVS requires PpAtg11 and PpVps15 (a subunit of the PtdIns-3-kinase). PpAtg2 and PpAtg7 are essential for PpAtg9 trafficking from the PVS to the vacuole and sequestering membranes, whereas trafficking of PpAtg9 proceeds independent of PpAtg1, PpAtg18, and PpVac8. How exactly PpAtg9 contributes to the formation of the MIPA and pexophagosome formation is less clear.

In *P. pastoris*, expression of dominant-negative forms (Sar1-T34N and Sar1-H79G) of the ER protein Sar1, impairs Atg8 lipidation and MIPA formation, but not the formation of the VSMs or the trafficking of Atg11 and Atg9 to these VSMs during micropexophagy [70]. During macropexophagy, the expression of Sar1-T34N inhibited the formation of the pexophagosome, whereas Sar1-H79G suppressed the delivery of the peroxisome from the pexophagosome to the vacuole. In this case, the pexophagosome contained Atg8 in wild-type cells, but in cells expressing Sar1-H79G these organelles contain both Atg8 and endoplasmic reticulum components, suggesting a defect in retrieval of components back to the ER, prior to pexophagosome/vacuole fusion.

The protein Atg25 has been described in *H. polymorpha* to be required for macropexophagy. It interacts with Atg11 and colocalizes with it at the PAS. In its absence, peroxisomes are constitutively degraded by nonselective microautophagy, a process that in wild-type *H. polymorpha* is only observed under nitrogen starvation conditions, suggesting that nonselective microautophagy is deregulated in *H. polymorpha atg*25Δ cells [39].

10. Requirement of Specific Proteins during the Final Stages of Pexophagy

Atg24, a molecule with a PtdIns3P-binding module (PX domain), is required for micropexophagy and macropexophagy, but not for general autophagy in *P. pastoris* and *S. cerevisiae* [33]. CFP-tagged PpAtg24 localizes to the vertex and boundary region of the pexophagosome-vacuole fusion complex during macropexophagy. Depletion of PpAtg24 blocked macropexophagy after pexophagosome formation and before its fusion to the vacuole. These results suggest that PpAtg24 is involved in the regulation of membrane fusion at the vacuolar surface during pexophagy via binding to PtdIns3P and could potentially be involved in pexophagosome fusion with the vacuole [33]. During micropexophagy, *Ppatg*24Δ cells form the MIPA and exhibit aberrantly septated vacuoles, reminiscent of other mutants defective in vacuolar fusion, but engulfment of peroxisomes is also impaired [33].

11. Pexophagy in Mammalian Cells

In contrast to yeast models, which have greatly contributed to the mechanistic understanding of pexophagy as outlined above, the molecular details of mammalian pexophagy are less well understood. This is partly based on fundamental differences between mammalian and yeast peroxisomes. While in yeasts the number of peroxisomes varies between 1–20 dependent on the species and growth conditions (see above), average mammalian cells contain between several hundred to thousands of peroxisomes (Figure 2(a)) [114]. Induction of peroxisome proliferation in rodents by phthalate esters [e.g., di-(2-ethylhexyl)phthalate; DEHP)], hypolipidemic drugs (e.g., fibrates) or nonclassical peroxisome proliferators (e.g., 4-PBA) results in a 2-3-fold increase of peroxisomal mass, which is a significantly smaller effect compared to the effects observed in yeasts. Consequently, quantitative analyses of peroxisome turnover in mammalian systems are limited by the detection method applied. Mammalian peroxisomes differ from those of yeast cells not only in number and induction mechanisms, but also by their modes of selective degradation. At least three independent degradation systems have been described: the Lon protease system, 15-lipoxygenase (15-LOX)-mediated autolysis and lysosomal degradation/pexophagy (Figure 6) [115]. Based on studies using *Atg7* conditional knockout mice it is estimated that up to 20–30% of the mass of liver peroxisomes is degraded by Lon protease-mediated mechanisms and 15-LOX-mediated autolysis of peroxisomes, whereas the remaining 70–80% are destroyed by autophagic mechanisms [115].

The peroxisomal isoform of the Lon protease is an ATP-dependent protease with chaperone-like activity that is involved in degradation of misfolded and unassembled peroxisomal proteins. Lon protease is upregulated in rats under peroxisome proliferation conditions (e.g., administration of DEHP) and further increases its levels after withdrawal of the inducing drug while peroxisomal enzymes are quickly degraded [116]. Subsequently, Lon protease activity catalyzes the breakdown of proteins resident in the peroxisomal matrix, indicating that it contributes to the reduction of peroxisome mass, if not quantity. Interestingly, the yeast ortholog of the Lon protease (encoded by the *PLN* gene in *H. polymorpha*) appears to be essentially involved in peroxisome quality control mechanisms, with only about a 25% contribution to reduction of peroxisome numbers, which increased only slightly from 2.6/cell to 3.3/cell in the absence of Pln, whereas the peroxisome number increased to 5.4/cell in the absence of the *ATG1* gene required for all forms of autophagy [117]. Assuming that the Lon protease has similar roles in yeast and mammals, it is conceivable that relative to autophagic mechanisms, it plays a relatively modest role (in the range of 25%) in reducing peroxisome number.

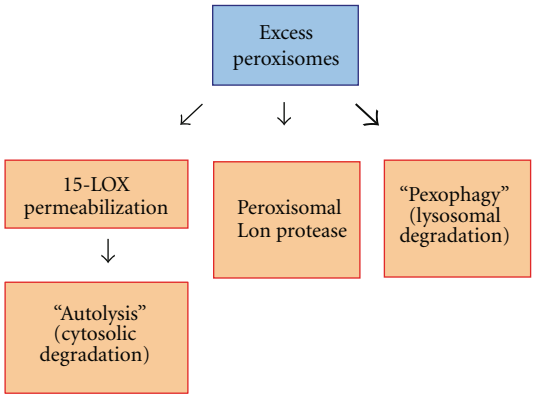

Figure 6: Peroxisome degradation pathways in mammalian cells. Surplus peroxisomes or their contents (e.g., peroxisomal matrix proteins) can be degraded by at least three distinct mechanisms: Lon protease-mediated proteolysis, 15-lipoxygenase (15-LOX)-mediated cytosolic degradation (autolysis), and pexophagy (autophagy-mediated lysosomal degradation). Current studies suggest that the majority of peroxisomes are degraded by pexophagy (indicated by bold arrow).

The cytosolic enzyme, 15-LOX, can associate with peroxisomal membranes leading to localized membrane disruption [118]. Structural breakdown subsequently exposes the peroxisomal content to cytosolic proteases resulting in its rapid degradation. This pathway appears to be initiated in parallel to pexophagy after drug-mediated accumulation of peroxisomes and accounts for removal of a limited fraction of excess peroxisomes.

While the abovementioned pathways contribute partially to peroxisome homeostasis under certain cellular conditions and other data argue for a role of the proteasome system by undefined mechanisms [119], the vast majority of selective peroxisome degradation is mediated by autophagosomal-lysosomal processes resembling yeast macropexophagy. As mentioned, early reports from the 1970s already noted the selective lysosomal degradation of mitochondria and peroxisomes during the diurnal cycle in rat inner organs [120], but it was only shown later that the autophagy machinery is specifically involved in degradation of surplus peroxisomes in mouse liver [121]. This was demonstrated by comparing abundance and degradation efficiency of peroxisomes after treatment with phthalate ester for 2 weeks and chase

after drug removal one week later in wild-type and autophagy-deficient Atg7$^{-/-}$ mice. The salient findings of this study emphasize mechanistic similarities to the above-mentioned yeast models used to study pexophagy: environmental conditions that require peroxisomal enzymes (e.g., oleate/methanol for yeasts, chemical peroxisome proliferators for rodents) lead to peroxisome proliferation, followed by pexophagic degradation when the organelles are no longer required or can be used as a resource for alternative pathways. This biological theme of a metabolic switch involving adaptation to changing external factors and thereby triggering pexophagy is also reminiscent of organelle remodeling in pathogenic fungi and parasitic protozoa as will be outlined in the next section.

A detailed functional analysis of peroxisome degradation using an *in vitro* cell culture system showed for the first time that peroxisomes are preferentially degraded over cytosolic proteins under starvation/recultivation conditions [119]. This study used Chinese Hamster Ovary (CHO) cells to describe autophagy-mediated peroxisome turnover when switching culture conditions from starvation in Hank's solution to reconstitution in nutrient-rich medium. The authors show convincingly that the peroxisomal membrane protein, Pex14, is bound by autophagosome-anchored LC3-II (i.e., the processed and lipidated form of LC3) under starvation conditions. Pex14 is an essential component of the peroxisomal translocon complex, which facilitates import of cytosolic proteins into the peroxisomal matrix. It is noteworthy that the dual role of Pex14 for both peroxisome assembly and selective degradation has also been shown for yeast systems [47]. Moreover, the study by Hara-Kuge and Fujiki points to an involvement of the cytoskeleton in this process by demonstrating the requirement of intact microtubules for the LC3-II/Pex14 interaction [119]. As an intriguing example of the competitive nature of the processes involved, binding of Pex14 to either LC3-II or the peroxisomal import receptor, Pex5, proved to be mutually exclusive. This might point to a general mechanism that ensures functional segregation of metabolically active and degradation-prone organelles. Although this study uses an unusual experimental setup by applying starvation followed by recultivation in rich medium, it opens the avenue for future studies addressing the question of how exactly PMPs contribute to physical interactions with the autophagy machinery. In line with these observations, a recent study describes the role of a Rab7-effector protein, FYCO1 (FYVE and coiled-coil domain-containing 1), as the physical link between LC3 family members, PtdIns3P and microtubule plus end-directed transport [122, 123], but the exact role of this mechanism for pexophagy in particular has not been addressed yet.

The dynamics of peroxisome turnover in mammalian cells under normal cultivation conditions have nicely been addressed in a recent publication [124]. Using HaloTag-labelled peroxisomal marker proteins to follow the long-term fate of peroxisomes in cultured CHO cells and mouse fibroblasts, the authors show that mammalian peroxisomes have a half-life of approximately 2 days under normal cultivation conditions and that peroxisomes of different age display a different capacity to import newly synthesized proteins. In addition, this study shows that even under normal growth conditions, pexophagy contributes to the majority of turnover of this organelle as demonstrated by sensitivity to 3-methyl adenine (3-MA, an autophagy inhibitor) treatment. These findings emphasize the dual role of autophagy-related pathways: while autophagy principally serves to ensure nutrient recycling under starvation conditions, the same machinery fulfills the purpose of a quality control and homeostasis mechanism even in the presence of all nutrients.

Because the autophagy machinery in mammalian cells targets ubiquitinated protein aggregates, experiments were designed to address whether monoubiquitination of peroxisomal proteins could cause the autophagic clearance of peroxisomes [125]. Using overexpression of PMPs, Pmp34 and Pex3, fused on the cytosolic side to a ubiquitin variant genetically tailored to block polyubiquitination, it was found that exposure of a single ubiquitin moiety on the cytosolic face of the peroxisomal membrane was sufficient to trigger turnover of this organelle. Specificity of this affect was demonstrated by analyzing sensitivity to protein topology and to the autophagy inhibitor, 3-MA, thus confirming the requirement of the autophagy machinery in degradation of the ubiquitin-labeled peroxisomes. Moreover, the study showed that the ubiquitin-binding autophagy adaptor, p62, is involved in selective degradation of peroxisomes under the chosen conditions. Although this study is primarily based on overexpression of ectopic proteins and the artificial placement of a ubiquitin tag on the peroxisomal membrane and does not identify the physiological target of this process, it has some interesting implications. The general requirement of p62 for mammalian peroxisome homeostasis was demonstrated even in the absence of ectopic ubiquitin tagging, since knockdown of p62 significantly increased endogenous peroxisome numbers under the experimental conditions. Furthermore, of all mammalian autophagy adaptors identified so far (e.g., p62/SQSTM1, NDP52, and NBR1), only p62 has as yet been shown to be involved in selective degradation of peroxisomes. Since these adaptors partly share mechanistic features such as bridging ubiquitinated cargo (e.g., cytoinvasive bacteria in the case of NDP52) to LC3 family members to link with the autophagy machinery, it is unclear to date how cargo selectivity is facilitated in mammals. An interesting finding on this theme comes from the field of xenophagy, the selective degradation of cytosolic pathogens (reviewed elsewhere in this special issue): As shown recently, the two ubiquitin-binding autophagy adaptors p62 and NDP52 are recruited independently to cytoinvasive *Salmonella sp*. and show distinct localization signals at the surface of the invaded pathogens [126]. The authors argue that two individual adaptor complexes are required for effective xenophagy of *Salmonella sp*. and that these two complexes organize distinct microdomains associated with bacteria. With respect to pexophagy, it has not been analyzed whether or not different adaptor proteins are involved in selective degradation of peroxisomes and what their respective contribution is. Answers to this type of question will be informative not only for pexophagy, but for the whole field of selective autophagy pathways. A hypothetical mechanistic model of mammalian pexophagy is illustrated in Figure 7.

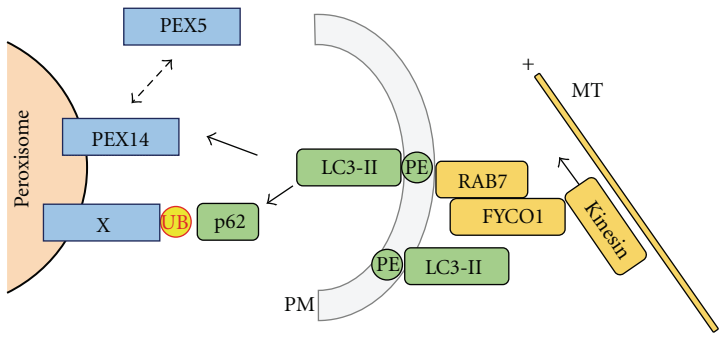

Figure 7: Hypothetical mechanistic model of pexophagy in mammalian cells. Processed and lipidated LC3 (LC3-II) is integrated into the expanding phagophore membrane (PM) and also may be involved in facilitating directed movement of the PM structure by interacting with microtubules (MT) via the RAB7 effector FYCO1 and motor protein Kinesin. Targeting of peroxisomes may either be accomplished by p62-mediated detection of ubiquitin (UB) motifs on still unknown peroxisomal membrane (or membrane associated) proteins (X) or by direct binding of LC3 to PEX14, a process which is discussed to compete with the binding of PEX5 to PEX14 (dotted arrow). See text for details.

12. Pexophagy in Plant and Human Pathogens

A very interesting and unexpected perspective originates from recent studies in the field of parasitology and infection biology showing that pexophagy is required for the phytopathogenicity of the cucumber anthracnose fungus, *Colletotrichum orbiculare* [127, 128]. This plant pathogen forms a specific structure termed the appressorium, which is required for penetration of the host epidermal cells in the course of infection. The authors used a random insertional mutagenesis screen to identify fungal genes that contribute to pathogenicity. They identified the *C. orbiculare* ortholog of *P. pastoris ATG26* to be essential for host cell infection. *PpATG26* is a well-characterized pexophagy gene encoding a sterol glucosyltransferase, which is essential for pexophagy in methylotrophic yeasts [46, 92]. In the case of *C. orbiculare*, the pathogen undergoes morphological changes reminiscent of pexophagy during development of its appressoria as indicated by vacuolar localization of peroxisomes and the requirement for the central autophagy protein, Atg8. While appressoria could still be formed in the *atg26* deletion mutant, the infection process was significantly delayed. Moreover, deletion of *atg8* completely abolished appressoria formation, suggesting an essential role of the autophagy machinery during infection. As the authors show, nonselective general autophagy is essential for early morphogenesis during pathogen development, while Atg26-dependent selective pexophagy is essential for later stages of direct host-pathogen infection steps. The authors conclude that Atg26-mediated pexophagy might be involved in maturation of the infection structures by providing molecular building blocks through organelle recycling.

Another report points to the role of pexophagy during developmental and environmental changes in the parasitic protozoan, *Trypanosoma brucei*. This human pathogen, which causes sleeping sickness and Chagas disease, harbors essential enzymes of glycolysis in its peroxisomal structures, which are therefore referred to as "glycosomes." The parasitic life cycle of this pathogen, which comprises different developmental stages in the Tse-Tse fly vector and the human host, requires adaptation of its metabolism to the changing environment. The necessary dynamic remodeling of glycosomal structures is facilitated by fusion of glycosomes with acidic lysosomes through autophagy-related mechanisms resembling pexophagy [129]. As shown recently, the acidic pH of the lysosomal compartment is responsible for inactivation of the key peroxisomal enzyme, Hexokinase (TbHK1), without affecting its protein level [130]. In addition, the pH change renders the enzyme sensitive to metabolic feedback regulation by both its substrate and product (ATP and ADP, resp.) and to modulation by other glycosomal metabolites, most likely by subtle changes in the protein tertiary structure. Thus, pexophagy appears to allow for a novel mechanism of regulating enzymatic activity by facilitating pH-dependent structural changes and concomitant feedback responses. These data point to an unexpected role of pexophagy as a regulator of essential enzyme activity in a parasitic protozoan during development and adaptation.

Moreover, the human fungal pathogen, *Candida glabrata*, requires adjustment of peroxisome number for survival after phagocytosis by immune cells [131]. The authors used fluorescent fusion proteins of transcription factors and peroxisomal enzymes to assess the metabolic status of the engulfed parasite. Using this approach, they showed that the pathogen responds to phagocytosis by increasing peroxisome number initially, most likely to fight phagocyte-induced oxidative stress. However, prolonged phagocytosis resulted in carbon starvation and a pexophagy-mediated decrease of peroxisomes. The requirement of this mechanism was shown by the dramatic loss in parasite survival during phagocytosis when the selective autophagy gene, *CgATG11*, or the general autophagy gene, *CgATG17*, were knocked out. The authors conclude that autophagy-related mechanisms, including pexophagy, represent important survival mechanisms for *Candida* after engulfment by phagocytes, pointing to the pivotal role of these pathways for providing essential cellular resources.

Kawaguchi et al. (2011) recently reported on a possible physiological role of pexophagy in yeast. This was achieved by exploring the relationship between the methylotrophic yeast *Candida boidinii* and the phyllosphere of growing *Arabidopsis thaliana* leaves [132]. The authors developed a methanol sensing assay in live *C. boidinii* cells using a PTS1-tagged fluorescent protein expressed from a methanol-inducible promoter, whereby an increase in environmental methanol concentrations resulted in enhanced fluorescence levels. They then used this assay to measure local methanol concentrations at the phyllosphere of growing *A. thaliana* leaves and showed that methanol concentrations at the phyllosphere change throughout the day corresponding to the light-dark cycle, whereby methanol concentration increased in the dark period, compared to the light period. In addition, they showed that autophagy as well as pexophagy are both required for yeast growth and survival at the phyllosphere, as autophagy and pexophagy mutants exhibited impaired proliferation on growing *A. thaliana* leaves. These results reveal interesting mechanisms used by methylotrophic yeast to survive at the phyllosphere, and how both autophagy and pexophagy are used to adapt to changes in environmental methanol dynamics, providing insight into plant-microbe interactions.

The common conclusion of the studies mentioned above is that pexophagy represents an important mechanism for survival and development under changing environmental conditions. Peroxisomes represent highly dynamic structures: Their biomass can easily be increased when peroxisomal functions are needed for specialized metabolic pathways or breakdown of damaging ROS, but they are quickly recycled when conditions change and they are not essential, so molecular building blocks and energy resources can be provided for alternative cellular functions.

Taken together, these studies point to a pivotal role of pexophagy in the development and morphogenesis of important plant and human pathogens.

13. Future Perspectives

Despite the great achievements of the last decade with respect to unraveling the molecular mechanisms contributing to pexophagy in various organisms, several aspects still remain to be resolved. The physiological role of pexophagy in model organisms such as yeast cells is still a matter of debate. With few exceptions, knockout of genes specifically involved in yeast pexophagy does not necessarily result in reduced viability or increased cell death. In fact, the role of pexophagy may rather be associated with quick adaptation to changing environmental conditions and thus may only emerge under cellular stress conditions like nitrogen starvation after growth under peroxisome proliferation conditions. In line with this view, pexophagy in other organisms appears to play a role for removal and recycling of unwanted or nonessential peroxisomes under condition when the cell is in need of molecular building blocks for alternative pathways, for example, for vital morphogenesis and development. In addition, the role of peroxisome turnover and linked changes in cellular redox state with cellular aging processes is increasingly recognized and warrants further investigation.

Although recent advances have pointed to a formerly unrecognized role of specific signal transduction pathways for the regulation of pexophagy (and mitophagy), the molecular framework of this process still remains to be elucidated. Which upstream events activate MAPKs differentially? Which are the pivotal targets of the protein kinase activity in this context, and how do these contribute to pexophagy regulation? Is there a molecular link between mitophagy and pexophagy? Moreover, it is not known if this mechanism is restricted to yeasts or if other organisms share the same regulatory circuits and if they have functional homologs of all the selectivity factors. Future work will therefore focus on elucidating the underlying conserved (or distinct) mechanisms.

The identity and mode of action of autophagy adaptors for yeast pexophagy is one major aspect of current research efforts. While Atg30 has been identified as an Atg11-binding pexophagy adaptor, the identity of the (proposed) Atg8-binding partner remains unresolved. In addition, while the requirement of phosphorylation for adaptor protein binding to Atg11 is well established [34, 96], it has not been addressed yet to what extent other posttranslational modifications, such as ubiquitination and/or alternative processing, of adaptor proteins contribute to the execution of pexophagy. Indeed, phosphorylation events in close proximity to Atg8-interaction motifs (AIMs) or LC3-interacting regions (LIRs) in the mammalian adaptor protein optineurin (OPTN) have been suggested as an important regulatory mechanism in xenophagy [133]. Unraveling the corresponding mechanism in yeast and mammalian pexophagy therefore represents an intriguing perspective.

The origin of membrane material for the autophagosome/phagophore membrane is still an unanswered question. Several current models argue for a contribution of the ER to provide membrane lipids and structural components. Sar1, an ER protein required for the secretory pathway, has been shown to have a role in pexophagosome formation, but the data do not unambiguously show that the pexophagosome membrane derives from the ER [70]. In addition, we have previously shown that Atg17 trafficks from the peripheral ER and colocalizes with Atg35, which regulates MIPA formation [43] but there is not a decisive mechanism of membrane trafficking as of yet.

In addition, the subcellular sorting mechanisms, which would be required to facilitate this process of membrane recruitment, are largely unknown yet recent advances towards membrane expansion and the requirement of SNAREs for autophagosome formation provide some insights [134]. However, we still need to understand how various membrane fusion events are orchestrated during pexophagy.

The role of cytoskeleton components for pexophagy is not yet fully understood. While pexophagy in yeast cells requires the actin skeleton [112], it appears that pexophagy in mammalian cells is dependent on tubulin-mediated interaction of LC3 family members with peroxisomal membrane proteins such as Pex14 [119]. Another form of selective autophagy, xenophagy of intracellular *Listeria* and

Salmonella, relies on components of the actin skeleton, a process mediated by the increasingly characterized class of septin proteins [135, 136]. Further work is needed to decipher the contribution of cytoskeleton elements and septins for different selective autophagy pathways.

While present studies have focused on experimental systems wherein pexophagy is induced by peroxisome proliferation followed by different starvation conditions, it will be a challenging task to analyze shared and distinct mechanisms for the degradation of damaged peroxisomes. Recent experiments have provided inroads to examine damage-induced pexophagy by destabilization of peroxisome membrane proteins [71]. Whether or not this interesting finding relates to physiological processes, and if the same mechanism is conserved in other yeasts and higher eukaryotes, remains to be unraveled.

Given the emerging link between peroxisome biology and the infection cycle of important viral pathogens (e.g., HIV, influenza, and rotavirus) on the one hand, and the contribution of peroxisomes to viral detection and innate immune responses on the other hand [137, 138], it will be of utmost importance to define the role of pexophagy in the context of these important human pathogens.

Future work will shed light on these and other unanswered questions addressing the molecular basis of peroxisome turnover pathways. The resulting insights are estimated to further our understanding of selective autophagy in general.

Abbreviations

15-LOX: 15-Lipoxygenase
3-MA: 3-Methyladenine
4-PBA: 4-phenylbutyrate
AIM: Atg8-Interaction Motif
ATG: Autophagy-related
CHO: Chinese Hamster Ovary cells
Cvt: Cytosol-to-Vacuole Targeting
DEHP: Di-(2-ethylhexyl) phthalate
FYCO1: FYVE and coiled-coil domain-containing 1 protein
GRAM: Domain found in glycosyltransferases, Rab-like GTPase activators and myotubularins
MAPK: Mitogen Activated Protein Kinase
MIPA: Micropexophagy-specific membrane Apparatus
PAS: Phagophore Assembly Site
PBD: Peroxisomal Biogenesis Disorders
PD: Peroxisomal Disorders
PE: Phosphatidylethanolamine
PED: Peroxisomal Enzyme/Transporter Deficiencies
PEX: Peroxin
PI3K: PtdIns-3-kinase
PM: Phagophore Membrane
PMP: Peroxisomal Membrane Protein
PPAR: Peroxisome-Proliferator Activated Receptor
PtdIns3P: phosphatidylinositol-3-phosphate
PtdIns4P: phosphatidylinositol-4-phosphate
PTS: Peroxisomal Targeting Signal
PVS: perivacuolar structures
PX: PtdIns3P-binding module
ROS: Reactive Oxygen Species
TbHK1: *Trypanosoma brucei* peroxisomal Hexokinase
VSM: Vacuole Sequestering Membrane.

Conflict of Interests

The authors declare no competing financial interests.

Acknowledgments

The authors would like to thank lab members Jean-Claude Farré, Taras Nazarko, and Katharine Ozeki for critically reviewing this paper. This work was supported by Grants GM069373 (to S. Subramani), a DFG (German Research Foundation) fellowship (Ti-640 1-1, to A. Till) and a Seed Grant from the San Diego Center of Systems Biology (SDCSB, to A. Till and S. Subramani). S. F. Burnett was supported by NSF GK-12 STEM Fellows in Education Grant 0742551 to Maarten Chrispeels and by funds from a Chancellors Associates Chair to S. Subramani.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 189794, 8 pages
doi:10.1155/2012/189794

Review Article

Alternative Macroautophagic Pathways

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Received 18 October 2011; Accepted 19 January 2012

Academic Editor: Kim Finley

Macroautophagy is a bulk degradation process that mediates the clearance of long-lived proteins, aggregates, or even whole organelles. This process includes the formation of autophagosomes, double-membrane structures responsible for delivering cargo to lysosomes for degradation. Currently, other alternative autophagy pathways have been described, which are independent of macroautophagic key players like Atg5 and Beclin 1 or the lipidation of LC3. In this review, we highlight recent insights in indentifying and understanding the molecular mechanism responsible for alternative autophagic pathways.

1. Introduction

Autophagy, which is highly conserved from yeast to human, is a cellular degradation pathway that delivers cytoplasmic substrates to lysosomes for subsequent degradation. In contrast to the Ubiquitin-Proteasome System (UPS), which directly degrades monomeric proteins in the cytoplasm or nucleus, autophagy targets a wide spectrum of substrates including long-lived proteins, protein aggregates, and organelles towards lysosomes for subsequent degradation. In mammalian cells, autophagy occurs under basal conditions but can be stimulated by various stress conditions including starvation, hypoxia, and treatment with apoptosis-inducing compounds like rapamycin. In addition to its role in maintaining cellular homeostasis, autophagy is implicated in a wide range of physiological and pathological conditions, including early embryological development, clearance of pathogens, tumor suppression, and antigen processing and presentation [1]. In order to target cytoplasmic proteins to the lysosomes, several autophagic pathways exist, including microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. While micro- and macroautophagy can occur both in eukaryotes, plants, and fungi, CMA has only been observed in mammals. Microautophagy is the direct engulfment of cytoplasm or whole organelles by invagination or protrusion of arm-like structures of the lysosomal membrane. Here, the sequestration of cytoplasmic cargo occurs directly at the vacuole surface [2–5]. The second type of autophagy is CMA, which selectively degrades specific cytosolic proteins containing a pentapeptide motif (KFERQ) that is recognized by the heat shock cognate protein 70 (Hsc70) [6, 7]. The chaperone-substrate complex subsequently binds the lysosome through interaction with the receptor Lamp-2a on the lysosomal membrane [8]. Upon delivery by Hsc70, the substrate protein is unfolded before crossing the lysosomal membrane and lysosomal Hsc70 pulls the substrate into the lysosomal matrix where it is degraded by proteases [9]. The last but main type of autophagy is macroautophagy. Here, double-membrane vesicles, termed autophagosomes, are formed and sequester portions of cytosolic content or intact organelles (such as mitochondria) [10]. These autophagosomes are subsequently transported in a dynein-dependent manner along microtubules and fuse with endosomes or directly with lysosomes to form autolysosomes, resulting in breakdown of their contents by hydrolytic enzymes [11]. Macroautophagy is the major cellular pathway to recycle cell components including long-lived proteins and organelles, thereby providing nutrients for the eukaryotic cell, and it is activated under nutrient starvation. Additionally, macroautophagy is essential for development, cell survival, and tissue-specific processes [12, 13]. The initiation of autophagosome formation starts with the phagophore (autophagosome precursor), and recent studies indicate that the source of the membrane is

the endoplasmatic reticulum (ER) [14, 15]. However, alternative sources for the autophagosomal membrane have been proposed, including the Golgi apparatus, and therefore the origin of the phagophore membrane still remains unresolved [16, 17].

2. Macroautophagy

Macroautophagy is a multistep process controlled by proteins termed autophagy-related (Atg) proteins [18]. The formation of the phagophore requires the class-III-phosphatidylinositol 3-kinase (PI3K) Vps34 that forms a complex with Beclin 1 (the mammalian orthologue of yeast Atg6). Inhibitors of Vps34 such as methyladenine (3-MA) or wortmannin can be used to inhibit macroautophagy since they prevent autophagosome nucleation [19–22]. The elongation of the autophagosomal membrane is dependent on two ubiquitin-like conjugation systems [23]. Atg5-Atg12 controls autophagy, where Atg12 is conjugated to Atg5 in a step that requires Atg7 (ubiquitin-activating-enzyme (E1)-like) and Atg10 (ubiquitin-conjugating-enzyme (E2)-like). The Atg5-Atg12 conjugation depends on Vps34 activity and is localized onto the phagophore where it dissociates upon formation of the autophagosome. Atg5-Atg12 forms a complex with Atg16L that modulates the next process, the ubiquitin-like conjugation of LC3-I (mammalian orthologue of Atg8). The protein LC3 is proteolytic activated by Atg4, which cleaves the C-terminus of LC3, thereby generating a cytosolic LC3-I, which subsequently conjugates with phosphatidylethanolamine (PE) to form membrane-associated LC3-II [24]. This process requires Atg7 and Atg3, and the Atg16L complex modulates the LC3-I lipidation by acting like an E3-like enzyme [25]. Although the Atg5-Atg12 conjugation dissociates upon completion of the autophagosome formation, LC3-II persists with the autophagosomal membrane even after fusion with a lysosomes and is regarded as a key marker for autophagosomes. Atg4 is also involved in the deconjugation reaction of LC3-II, as Atg4 delipidates LC3-II and removes it from the autophagosomal membrane [24, 26]. A pathway that negatively regulates macroautophagy is controlled by mTOR (mammalian target of rapamycin). mTOR activity is inhibited under starvation conditions, which activates starvation-induced macroautophagy. Recently, two new key regulators of macroautophagy, named NIX and DOR, which directly interact with the autophagosome-membrane-associated protein LC3, were identified [27]. Nix, a Bcl2-related protein localized the outer mitochondrial membrane, has a function as an adaptor protein and recruits autophagic components to mitochondria via its WXXL-like domain facing the cytoplasm [28–30]. NIX is upregulated during erythroid differentiation where a lack of mitochondria is achieved by mitophagy [27, 31, 32]. Interestingly, NIX-deficient mice show remaining mitochondria in matured red blood cells suggesting that NIX is a selective autophagy receptor that mediates mitochondrial clearance, as it directly binds LC3, but it may also target mitochondria for degradation in an LC3-independent manner [27, 33, 34]. Intriguingly, in the same issue of EMBO reports, another new autophagy-related protein was reported. Mauvezin et al. identified the nuclear cofactor of thyroid hormone receptors, termed DOR (diabetes- and obesity-regulated gene), as a new player of macroautophagy [35]. Stress-induced macroautophagy by starvation or rapamycin leads to release of DOR from the nucleus in DOR-transfected HeLa cells. This relocalization was not observed in the absence of cellular stress, indicating that cellular stress is essential to trigger DOR recruitment to the cytoplasm. DOR is associated with early autophagosomes via interaction with LC3 and GATE16 but does not colocalize with autolysosomes suggesting that DOR has a regulatory role in recruiting substrates for autophagic clearance. In addition, DOR-transfected HeLa cells show increased turnover of proteins and elevated numbers of autophagosomes compared to untreated cells. It has yet to be discovered which role DOR is playing, as it may be involved in targeting proteins to autophagy or in the formation and nucleation of the autophagosome. Whether DOR activation affects autophagy-induced alterations in cell survival remains to be established.

Macroautophagy was originally described to target intracellular organelles such as mitochondria and big protein complexes, but over the years it became clear that also most long-lived proteins are degraded via autophagic pathways. In contrast, the other main degradation machinery in the cell, the UPS, degrades mainly soluble short-lived and misfolded proteins that are targeted to the proteasome following ubiquitination (using a series of E1-E2-E3 enzymes to specifically target proteins for destruction). The proteasome is present in both the cytoplasm and the nucleus and can unfold and degrade single proteins into small peptide fragments that are subsequently recycled by peptidases. Interestingly, impairment of the proteasome leads to an increase in macroautophagy, indicating that macroautophagy can target accumulating ubiquitinated proteasomal clients when required [36–39]. In contrast, impairment of macroautophagy does not lead to increased proteasome activity. Inhibition of macroautophagy does not affect the catalytic activity of the proteasome but results in the accumulation of the macroautophagy cargo receptor p62 (also termed SQSTM1) which competes with the proteasome for ubiquitinated substrates. Indeed, silencing of p62 increases the amount of UPS clients, whereas overexpression of p62 inhibits degradation of the proteasomal substrates p53 and Ub^{G76V}-GFP [40, 41]. As p62 links ubiquitinated proteins via its ubiquitin-associated (UBA) domain to the autophagic protein LC3-II and is itself degraded in the process, inhibition of macroautophagy leads to p62 accumulation which will compete and frustrate other ubiquitin-binding proteins that participate in proteasome-mediated degradation.

3. Alternative Autophagic Pathways

Failure of the UPS or autophagic pathways to efficiently clear proteins leads to the accumulation and subsequent aggregation of these proteins, which is a hallmark of various neurodegenerative disorders including polyglutamine (polyQ)

disorders such as Huntington's disease. Here, fragments of the disease-related protein containing the polyQ tract initiate aggregation and toxicity, which can be mimicked by expressing the expanded polyQ sequence as a peptide [42]. Apparently, not all peptides are efficiently degraded by peptidases, which led to our recently published study where we examined potential alternative degradation machineries when peptidases would fail in degrading protein fragments [43]. In this study, we introduced peptidase-resistant peptides into living cells and observed a perinuclear accumulation of these peptides in time. Surprisingly, these structures did not represent aggregates or inclusion bodies as observed previously for aggregation-prone protein fragments, as no UPS components or chaperones were recruited. Although initially present in the nucleus and cytoplasm, the peptides were efficiently targeted to lysosomes within a few hours upon introduction into cells, and subsequently degraded. Our results indicate, therefore, that similar to the described increase in autophagy upon proteasome impairment, a backup mechanism exists for small protein fragments that show peptidase resistance. Intriguingly, this mechanism was very efficient for peptides of the average size of proteasomal products (6–9 amino acids), but far less for extended peptides over 25–30 amino acids which remained cytoplasmic for prolonged periods [43]. Similar to expanded polyQ peptides of disease-related lengths, these expanded peptidase-resistant peptides were more resistant to clearance by lysosomes suggesting that this pathway is particularly efficient for small peptides generated by the proteasome. It is tempting to speculate that this mechanism evolved as a backup to peptidases in the clearance of proteasome-derived peptides and emphasizes the need to identify the involved proteins. Using correlative microscopy, we mainly observed double-membrane vesicles that contained peptides and that colocalized with LC3. The colocalization increased when we used Bafilomycin A1 to impair maturation into autolysosomes. In contrast, we could prevent colocalization of LC3 with the macroautophagy inhibitor 3-MA, suggesting that the macroautophagic pathway took over the clearance of these peptides. Unexpectedly, inhibition of macroautophagy by inhibitors such as 3-MA or knockdown of Atg5 prevented recruitment of LC3 but did not affect the trafficking of these peptides into lysosomes or their subsequent degradation. Apparently, LC3 was recruited during the trafficking of peptides towards lysosomes yet was not essential. Similar to the knockdown of the various LC3 isoforms (LC3A-C), knockdown of the Atg8-related GABARAP proteins, that can interact with autophagosomes, did not affect the targeting of peptides towards lysosomes [44, 45]. As knockdown of Atg5 or WIPI-1 did not affect the trafficking and subsequent degradation of peptides in lysosomes, we concluded that these peptides entered lysosomes via a pathway different from macroautophagy. CMA is also unlikely to contribute to this pathway as the peptides lack a CMA motif and peptides composed of D-amino acids, which are unable to bind chaperones like Hsc70, were also trafficking via this pathway. Finally, we also examined endosomal microautophagy, a process that delivers soluble cytosolic material to vesicles of late endosomes or multivesicular bodies (MVBs) [46, 47]. Although accumulated peptides colocalized with internalized MHC class II molecules which may lead to so-called cross-presentation to the immune system (unpublished observation), knockdown of the sorting complexes required for transport (ESCRTs) I and III showed no effect on peptide accumulation in lysosomes. As no recruitment of ESCRT regulators towards accumulated peptides was observed, this indicates that the endosomal microautophagy pathway is not involved in the trafficking and clearance of the peptidase-resistant peptides.

The accumulation and subsequent lysosomal degradation of cytoplasmic proteins independent of known autophagy pathways have been previously observed in several studies (as described below), although in each case differences in sensitivity to autophagy inhibitors and the involvement of various Atg proteins were reported. Interestingly, in a study using Green Fluorescent Protein (GFP) like fluorophores, a pathway reminiscent of that we observed for the peptidase-resistant peptides was observed [40]. Various GFP-like fluorophores have been shown to form dimers, tetramers, or even larger complexes. Upon expression, these fluorescent proteins formed cytoplasmic fluorescent puncta that resembled lysosomes, similar as observed for the peptidase-resistant peptides [48]. However, the accumulating fluorophore proteins including monomeric RFP1 (mRFP1) showed resistance to lysosomal degradation and retain fluorescence, in contrast to the peptides. Trafficking of the GFP-like proteins and the peptidase-resistant peptides was not affected in Atg5-deficient mouse embryonic fibroblasts, suggesting that they may be targeted to lysosomes by a similar pathway (although no other macroautophagy markers were examined for the fluorescent proteins). So is the constitutive macroautophagy-independent targeting of cytoplasmic proteins and peptides to autolysosomes restricted to introduced peptides and GFP-like fluorophores?

At least two alternative autophagy pathways have been described: an Atg5/Atg7-independent pathway and the so-called noncanonical autophagy pathway, which is independent of Beclin 1 (Table 1). The Atg5/Atg7-independent autophagic pathway was recently discovered in mouse embryonic fibroblasts (MEF) lacking Atg5 and Atg7 that were treated with the cytotoxic stressor etoposide, which caused an equivalent appearance of autophagic vacuoles when compared to wild-type cells [49]. Moreover, autophagic vacuoles were also found in starved Atg5-/- cells. The Atg5/Atg7-independent form of autophagy does not involve the lipidated conjugate LC3-II, which is membrane associated. Interestingly, equivalent numbers of LC3-positive and LC3-negative autophagosomes were observed in etoposide-treated wild-type cells, suggesting that conventional and alternative autophagic pathway occur at the same time. The proteins Atg5, Atg7, and LC3, which are important in the ubiquitin-like conjugation system for the autophagosome elongation, are not involved in this alternative form of autophagy. However, silencing of Beclin 1 and Vps34 decreased the amount of autophagosomes, indicating that the PI3K complex, which acts upstream of initiation of autophagosome formation, is still required in etoposide- or starvation-induced autophagy in Atg5-/- cells. Accordingly,

Table 1: Types of alternative macroautophagic pathways.

Alternative macroautophagic pathways	Macroautopagic molecules involved	Macroautopagic molecules not involved	Induction	Cell type	Reference
Beclin 1-independent	Atg5 Atg7 Ulk1/2 LC3	Beclin 1 (Vps34)	Resveratrol	MCF-7 (breast cancer cells)	[50]
			Staurosporine Etoposide MK801	primary cortical neurons	[51]
			H_2O_2	RAW 264.7 (macrophage cells)	[52]
			MPP+	SH-SY5Y (neuroblastoma cells)	[53]
				primary dopaminergic neurons	
			As_2O_3	ovarian cells	[55]
Atg5/Atg7-independent	Beclin 1 Vps34 Ulk1 Fip200	Atg5 Atg7 Atg9 Atg12 Atg16 LC3	Etoposide Staurosporine Starvation	Atg5-/- MEF Atg7-/- MEF wt MEF	[49]
Degradation of peptidase-resistant peptides	LC3 (but not essential)	Atg5 WIPI-1 p62 Tsg101 Vps24	Resistance against cytoplasmic peptidases	HeLa Atg5-/- MEF wt MEF	[43]

protein degradation via this pathway was inhibited by the PI3K inhibitor 3-MA. Furthermore, silencing of components of the Ulk1 complex, a mammalian serine/threonine protein kinase that plays a key role in the initial stages of autophagy, decreased autophagic vacuoles, suggesting that the Ulk1 complex is needed for Atg5/Atg7-independent autophagy [49].

Apoptosis-induced stress, for example, by staurosporine, resveratrol, or H_2O_2 can also induce the so-called non-canonical autophagy pathway, where autophagosomes can be formed independent of Beclin 1 or Vps34 and with an insensitivity to 3-MA [50–52]. However, this specific pathway still requires Atg7-activity for LC3-I lipidation and is, therefore, different from the Atg5/Atg7-independent pathway described above [49]. Furthermore, Scarlatti et al. have shown that resveratrol inhibits the mTOR activation by a direct inhibitory effect on the upstream class 1A PI3K [50]. Similarly, a Beclin 1-independent pathway has been reported in neuronal cells treated with the neurotoxin 1-methyl-4-phenylpyridinium (MPP+) [53] and in other cellular systems in response to various drugs [54, 55]. These studies have shown that several agents stimulate autophagic cell death through Beclin 1 in canonical autophagy pathways [56]. Recently, evidence emerged that autophagy and cell death are induced independent of Beclin 1 and Vps34. In breast cancer cells, resveratrol induces autophagic cell death in a Beclin 1-independent manner [50]. Silencing of Atg7 impairs the cellular death elicited by resveratrol. In dopaminergic neuronal cells, the neutotoxin MPP+ induces Beclin 1-independent autophagy and cell death [53]. As most studies on the noncanonical pathway used compounds to induce cell death, it is tempting to link the noncanonical autophagy pathway to a death execution mechanism or cell survival. However, it has also been suggested that the independency of the noncanonical autophagy pathway may provide an adaptation to loss of Beclin 1, for example, in various tumors where Beclin 1 is deleted, in immune cell development, and may even be an evolutionary way to circumvent inhibition of Beclin 1 by various viruses in order to prevent autophagy [57–59].

None of these alternative autophagy pathways seem to correspond to the trafficking we observed for the peptidase-resistant peptides, as the Atg5/Atg7-independent pathway is still 3-MA sensitive (in contrast to the peptide targeted to lysosomes), while the noncanonical pathway (Beclin 1-independent) is 3-MA insensitive but still depends on LC3. Thus, lysosomal degradation of peptidase-resistant peptides and proteins, as we and others have demonstrated [35, 41–45], defines a novel authophagy route independent of known regulators of the constitutive macroautophagic pathway like Beclin 1, Atg5 or LC3. A better understanding of the role of these alternative autophagic pathways and their molecular regulators raise to two crucial questions: (1) What is the origin of the autophagic membrane in the different autophagic routes, and (2) Which stimuli trigger the different autophagic pathways?

In mammalian macroautophagy, various sources for the origin of the autophagosome membrane have been proposed including the ER, the Golgi complex, the plasma membrane, and the mitochondria [17, 60–66]. Alternatively, *de novo* synthesis of a nucleating structure, the phagophore, is proposed to elongate by the addition of lipids via the integral

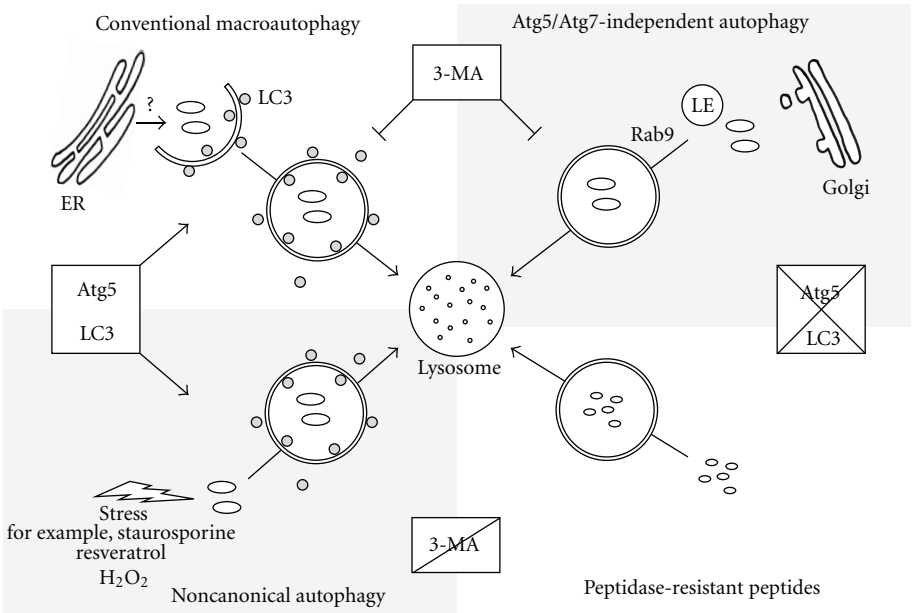

Figure 1: Alternative macroautophagic pathways lead to lysosomal degradation. At least four autophagic pathways can be distinguished that all show double-membrane autophagic structures and end in lysosomal degradation of cytoplasmic cargo. Conventional macroautophagy is hallmarked by the recruitment of lipidated LC3 to autophagosomal membranes that may origin from the endoplasmatic reticulum (ER). This process is dependent on Atg5 and Beclin 1 and can be inhibited by 3-methyladenine (3-MA). In contrast, the observed Atg5/Atg7-independent autophagy pathway forms Rab9-positive double-membrane vesicles derived from the *trans*-Golgi network and late endosomes (LE), and while it can be inhibited by 3-MA and is dependent on Beclin 1, the process is independent of Atg5 and LC3. Almost similar, the degradation of accumulated peptidase-resistant peptides is independent of Atg5 and LC3 and is also insensitive to 3-MA treatment. Finally, the noncanonical autophagy pathway induced by different stress factors is dependent on Atg5 and LC3 and independent of Beclin 1 but cannot be impaired by 3-MA.

membrane protein Atg9 [67–70]. Atg9 seems to be a key regulator in regulating the formation and expansion of nascent autophagosomes. Unfortunately, the identity of proteins that partition to the autophagosomal membrane remains largely unknown. Therefore, attempting to determine the origin of the autophagosomal membrane based on the associated proteins remains a challenge [71]. Alternatively, others attempted to determine the source of the autophagosomal membrane by inspecting its thickness and lipid composition [15]. Several studies reported that the autophagosomal membrane can be classified as of a thin type (6–8 nm), similar to membranes of the ER and mitochondria [60, 72–75]. Furthermore, lipid structures enriched in PI3P (known as omegasomes) were formed in the vicinity of ER membranes after amino acid starvation, suggesting that these omegasomes originate from the ER [76–79]. As the omegasomes carry autophagosomal proteins like Atg5 and LC3, they may represent the source of isolated membranes required for autophagosome expansion. In contrast, in the Atg5/Atg7-independent autophagic pathway, autophagosomes with membranes of the thick type (9-10 nm) were observed, similar to membranes of lysosomes and the *trans*-Golgi network [49]. Intriguingly, unlike the conventional pathway the alternative Atg5/Atg7-independent form of autophagy is blocked by brefeldin A, indicating that autophagosomes are derived from the Golgi-apparatus. Etoposide-induced Atg5/Atg7-independent autophagy is accompanied by colocalization of markers of the *trans*-Golgi and late endosomes (such as the mannose 6-phosphate receptor, TGN38, and Rab9) with Lamp-2-positive autolysosomes, further pointing to the requirement of the *trans*-Golgi or late endosomes in this alternative form of autophagy. Indeed, silencing of Rab9 or expression of a Rab9 dominant negative mutant established an essential role for Rab9 in membrane expansion from isolated membranes and led to an accumulation of isolated membranes after silencing of Rab9 but not upon inhibition of Ulk1 or Beclin 1. Since the Atg5/Atg7-independent type of alternative autophagy is activated by starvation and the stress-inducing reagent etoposide, but not by rapamycin, this suggests that a specific stimulus for induction of autophagy activates nonconventional macroautophagy with different lipid structures compared to conventional macroautophagy. To the best of our knowledge, there is no clear data on the source of membrane for the Beclin 1-independent noncanonical autophagy pathway.

So far, several sources have been proposed to provide the putative moiety of autophagosomal membranes. However, autophagosomal membranes could derive from multiple membrane sources and the origin of lipids may vary dependent on the cell type, the stimulus that triggers the degradation, and the type of cargo for autophagic destruction (proteins, aggregates or even whole organelles). As shown in Figure 1, there are now at least three alternative pathways that target cytosolic content to lysosomes, which can be discriminated by their dependence on Atg5 and 3-MA (Figure 1). The identification of key players and the origin of membrane

structures involved in alternative autophagic pathways will be important for the understanding of molecular mechanism regulating these various types of autophagy.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 736905, 21 pages
doi:10.1155/2012/736905

Review Article

Aggrephagy: Selective Disposal of Protein Aggregates by Macroautophagy

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Received 1 December 2011; Accepted 6 January 2012

Academic Editor: Masaaki Komatsu

Protein aggregation is a continuous process in our cells. Some proteins aggregate in a regulated manner required for different vital functional processes in the cells whereas other protein aggregates result from misfolding caused by various stressors. The decision to form an aggregate is largely made by chaperones and chaperone-assisted proteins. Proteins that are damaged beyond repair are degraded either by the proteasome or by the lysosome via autophagy. The aggregates can be degraded by the proteasome and by chaperone-mediated autophagy only after dissolution into soluble single peptide species. Hence, protein aggregates as such are degraded by macroautophagy. The selective degradation of protein aggregates by macroautophagy is called aggrephagy. Here we review the processes of aggregate formation, recognition, transport, and sequestration into autophagosomes by autophagy receptors and the role of aggrephagy in different protein aggregation diseases.

1. Introduction

Misfolded proteins result from mutations, incomplete translation giving defective ribosomal products (DRiPs), misfolding after translation, aberrant protein modifications, oxidative damage, and from failed assembly of protein complexes. Misfolded proteins expose hydrophobic patches that are normally buried internally in the native folded state. These hydrophobic surfaces trigger aggregation and can sequester normal proteins compromising their functionality [1]. To defend cells against the hazards caused by accumulation of misfolded proteins, different protein quality control machineries are active at several levels. Molecular chaperones, like the heat shock proteins (Hsp), recognize, assist folding, prevent aggregation, and attempt to repair misfolded proteins. However, if the damage is beyond repair, chaperone complexes, often in conjunction with interacting ubiquitin E3 ligases, channel the misfolded protein or protein aggregates to degradation pathways.

1.1. The UPS. The two major degradation systems in the cell are the ubiquitin-proteasome system (UPS) and the lysosome (Figure 1). The UPS comprises the proteasome and the enzymatic cascade catalysing the ubiquitination of substrates destined for degradation in the proteasome. The prime tag for proteasomal degradation is a chain of 4 or more ubiquitin moieties covalently linked to lysine residue(s) of the target. Ubiquitin has 7 internal lysines (K6, K11, K27, K29, K33, K48, and K63) that can be linked, forming polyubiquitin chains [2, 3]. K48-linked polyubiquitin chains represent the canonical proteasomal degradation tag, but also K11-linkages are used and some substrates with K63-linked polyubiquitin can be degraded by the proteasome [4]. An enzyme cascade of E1 activation, E2 conjugation, and E3 ligation enzymes mediates the ubiquitination of target proteins [5]. The human repertoire consists of two ubiquitin-specific E1 activation enzymes, about 30 E2 conjugation enzymes, and more than 1000 E3 ligases providing a great versatility in substrate recognition and enabling diversity in ubiquitin chain linkages added to substrates [6–9].

The proteasome consists of a barrel-shaped catalytic core particle, called the 20S proteasome, and the regulatory particle [10, 11]. The cylindrical catalytic particle has a central channel with a diameter of only ~1.5 nm with three proteolytically active proteasomal subunits facing the inside of this channel. Hence, the digestion chamber is inaccessible

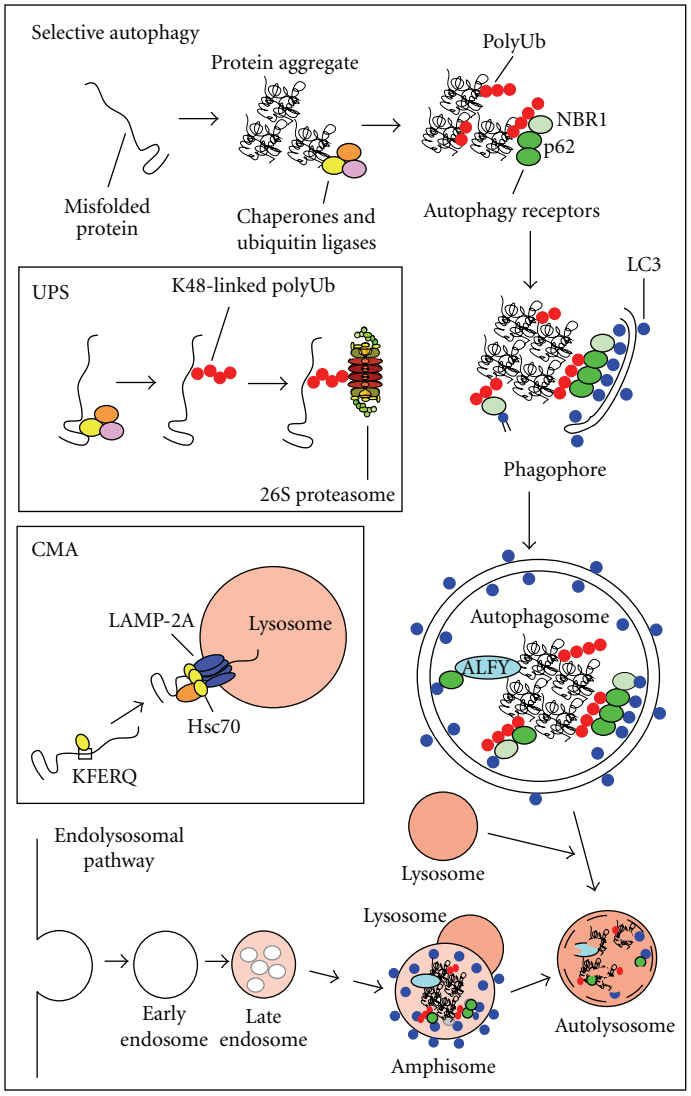

Figure 1: Proteins recognized as misfolded by molecular chaperones can be degraded by selective autophagy, the ubiquitin-proteasome system (UPS) or chaperone-mediated autophagy (CMA). In selective autophagy, misfolded proteins are often assembled into aggregates before they are degraded. They are also often ubiquitinated, and this induces the recruitment of ubiquitin binding cargo receptors such as p62 and NBR1. These cargo receptors bind to ubiquitinated cargos (in this case a protein aggregate) and to ATG8 homologues conjugated to the inner surface of the phagophore (LC3 indicated as blue dots). This way, cargos are selectively delivered to the inner surface of the phagophore. An autophagosome is formed by closure of the phagophore. The autophagosome fuses with a late endosome or with a lysosome, but the end point is in both cases the formation of an autolysosome where the contents are degraded. Substrates for the UPS and CMA degradation pathways need to be in a soluble and monomeric form. Degradation by the UPS depends on K48-linked polyubiquitination of the misfolded substrate. The substrate is then delivered to the 26S proteasome, where it is deubiquitinated and degraded. Degradation by CMA depends on an Hsc70-mediated recognition of a KFERQ motif on the misfolded substrate. The substrate is then delivered to the lysosomal receptor LAMP-2A, transported into the lumen of the lysosome, and degraded.

for folded proteins. Substrate access is regulated by "gates" on both sides of the 20S proteasome. The complete 26S proteasome contains two 19S regulatory subunits, one on each side, mediating substrate recognition, unfolding, and transfer into the catalytic chamber of the 20S proteasome [10–12]. The 19S regulatory particle consists of the base and the lid. The base has six AAA-type ATPases (Rpt1–Rpt6) forming the hexameric ring and four non-ATPase subunits (Rpn1, Rpn2, Rpn10, and Rpn13). The hexameric ring unfolds proteasomal substrates and together with Rpn1-Rpn2 helps open the gate into the catalytic chamber of the 20S proteasome. Rpn10 and Rpn13 recognize and recruit proteasomal substrates by binding to the K48-linked polyubiquitin degradation tag [13]. The lid has nine Rpn subunits (Rpn3, Rpn5–9, Rpn11-12, and Rpn15). Rpn11 is a de-ubiquitination enzyme (DUB) responsible for recycling of ubiquitin [10, 11, 13].

1.2. Autophagy. The lysosomal degradation of intracellular contents, such as misfolded proteins, protein aggregates,

and organelles, is mediated by autophagy [14, 15]. Three major types of autophagy have been described in mammalian cells, that is, macroautophagy [14–16], microautophagy [17], and chaperone-mediated autophagy (CMA) [18, 19]. Of these, macroautophagy (hereafter referred to as autophagy) is the only process that can mediate the degradation of larger substrates such as organelles, microbes, and protein aggregates (Figure 1). The UPS and CMA are only capable of degrading one extended polypeptide at the time. Autophagy is initiated by the formation of a double-membrane structure, the phagophore. The source of the phagophore membrane is still under debate, and both the ER, mitochondria, plasma membrane, and the Golgi apparatus have been implicated [20]. Elongation of the phagophore depends on two ubiquitin-like conjugation reactions. First, autophagy-related gene 12 (ATG12) is conjugated to ATG5 resulting in the formation of an oligomeric ATG5-ATG12-ATG16L complex. This complex is then needed for the conjugation of ATG8 homologues to phosphatidylethanolamine (PE) on the phagophore membrane [21]. Mammalian ATG8 homologues are grouped into three subfamilies, that is, the LC3 subfamily (LC3A, B, and C), the GABARAP subfamily (GABARAP and GABARAPL1/GEC1), and GABARAPL2/GATE-16 [22]. Conjugation of ATG8 homologues to both sides of the phagophore enables them to act as surface receptors for the specific recruitment of other proteins. Lipidated ATG8 proteins are also involved in membrane biogenesis of autophagosomes via their membrane fusion activity [23]. Autophagosomes are formed by closure of the phagophore into a double-membrane vesicle. Lipidated ATG8 homologues on the outer membrane are released by ATG4B upon completion of autophagosome formation [24]. In mammalian cells autophagosomes often form at the cell periphery and are transported along microtubules and fuse with late endosomes or lysosomes at the microtubule-organizing centre (MTOC) area of the cells finally resulting in degradation of their contents.

1.3. Selective Autophagy. Autophagy has been considered as a bulk degradation system with little or no selectivity that is induced to replenish energy stores upon starvation. However, there is now considerable evidence to support the notion that the process may also be highly specific [25–27]. The term selective autophagy refers to the selective degradation of organelles, bacteria, ribosomes, specific proteins, and protein aggregates by autophagy. In selective autophagy, an important role is played by proteins acting as autophagy receptors such as p62 and NBR1 that bind directly to ATG8 homologues (Figure 1). The autophagy receptors are themselves degraded by autophagy, and they mediate selective autophagy via interactions with substrates that are simultaneously degraded [26, 28–31]. Selective autophagy is an important quality control system and is part of a basal constitutive autophagy that can also be induced or boosted by various stressors including oxidative stress, infections, protein aggregation, and proteasomal inhibition [26, 32].

The formation of larger protein aggregates is regarded as a cellular defense mechanism [33, 34]. The large aggregates or inclusions are less toxic to the cell than the presence of smaller microaggregates dispersed throughout the cell [33, 35–38]. Since the large inclusions are usually readily visible in the light microscope, while the more toxic soluble species are not, the inclusions can also be used to distinguish between different neurodegenerative disorders involving aggregation of specific, often mutant, proteins. The protein aggregates may also represent intermediates in autophagic degradation of aggregation-prone proteins [39]. The assembly of autophagy substrates into larger aggregates or clustered structures is a common feature of selective autophagy [26]. It may facilitate their uptake into autophagosomes, and aggregates may work as nucleation sites for the phagophore, the forming isolation membrane [40].

Proteins damaged beyond repair are recognized and sorted by chaperone and co-chaperone complexes containing chaperone-assisted ubiquitin E3 ligases to three different degradation pathways: the UPS, CMA, and/or aggrephagy. The term aggrephagy was introduced by Per Seglen to describe the selective sequestration of protein aggregates by autophagy [41]. In the following we will review the current knowledge on how protein aggregates are recognized, sorted, and degraded by aggrephagy.

2. Crosstalk between Degradation Pathways: Hsp70/Hsp90 and Co-Chaperones

2.1. Quality Control of Newly Synthesized Proteins. A complex consisting of Hsp70, Hsp40, and several co-chaperones including Cdc37 mediates the protein quality control of newly synthesized proteins in the cytosol (Figure 2(a)). In this process, DRiPs and aggregation-prone translational products are degraded. Functional products are released or delivered to the Hsp90 chaperone complex. In ER and mitochondria, homologs of Hsp70 play a similar role in the quality control of newly synthesized proteins. The protein quality control in ER (reviewed in [42]) begins when a nascent chain enters ER through the translocon. Newly synthesized proteins transiently undergo cycling with the ER luminal Hsp70 paralog BiP/GRP78 which is associated with several co-chaperones. Proteins that are recognized as misfolded or not properly processed are delivered for ER-associated degradation (ERAD). ERAD substrates are retranslocated into the cytoplasm where they are degraded mainly by the UPS (Figure 3(a)). A chaperone holdase activity mediated by an associated BAG6 complex is needed to keep ERAD substrates unfolded, yet soluble, until they are degraded [43].

2.2. Selective Degradation of Damaged Proteins. Quality control of mature proteins is another important role of Hsp70/Hsp90 chaperone complexes (Figure 2(a)). There is considerable crosstalk between the Hsp70 and Hsp90 chaperone complexes, but in general Hsp90 protects proteins from unfolding and aggregation, whereas Hsp70 is responsible for their degradation in cases when unfolding or aggregation cannot be prevented. The classic clients of Hsp90 are unstable proteins that undergo tight cycling with the chaperone, and in response to Hsp90 inhibition, these proteins are rapidly delivered to Hsp70 and degraded. Other more stable proteins

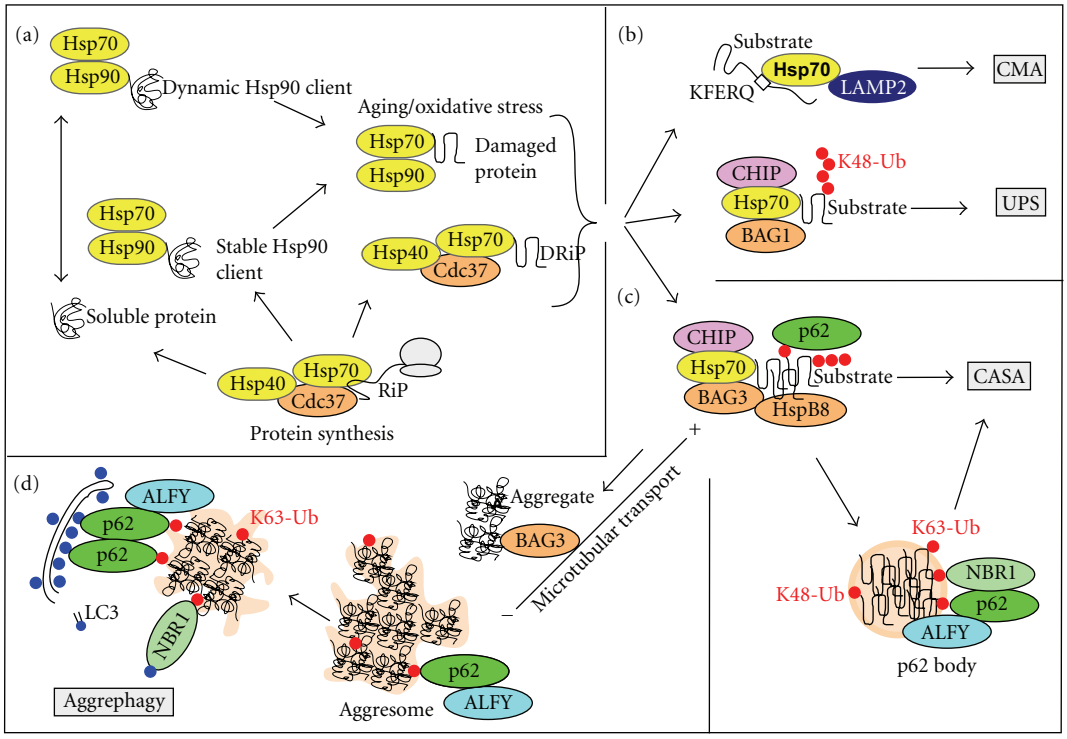

Figure 2: Protein degradation assisted by heat shock proteins and their co-chaperones. (a) Substrates selected for degradation by heat shock proteins are either defective ribosomal products (DRiPs) or Hsp90 client proteins that start to unfold or aggregate. Formation of the latter type of substrate is increased under conditions of oxidative stress or during aging. (b) Misfolded and monomeric substrates bound to Hsp70/Hsc70 are preferentially degraded by CMA or by the UPS. (c) In response to aggregation, or if the capacity of CMA and the UPS is insufficient, substrates are degraded by chaperone-assisted selective autophagy (CASA). This process relies on the co-chaperones BAG3 and HspB8, the E3 ubiquitin ligase CHIP, and autophagy receptors such as p62. The process may also rely on the assembly of the misfolded substrates into p62 bodies. (d) If degradation of misfolded substrates is impaired, BAG3 interacts with dynein and transport protein aggregates along microtubules to the aggresome. The contents of aggresomes may subsequently be degraded by aggrephagy.

may be less dependent on Hsp90, but they may still undergo dynamic cycling with the chaperone complex [44].

If a misfolded protein cannot be refolded by chaperones, this normally results in its degradation by the UPS, CMA, and/or selective autophagy. Since Hsp70 can mediate the delivery to all three degradation pathways, the same substrate can in principle be degraded by all three systems (Figures 2(b) and 2(c)). Inefficient degradation by one system is often compensated by increased degradation by another system. Impairment of the UPS or CMA leads to activation of autophagy [45–49]. Vice versa, in cells where autophagy is inhibited, CMA is increased to compensate [50].

Previously, autophagy was considered to act only as a back-up system when the capacity of UPS and CMA is overwhelmed. However, selective autophagy is active also under normal conditions, and tissues such as brain, liver, and muscle have a constitutive need for selective autophagy [51–55]. An obvious role for selective autophagy under normal conditions is to degrade substrates that are not solubilized or unfolded and exist as some form of aggregated structure.

2.3. Degradation by CMA or the UPS. In CMA, cytosolic substrates with a KFERQ-like motif are degraded in lysosomes without the formation of autophagic vesicles (Figure 1). Substrates are recognized by an Hsc70 complex, delivered to the lysosomal receptor LAMP-2A, and transported into the lumen of the lysosome where they are degraded [18, 19]. The KFERQ-like motif is present in 30% of cytosolic proteins, and the fraction may be higher than this due to posttranslational modification [56]. CMA activity is proportional to the level of LAMP-2A at the lysosomal membrane. Expression of LAMP-2A is upregulated, and CMA therefore increased under oxidative stress conditions [57].

In order to be degraded by the UPS, a substrate must be polyubiquitinated with chains consisting of four or more preferably K48-linked ubiquitin moieties. CHIP (carboxyl terminus of constitutive Hsc70-interacting protein) is a cofactor for Hsp70 and Hsp90 and a prototype of the chaperone-dependent ubiquitin E3 ligases involved in proteasomal degradation of Hsp90 client proteins [58–60]. The DUB ataxin-3 regulates the length of ubiquitin chains added to CHIP substrates, and it is likely that this ubiquitination is not only regulated by CHIP but also by other chaperone-assisted E3 ligases and DUBs [61].

2.4. Chaperone-Assisted Selective Autophagy (CASA). The group of Hohfeld has introduced the term chaperone-assisted selective autophagy (CASA) to describe selective

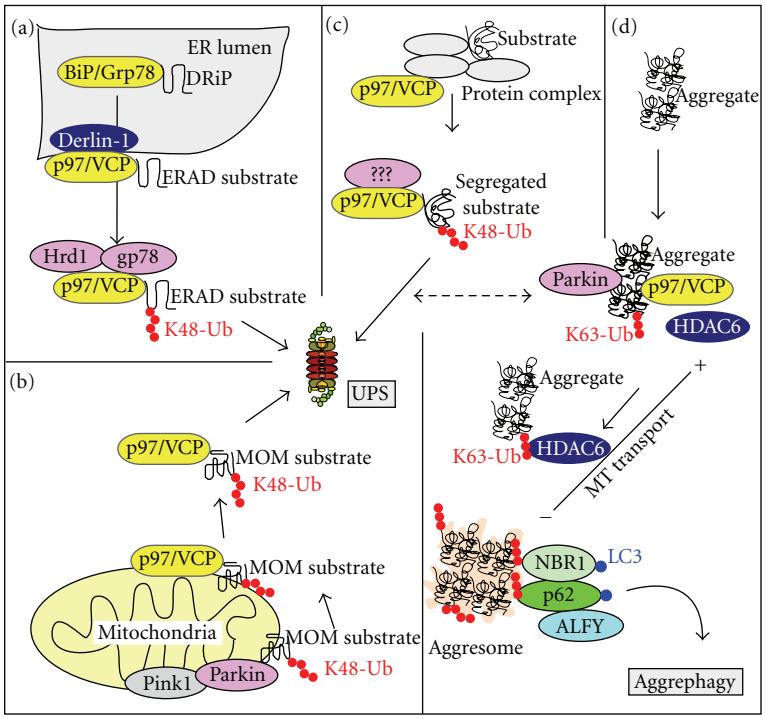

Figure 3: Protein degradation assisted by p97/VCP and HDAC6. (a) Misfolded substrates located in the ER lumen or at the ER membrane are recognized by the ER luminal Hsp70 homologue BiP/Grp78 and degraded by ER-associated degradation (ERAD). A complex of p97/VCP and Derlin-1 mediates the transport of ERAD substrates into the cytoplasm where they are ubiquitinated by E3 ligases such as Hrd1 and gp78 and degraded by the UPS. (b) p97/VCP mediates the segregation of ubiquitinated mitochondrial outer membrane (MOM) substrates into the cytoplasm, where they are degraded by the UPS. (c) p97/VCP mediates the segregation of selected substrates from nuclear or cytoplasmic protein complexes, followed by their degradation by the UPS. (d) p97/VCP is also required for the transport of protein aggregates to the aggresome. This depends on ubiquitination of the aggregate by an E3 ligase such as Parkin, and the delivery of the ubiquitinated aggregate to HDAC6. HDAC6 binds to K63-linked polyubiquitin chains and to dynein, and it is responsible for the transport of ubiquitinated protein aggregates along microtubules to the aggresome. The contents of aggresomes may subsequently be degraded by aggrephagy.

autophagy of misfolded proteins following a chaperone-mediated formation of protein aggregates that are targeted to form autophagosomes [62]. The dedicated chaperone in CASA is BAG3 (Figure 2(c)). The BAG (Bcl2-associated athanogene) family (BAG1-6) of co-chaperones uses their BAG domain to interact with the ATPase domain of Hsp70. BAG1 competes with Hip for interaction with Hsp70, and binding of BAG1 induces proteasomal degradation of misfolded Hsp70 substrates (Figure 2(b)). Alternatively, a multi-chaperone complex of Hsp70, BAG3, and HspB8 induces selective degradation of misfolded proteins by autophagy. Substrates shown to be degraded by this complex include polyQ-expanded huntingtin [63] and SOD1 [64]. CASA is important also under normal growth conditions, and mice deficient for BAG3 die shortly after birth due to the development of a progressive muscle weakness [65]. In muscles, a complex containing BAG3, its partner HspB8, CHIP, and Hsp70 is constitutively needed for the maintenance of Z-disks [66]. Loss of BAG3 activity in patients or transgenic animals leads to a contraction-dependent disintegration of Z-discs [65, 67]. The BAG3 complex is here needed for clearance of damaged components such as filamin [66].

2.5. p62 Bodies, DALIS, and ALIS. There is an intimate relationship between CASA and the formation of p62 bodies (Figure 2(c)), but more studies are needed to verify whether their formation is required for CASA or not. The contents of p62 bodies are degraded by selective autophagy, and this depends on a direct interaction of its major constituent p62 and its interaction partner NBR1, with ATG8 homologues on the phagophore [30, 31]. The decision to form p62 bodies and to degrade misfolded substrates by CASA may be decided by the BAG3 : BAG1 ratio within the specific cell. The link between BAG3 and the formation of p62 bodies was initially described by the group of Christian Behl [68]. Strikingly, in aging cells, an increased level of BAG3 relative to BAG1 is responsible for a shift from proteasomal towards autophagic degradation of misfolded proteins. This correlates with an increased formation of p62 bodies [68].

A specialized type of protein aggregate clearly related to p62 bodies is the dendritic cell aggresome-like induced structures (DALISs) initially studied by the group of Philippe Pierre [69, 70]. This type of ubiquitinated structure is transiently formed in antigen-presenting cells such as dendritic cells and macrophages during immune cell maturation. By

using puromycin to induce the formation of DRiPs, they showed that misfolded proteins accumulate in DALIS and become ubiquitinated within these structures. DALIS is an ordered type of structure distinct from aggresomes. The formation of DALIS is stress-induced and transient and does not depend on transport along microtubules [69, 70]. Later studies showed that similar structures can be formed in many cell types in response to stressors like puromycin, oxidative stress, starvation, and transfection, and they were therefore given the name ALIS [71]. We noted that p62 is a major protein in these structures and realized that ALIS and p62 bodies are indistinguishable structures [31]. The relationship between p62 bodies and DALIS needs to be analyzed more carefully. p62 bodies have been used by us as a term to describe aggregates formed by p62 in response to various stressors. A major role of p62 bodies is to serve as substrates for selective autophagy. It is important to realize that some types of p62 bodies may not be true ALIS, in the sense that they may not fulfill the criteria as has been described for the DALIS of dendritic cells [69, 70, 72]. We therefore consider p62 bodies to represent a more broad type of structure, also including aggregates that are different from DALIS/ALIS.

An important role of DALIS during immune cell maturation is MHC class I presentation, and this depends on proteasomal degradation [73]. This actually also occurs for DRiPs accumulated in ALIS in HeLa cells during autophagy inhibition, although these DRiPs are normally also autophagy substrates [74]. A recent study explored the degradation of DALIS formed in dendritic cells, and this study revealed that the contents of DALIS can be degraded both by the proteasome and by selective autophagy [72]. In line with other studies, BAG3 is needed for selective autophagy of these structures, but not for their proteasomal degradation [72]. Degradation by the UPS is probably not a specific feature of the DALIS but may occur with p62 bodies of other cell types as well [71]. However, oxidative stress-induced p62 bodies in pancreatic cells of diabetic rats are only cleared by autophagy [75]. Possibly, the aggregation status of proteins within the p62 body is an important parameter that may regulate the recruitment of BAG3 to the aggregate.

3. The Decision to Form Aggregates

3.1. The Aggresome. The aggresome, formed in response to proteasomal inhibition or overexpression of aggregation prone proteins, is currently the best-studied protein aggregate with respect to formation and degradation mechanisms. The aggresome is located close to the nuclear envelope at the microtubule organizing center (MTOC), and its formation depends on microtubule-dependent transport of protein aggregates [34, 76]. It is insoluble and metabolically stable. The proteins of an aggresome are normally ubiquitinated [77], and they are enclosed by intermediate filaments such as vimentin and keratin, depending on cell type [34, 76]. Other types of inclusions observed in proteinopathies may have a nuclear or more dispersed cytoplasmic location. The formation of a specific type of inclusion is often associated with the formation of a variety of smaller intermediates that can be unstructured or have a variety of different types of structures [1]. A study reported two different types of aggresome-like structures found both in yeast and mammalian cells called the "juxtanuclear quality control" (JUNQ) and "insoluble protein deposit" (IPOD) that differ from the classical definition of an aggresome since they do not localize to the MTOC [78]. Both of these structures require microtubular transport for their formation. IPODs are localized to the cell periphery often near vacuoles, and the aggregates do not contain ubiquitin. JUNQ, on the other hand, is localized close to the nucleus and contains ubiquitinated proteins and associated proteasomes. More studies are required to determine the relationships between these different "aggresomes."

What should be noted is that aggregation may also be part of an important functional state of some proteins. One example is the autophagy receptor p62 which is present in almost all types of protein aggregates. p62 is continuously degraded by autophagy, and this relies on its ability to polymerize [29]. What type of overall structure p62 forms in order to be degraded is not known, but this is a relevant question since this intrinsic structure may also be essential for the structural and functional role of p62 in protein inclusions. Proteins like p62, ALFY (autophagy-linked FYVE protein), and likely also NBR1 (neighbor of BRCA1 gene), are general contents of protein inclusions and are believed to be there because they are involved in both their construction and their degradation by autophagy [26, 28, 30, 31, 79–81]. There is also an ongoing discussion whether it is the mature inclusions or the intermediate precursors that are degraded by autophagy. Two independent pathways have so far been described for the formation of an aggresome, distinguished by whether it is histone deacetylase 6 (HDAC6) or BAG3 that mediates the actual transport of aggregates to the aggresome (Figures 2(d) and 3(d)).

3.2. HDAC6: Transport of Aggregates and Autophagosomal Maturation. HDAC6 facilitates dynein-mediated transport of ubiquitinated substrates to the aggresome, and it is also important for the clearance of aggresomes by autophagy [77, 82, 83] (Figure 3(d)). These roles of HDAC6 are in particular important under conditions when proteasomal degradation is impaired and misfolded proteins are preferentially degraded by autophagy [46, 84]. HDAC6 interacts directly with dynein and with ubiquitinated substrates and has a preference for K63-linked polyubiquitin chains [83, 85]. In addition to a role in aggregate formation and aggrephagy, HDAC6 has a role in maturation of autophagosomes and knockdown of HDAC6 results in the accumulation of autophagosomes [86]. These autophagosomes contain ubiquitinated proteins, demonstrating a role for HDAC6 in the maturation of a subset of autophagosomes involved in selective autophagy of misfolded proteins. The role of HDAC6 in this process is to regulate the actin cytoskeleton [86]. HDAC6 and p62 may act sequentially in the degradation of ubiquitinated protein aggregates with p62 recruiting phagophores to the aggregates for autophagosome formation and HDAC6 acting at the maturation step enhancing fusion between autophagosomes and lysosomes by remodeling actin [83].

3.3. BAG3-Mediated Formation of Aggresomes. BAG3 and CHIP are both needed for targeting of Hsp70 substrates to the aggresome [38] (Figure 2(d)). BAG3 interacts directly with dynein, and this directs transport of Hsp70 substrates to the aggresome [87]. This transport does not depend on ubiquitination of substrates, although the E3 ubiquitin ligase CHIP is required [38]. Depletion of CHIP inhibits aggresome formation in response to proteasomal inhibition, whereas expression of a dominant negative CHIP that does not interact with E2 conjugation enzymes induces aggresome formation [38]. Hence, in the absence of proteasomal degradation or CHIP-mediated ubiquitination, CHIP induces aggregation and BAG3-mediated transport of misfolded substrates, resulting in the formation of aggresomes. Hence, BAG3 may play an important role in recruiting non-ubiquitinated substrates to the aggresome [87].

3.4. p97/VCP: An Ubiquitin-Associated Hsp-Independent Molecular Chaperone. The AAA-ATPase family protein p97/VCP (valosin-containing protein) is a molecular chaperone with important roles in cell division, organelle biogenesis, nuclear envelope formation, and protein degradation [88]. To understand the diversity of cellular roles displayed by p97/VCP, it is important to look at the roles of the various cofactors it interacts with. Most of these interactions are mediated by the N-terminal domain, while a few are mediated by the C-terminal 10 amino acids [89]. Functional roles are known only for a subset of these interactions, but the majority of p97/VCP cofactors have a clear connection to ubiquitin. Loss of p97/VCP in mammalian cells results in accumulation of insoluble ubiquitinated proteins [90–92]. Functional p97/VCP is a homohexamer, and ATP hydrolysis is associated with conformational changes and release of substrates and cofactors [93]. Several studies support a "segregase" activity of p97/VCP, in which ATP hydrolysis is used to segregate ubiquitinated substrates from protein complexes, cell membranes, and chromatin [94–96] (Figures 3(a)–3(c)). p97/VCP is located in the cytoplasm and nucleus and is recruited to the ER membrane in response to ER stress. In ERAD, p97/VCP interacts with the integral ER membrane protein Derlin-1 to unfold, transfer, and extract UPS substrates from the ER membrane [97] (Figure 3(a)). Several ERAD-directed E3 ligases have been detected in mammalian cells, including Hrd1 and gp78 [98]. p97/VCP also plays a role in autophagic degradation of damaged mitochondria after treatment with CCCP (Figure 3(b)). In this case, it is needed for the extraction and delivery of mitochondrial mitofusins to the UPS, after they first have been ubiquitinated by Parkin [99, 100].

No crosstalk between p97/VCP and the Hsp70/Hsp90 molecular chaperones is reported, and cellular roles mediated by p97/VCP may therefore be distinct from those displayed by the other group of molecular chaperones. Interestingly, overexpression of p97/VCP inhibited accumulation of ubiquitinated proteins in autophagy deficient cells overexpressing p62, indicating that there may be a competitive relation between p62 and p97/VCP for the binding to ubiquitinated substrates [101]. p97/VCP is important for aggresome formation in mammalian cells in response to proteasomal inhibition [92, 102–104]. p97/VCP is proposed to induce aggresome formation via a delivery of ubiquitinated protein aggregates to HDAC6 (Figure 3(d)), but the relation between p97/VCP and HDAC6 in this process is only partially understood [105]. Similar to HDAC6, p97/VCP is involved in maturation of autophagosomes. Knockdown of p97/VCP leads to accumulation of autophagosomes containing ubiquitinated substrates [106].

Mutations in p97/VCP cause inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia (IBMPFD) [107]. Structural data for p97/VCP mutants reveals conformational changes in the N-terminal domain [108]. This has a strong effect on cofactor interactions, and some interactions like binding of the ubiquitin ligase E4B are reduced whereas others, like binding of the DUB ataxin 3, are increased [109]. Myoblasts expressing IBMPFD mutants of p97/VCP have defects in degradation of ERAD substrates [110], and their expression in myoblasts or transgenic mouse muscle leads to accumulation of ubiquitinated aggregates [110, 111]. Aggresome formation in cell culture is also affected by mutant p97/VCP expression [102, 103]. FRAP analyses suggest that it is the release of substrates from p97/VCP that is impaired, so that aggregation-prone proteins are not delivered to HDAC6 and therefore accumulate in peripheral aggregates lacking p62 and LC3 [102]. The failure to form aggresomes could be rescued by HDAC6 overexpression [102], suggesting that HDAC6 has a protective role.

3.5. Ubiquilin-1: A Ubiquitous Distributor and Chaperone. Ubiquilin-1 is another protein linked to the sorting of misfolded proteins to different degradation systems. But ubiquilin-1 is also a chaperone needed for folding and stabilization of specific client proteins. The four mammalian ubiquilins have a domain structure reflecting that of p62, with a ubiquitin binding C-terminal UBA domain and an N-terminal UbL domain interacting with the Rpn10/S5A proteasomal subunit [112, 113]. Ubiquilin-1 is involved in ERAD as part of a complex with erasin and p97/VCP [114]. More recent studies have indicated a role for ubiquilin-1 in delivery of proteins to CMA or to autophagy. Ubiquilin-1 is itself degraded by both pathways [115]. Ubiquilin-1 is also involved in the delivery of proteins to the aggresome [116–118], and it protects against polyQ-induced cell death in cellular and invertebrate models of Huntington's disease [119, 120]. It may also promote autophagic degradation of protein aggregates [121, 122]. Ubiquilin-1 has an intrinsic chaperone activity in vitro [123], and it seems to act as a chaperone for the aggregation-prone amyloid precursor protein (APP) [123]. In HeLa cells, expression of ubiquilin-1 reduces toxicity associated with APP and protects against aggregation of APP [123]. Brains of Alzheimer's disease (AD) patients often have a decreased level of ubiquilin-1 which may contribute to late-onset AD [123].

4. Linking Protein Aggregates with the Phagophore

4.1. p62 and NBR1. Selective autophagy depends on autophagy receptors like p62 and NBR1. These proteins are themselves degraded by autophagy due to a direct interaction with ATG8 family proteins conjugated to PE and bound to the phagophore membrane [26, 28–31]. p62 and NBR1 share a similar domain architecture, both containing an N-terminal PB1 domain and a C-terminal UBA domain [124]. The interaction between p62 or NBR1 and ATG8 homologues is mediated via a short, linear LIR motif in p62 and NBR1 [29–31, 125, 126]. The p62 LIR has the core motif DDDWTHL. Following the initial discovery of an LIR in p62 [31], this motif has been identified in an increasing list of proteins. Based on characterized LIR motifs, the present consensus sequence is D/E-D-W/F/Y-x-x-L/I/V [26]. The LIR interaction surface of ATG8 proteins has two hydrophobic pockets accommodating the aromatic (W/F/Y) and the hydrophobic side chains (L/I/V) of the core motif, and the acidic residues often interact with basic residues of the N-terminal arm of the ATG8s [29, 125–127]. Lipidated ATG8 proteins are located both on the inner and outer surfaces of autophagic vesicles, and they therefore make a perfect scaffold for specific recruitment of proteins to the phagophore or the autophagosome. p62 is a polymeric protein, and polymers are made via head-to-tail interactions between PB1 domains [124, 128]. The PB1 domain-mediated polymerization is essential for the selective degradation of p62 by autophagy [29], and it is required for the targeting of p62 to the autophagosome formation site at the ER [40]. It is also crucial for the ability of p62 to assemble proteins into aggregates [28]. p62 and NBR1 have a very different primary sequence, and NBR1 contains several domains that are not present in p62. Homologs of NBR1 are found throughout the eukaryotic kingdom, whereas the presence of p62 is unique for metazoans and likely the result of a duplication event early in the metazoan lineage [129]. Plant NBR1 is able to polymerize via the PB1 domain, and this is required for autophagic degradation similar to metazoan p62 [129]. During evolution, NBR1 has lost the ability to polymerize via the PB1 domain, while p62 has lost several domains like the FW domain. Therefore these two proteins may have independent roles in selective autophagy, but they may also cooperate as indicated by the fact that p62 interacts directly with NBR1 [124]. Recently, several other autophagy receptors in addition to p62 and NBR1 have been identified. These are ATG32 and NIX/BNIP3L acting in mitophagy [130–132], NDP52 and optineurin in xenophagy [133, 134], and Stbd1 in degradation of glycogen [135]. However, only p62 and NBR1 have so far been linked to degradation of protein aggregates.

It should be noted that p62 is also involved in selective autophagy of non-ubiquitinated substrates, and selective autophagy of a mutant superoxide dismutase 1 (SOD1) causing ALS depends on a direct and ubiquitin-independent interaction between SOD1 and p62 [136]. Recently, p62 was found to be required for selective autophagic clearance of a non-ubiquitylated substrate, an aggregation-prone isoform of STAT5A (STAT5A_ΔE18) that formed aggresomes and/or aggregates by impairment of proteasome functioning or autophagy [137]. Different domains of p62 interacted with SOD1 and STAT5A in these cases. A third example of p62-mediated selective autophagy of non-ubiquitin substrates is the p62-mediated autophagic clearance of Sindbis virus capsids from neurons of infected mice [138]. A bona fide example of ubiquitin-independent aggrephagy is seen in *C. elegans* where the polymeric autophagy receptor SEPA-1 binds to the P granule component PGL-3 and to the ATG8 homologue LGG-1 to mediate the selective autophagic degradation of P granules [139]. This way the maternally derived germ P granule components are degraded by aggrephagy in somatic cells during embryogenesis. However, in most cases ubiquitin binding seems to be important and a study on the role of p62 and ubiquitin in pexophagy clearly indicates that ubiquitin may serve as a label that is recognized by p62 [140]. Less is known about the role of NBR1 in selective autophagy, mainly because it is less studied. p62 has been implicated in the autophagic clearance of midbody ring complexes [141], and recently NBR1 was found to be required and more important than p62 for clearance of midbody derivatives by autophagy [142]. It was found that disposal of midbody derivatives accompanied stem-cell differentiation and that the autophagy receptor NBR1 bound to the midbody protein CEP55 to mediate the autophagic degradation.

4.2. ALFY. ALFY is a 400 kDa scaffold protein with an ensemble of domains located to its C-terminal region. This part of ALFY contains a BEACH domain, an ATG5 interacting WD40 repeat region [80], and a PtdIns(3)P-binding FYVE domain [143]. Co-immunoprecipitation experiments indicate that the BEACH domain in ALFY is important for its ability to form complexes in vivo with p62 [79]. ALFY and p62 colocalize strongly in cytoplasmic and nuclear protein aggregates in cell culture [79, 80], and they are both degraded by autophagy in response to the formation of p62 bodies in HeLa cells [79]. In fact, p62, NBR1, and ALFY are all important for selective autophagy of p62 bodies in HeLa cells [30, 31, 79].

ALFY is under normal conditions mainly nuclear. In response to amino acid starvation or puromycin-induced accumulation of DRiPs, ALFY is redistributed to cytoplasmic p62 bodies [79] (Figure 2(d)). It is also redistributed to cytoplasmic polyglutamine inclusions [80] and to aggregates induced in response to proteasomal inhibition [143]. The redistribution of ALFY in HeLa cells depends on p62 and seems to depend on the ability of p62 to shuttle between the cytoplasm and the nucleus [79]. Autophagic degradation of ALFY most likely depends on its association with p62 and/or cytoplasmic protein aggregates [79, 80].

ALFY is required for aggrephagy, but not for starvation-induced autophagy [80]. Knock-out studies have revealed an important role of ALFY in constitutive autophagy of misfolded proteins, both in mammals and in flies [80, 144]. In flies, knockout of the *Drosophila* homologue *blue cheese* (bchs) results in accumulation of ubiquitinated protein inclusions, neurodegeneration, and a reduced life-span

[144]. Furthermore, overexpression of Bchs reduces neurotoxicity in a *Drosophila* eye model of polyglutamine toxicity [80]. In mammals, ALFY is recruited to cytoplasmic and nuclear protein inclusions as part of a complex containing p62, NBR1, LC3, ATG5, ATG12, and ATG16L [80]. In mammalian cell culture, ALFY is required for efficient degradation of polyglutamine and α-synuclein inclusions. This depends on a direct interaction between ALFY and ATG5 [80]. Notably, overexpression of the C-terminal part of ALFY alone promoted degradation of polyglutamine inclusions in a neuronal lentiviral model [80]. Very likely, co-recruitment of p62, NBR1, and ALFY and their interaction partners, to a protein aggregate, initiates the formation of autophagy membranes. However, more studies are needed to identify the specific roles mediated by each of these proteins recruiting different components of the autophagy machinery for autophagosome formation at the protein aggregate.

5. Regulation of Aggrephagy by Posttranslational Modifications

The selective autophagy of protein aggregates is regulated at the level of the autophagic machinery, the level of autophagy receptors, like p62, NBR1, and ALFY, and the level of the protein aggregates [81, 145]. Hitherto, there is a scarcity of data available to illuminate the mechanisms involved in regulating aggrephagy. However, both autophagy receptors and substrates are regulated by posttranslational modifications including ubiquitination, phosphorylation, and acetylation [146].

K63-linked polyubiquitin chains have been associated with autophagic degradation [147, 148], and this may clearly have a role in recruiting autophagy receptors like p62 and NBR1 [30, 149] or HDAC6 [85]. So, is there a simple ubiquitin-code where substrates tagged with K48-linked ubiquitin chains are degraded by the UPS while aggregated substrates tagged with K63-linked ubiquitin are degraded by autophagy? Several of the proteins involved in aggrephagy, like p62 and HDAC6, bind preferentially to K63-linked ubiquitin [85, 149]. The E3 ligase TRAF6 interacts with p62, and it also catalyzes K63-linked ubiquitination of its substrates [150]. Formation of aggresomes requires the activity of the deubiquitinating enzyme ataxin-3 which can bind to HDAC6 and trims both K48- and K63-linked ubiquitin chains [151, 152]. Hence, ataxin-3 (and other DUBs) may be required for editing the ubiquitin code to one favouring aggrephagy [83]. Note that mutant polyQ-expanded ataxin-3 is an aggregate-prone protein that causes spinocerebellar ataxia type 3 that is degraded by autophagy in a mouse model of this disease [153]. The DUB cylindromatosis tumor suppressor (CYLD) interacts with TRAF6 to remove K63-linked ubiquitin in a p62-dependent manner [154]. Hence, in addition to binding to ubiquitinated aggregates, p62 may also be involved in regulating the K63-linked ubiquitination of aggregates acting as autophagy substrates through its interactions with TRAF6 and CYLD. In brains of p62 KO mice, there was a hyperaccumulation of K63-linked ubiquitin in the insoluble fraction suggesting accumulation of substrates and also dysregulation of the TRAF6-CYLD interplay in the absence of p62 [154]. These mice showed AD-like symptoms, and aggregated K63-ubiquitinated tau protein was recovered from brain fractionation experiments [155]. Not surprisingly, TRAF6 is found in the Lewy bodies in sporadic Parkinson's disease (PD) brains [156]. TRAF6 also regulates autophagy positively by mediating K63 ubiquitination of beclin 1, and this is opposed by the DUB A20 [157].

The autophagy pathway is directly regulated by several kinases including Ulk1/2, mTOR, AMPK, and PKA. In addition, autophagy receptors like p62 and optineurin, as well as LC3B, have recently been shown to be regulated by phosphorylation [134, 158, 159]. PKA-mediated phosphorylation of a site in the N-terminal arm of LC3 inhibited its recruitment to autophagosomes [158]. TANK binding kinase 1 (TBK1) phosphorylated optineurin on Ser-177 in the LIR motif, enhancing LC3 binding affinity and autophagic clearance of cytosolic *Salmonella* showing that the LIR-LC3 interaction can be regulated by phosphorylation [134]. Phosphorylation of p62 on Ser-403 in the UBA domain increased the affinity for polyubiquitin and stimulated aggrephagy of ubiquitinated proteins [159]. Interestingly, recently a number of reports show that, similar to p62, also optineurin is found in ubiquitin-positive inclusions in sporadic and familial ALS, neurofibrillary tangles and dystrophic neuritis in AD, and LBs in PD and more neurodegenerative diseases (see that is, refs. [160–162]). Optineurin was recently found to be mutated and causatively linked to the disease in some cases of familial ALS [160]. Mutations of optineurin have also been found in sporadic ALS [163].

The aggregating substrates can be phosphorylated in a manner affecting their clearance. A number of studies report on phosphorylations affecting cleavage, aggregation, and clearance of aggregation-prone polyQ-expanded proteins including huntingtin and ataxin-1 and 3 (see [81]). It has long been recognized that phosphorylation of tau affects its aggregation. In the brains of adult p62 knock-out mice, an age-dependent increase in the activity of several kinases, including glycogen synthase kinase 3β (GSK3β), protein kinase B (PKB), mitogen-activated protein kinases, and c-Jun-N-terminal kinase, results in hyperphosphorylated tau and formation of neurofibrillary tangles [155].

Members of the basic autophagy apparatus including Atg5, 7, 8, and 12 can be acetylated by the acetyltransferase p300, and p300 binds directly to Atg7 [164]. Acetylation of these proteins mediated by p300 inhibits autophagy, and silencing of p300 increases autophagy flux [164]. Acetylation was also recently shown to affect the autophagic clearance of a fragment of mutant huntingtin and an N-terminal caspase-7 cleavage fragment of ataxin-7. Acetylation of lysine 444 (K444) increased autophagic degradation of mutant huntingtin [165]. This acetylation also mitigated the toxic effects of mutant huntingtin in primary striatal and cortical neurons and in a transgenic *C. elegans* model of Huntington's disease. Mutant huntingtin resistant to acetylation accumulated and led to neurodegeneration in cultured neurons and in mouse brain [165]. The opposite effect of acetylation was seen for ataxin-7 [166]. Cleavage of ataxin-7 by caspase-7 generates toxic N-terminal polyQ-containing fragments that accumulate with disease progression. Acetylation of

lysine 257 (K257) adjacent to the caspase-7 cleavage site of ataxin-7 promotes accumulation of the fragment, while the unmodified ataxin-7 fragments are degraded by autophagy [166].

6. Other Regulatory Aspects

So far very little is known about differential gene regulation occurring as a result of aggregate formation. There is clearly a link between aggregate formation and oxidative stress responses. p62 binds to the cytoplasmic inhibitor KEAP1 to stabilize the oxidative stress response transcription factor Nrf2, which then induces a repertoire of oxidative stress response genes [167–170]. The p62 gene is itself one of the targets of Nrf2 enabling p62 to set up a positive feedback loop [168]. Deprenyl, which is a candidate neuroprotective drug in PD, can also lead to nuclear accumulation of Nrf2 and induction of oxidative stress response genes [171]. Transcription of the p62 gene is increased during aggregate formation induced by proteasomal inhibition [172]. Activation of the p62-Nrf2 pathway may therefore be an important protective response during aggregate formation and a target for development of neuroprotective drugs.

Several central proteins involved in aggrephagy including Beclin 1, diabetes- and obesity-regulated gene (DOR), p62, and ALFY shuttle between the nucleus and cytosol (see [81]). The nucleocytoplasmic shuttling of p62 is regulated by phosphorylation sites at or C-terminal to the major nuclear localization signal [173]. In the nucleus both p62 and ALFY may be involved in collecting ubiquitinated proteins in the PML (promyelocytic leukemia) nuclear bodies for proteasomal degradation [173]. Whether substrates can be transported out of the nucleus for degradation by autophagy is an open question.

7. Is the Aggregate Eaten in One Big Bite or in Smaller Pieces?

It is now well established that autophagy is needed for the removal of cytoplasmic protein inclusions [80, 82, 174–176]. But are the insoluble inclusions solubilized or modified before engulfment? There seems to be a putative conflict between the caging of protein aggregates within intermediate filaments such as vimentin or keratin [34, 76] and the degradation of these aggregates by autophagy. However, knock-out studies of genes associated with aggregation indicate a positive role for aggregation in autophagy [82]. In AD, autophagosomes with electron dense amorphous or multilamellar contents accumulate in massive numbers [177]. The accumulation of protein aggregates or inclusions has also been demonstrated in cell culture. Immunoelectron microscopy on mammalian cells stably expressing Htt103Q revealed that insoluble polyglutamine inclusions are found within autophagosomes [80]. SDS-insoluble Htt103Q was in this case also found in autophagosome fractions after cell fractionation, clearly demonstrating that insoluble inclusions are indeed engulfed by autophagic structures. Also p62 bodies have been shown by immunoelectron microscopy to accumulate inside autophagosomes [31]. It is in this context relevant that other large cellular structures such as organelles and bacteria are degraded by selective autophagy [26], and there is no evidence that these structures are cut into smaller pieces before they are engulfed.

In non-metazoan species, including plants and fungi, Hsp104 forms a complex with Hsp70 and Hsp40 that has disaggregation activity capable of dissolving amyloid-like structures [178]. No homolog of Hsp104 exists in metazoans, but a less potent disaggregation activity was recently shown for a mammalian complex consisting of Hsp110, Hsp70, and Hsp40 [179]. The observed presence of insoluble inclusions within autophagic vesicles does not exclude that another route of degradation is more important, and studies on degradation of aggregation-prone proteins are often confused by the fact that their soluble forms can also be degraded by CMA or the UPS. The long-standing question whether it is the large aggregate which is degraded wholesale or if it is dismantled into smaller aggregates that are engulfed by forming autophagosomes still remains unanswered.

8. Dysfunction of Autophagy in Proteinopathies

Macroautophagic stress indicates a situation when the normal flow of autophagic degradation is impaired [180]. If macroautophagic stress is caused by defects in protein degradation pathways, the effect is impaired degradation of misfolded proteins and accumulation of protein aggregates. But often, the first indication that autophagy is affected in neurodegenerative diseases and disease models is an abnormal number of autophagosomes and/or amphisomes (fusion product of autophagosomes and late endosomes) (reviewed in [181]). In this case, the defect in autophagy is caused by impaired endocytosis at the late endosome-lysosome level, inhibition of autophagosome maturation, and/or inhibition of lysosomal degradative functions. The pressure on autophagy is increased during aging, in part because CMA activity declines. This is mainly caused by decreased levels of LAMP-2A at the lysosomal membrane [182]. Autophagosome formation declines during aging due to decreased expression of some of the vital autophagy proteins like ATG8 family proteins and Beclin1 [183, 184]. Aging is associated with increased intracellular oxidative stress leading to increased unfolding of proteins. Combined with a decline in autophagosome maturation and/or lysosomal degradation, this helps explaining the late-onset phenotypes often observed for several of the proteinopathies. Induction of autophagosome formation is often suggested as a solution to the problem of macroautophagic stress, and it has shown promising results in cell culture and in vivo models [39, 185]. But in other cases autophagosome formation is not a solution, since maturation of autophagosomes or lysosomal degradation is impaired. Caution is therefore required before trying to boost autophagy as a therapeutic strategy in neurodegenerative diseases. It is imperative to first determine the main cause of dysfunctional autophagy in the different types of proteinopathies before trying to boost or inhibit autophagy/aggrephagy as a therapy.

8.1. Accumulation of p62: Proteinopathies in Liver and Muscle. p62 is present in almost all cytoplasmic and nuclear inclusions found in human diseases [186–189]. In most proteinopathies, another aggregation-prone protein is responsible for the formation of the aggregate, and p62 is recruited later and possibly in response to ubiquitination of the aggregate. An example is polyglutamine inclusions that are formed independently of p62 [190]. However, p62 is together with NBR1 and ALFY important for the formation and degradation of p62 bodies in response to puromycin treatment or starvation of HeLa cells [30, 31, 79]. These structures are highly ubiquitinated and substrates for selective autophagy [28, 31]. p62 is also crucial for the formation of two types of pathogenic aggregates found in chronic liver diseases [188, 191], that is, intracellular hyaline bodies found in hepatocellular carcinoma and Mallory-Denk bodies (MBs) found in alcoholic and nonalcoholic steatohepatitis. Similar to p62 bodies, p62 and ubiquitin are major constituents of these structures, but MBs in addition contain abnormal keratins [191]. Most likely, their formation is initially caused by insufficient degradation of p62 bodies by autophagy. What should be noted is that the level of ALFY in the liver is very low [143], and it is tempting to speculate that the tendency of p62 to form aggregates in hepatocytes is caused by this. Apart from Paget's disease of the bone affecting the skeleton, liver is the only organ where p62 has been shown to play a main role in the formation of protein aggregates in human disease.

Autophagy knock-out studies support the hypothesis that p62 bodies develop into stable aggregates if autophagy is impaired. In mice, tissue-specific knockout of autophagy causes accumulation of p62-containing aggregates in neurons [53, 54, 192], hepatocytes [55], skeletal muscle [51, 52], cardiac muscles [193], pancreatic β cells [194, 195], and kidneys [196]. A similar accumulation of ubiquitinated aggregates is seen after knockout of autophagy in flies [184, 197]. Importantly, p62, or the *Drosophila* homologue Ref(2)P, is required for the formation of ubiquitinated protein aggregates under autophagy knock-out conditions, both in cell culture and in vivo [28, 31, 55, 198]. The most likely interpretation is that p62-mediated accumulation of ubiquitinated proteins in p62 bodies results in the formation of aggregates that in the absence of autophagy cannot be degraded [55]. In cells lacking p62, the contents of the aggregates are likely to be degraded by the UPS or CMA and the effect of autophagy inhibition is therefore less pronounced. Blockade of autophagy may in fact inhibit the UPS if the p62 level gets very high because p62 may inhibit substrate delivery to the proteasome [101].

NBR1 colocalizes with p62 in the Mallory-Denk bodies in patients with alcoholic steatohepatitis [30], and may therefore contribute to the formation of aggregates in liver. NBR1 also colocalizes with p62, LC3, and phosphorylated tau in ubiquitinated protein aggregates of sporadic inclusion-body myositis (s-IBM) that is the most common degenerative myopathy associated with aging [199, 200]. Hence, it is very likely that p62 and NBR1 cooperate in clearance of protein aggregates by autophagy.

8.2. Proteinopathies in Neurodegeneration. There is very little LC3-II or autophagosomes in healthy neurons, but this is due to a very rapid turnover of autophagosomes [177]. Neurons may therefore be vulnerable to inhibition of the flow of autophagosomes at any step downstream of autophagy formation. Autophagy of proteins is part of the normal function of postmitotic neurons and is constitutively needed. Conditional knockout of autophagy in mice causes neuronal degeneration and accumulation of ubiquitinated protein aggregates [53, 54]. This clearly demonstrates that autophagy delays the onset of neurodegenerative diseases. The major component of inclusions formed in neurodegenerative diseases is often a single protein, and the most common intracellular neuronal proteinopathies are formed by α-synuclein, tau, TDP-43 (transactive response DNA-binding protein-43), or a mutated protein with extended polyglutamine repeats (see refs. [81, 145, 181, 201]). Aggregation-prone proteins that are mutated in disease are often used as models to study protein aggregation and aggrephagy. Among the best studied neurodegenerative diseases are the α-synucleinopathies caused by aggregation of α-synuclein and responsible for diseases like Parkinson's disease (PD) and dementia with Lewy bodies [202]. These diseases are characterized by the aggregation of α-synuclein into so-called Lewy bodies (LBs), but PD is also associated with lysosomal dysfunction and mitochondrial dysfunction [180, 203]. The LBs of PD and dementia with LBs are likely the disease-associated aggregates morphologically most similar to a "classical" aggresome [204–206]. HDAC6 is a component of LBs, and the formation of LBs depends on ubiquitination of substrates by Parkin and transport mediated by HDAC6 [77, 85]. The α-synuclein is degraded by CMA or autophagy [207–209]. In PD and certain tauopathies, there is a block in CMA because accumulation of α-synuclein or toxic forms of tau inhibit the CMA translocation complex [207, 210, 211]. Such inhibition of CMA may play a key role in the development of these disorders [181], and it may be responsible for the observed activation of autophagy in PD [209]. Induction of autophagy may have a protective effect on α-synuclein-related diseases [212–214]. However, too much autophagy may be toxic if maturation of autophagosomes is impaired. Activation of autophagosome formation may therefore be beneficial at early stages of the disease but may lead to enhanced neuronal degeneration in other settings [180].

Another group of neurodegenerative diseases are 10 different autosomal dominant disorders caused by aggregation of polyglutamine stretches on proteins [201]. These are caused by genes that contain a stretch of repetitive CAG glutamine codons that is unstable and tends to expand. The tendency of polyglutamine stretch containing proteins to aggregate is proportional to the number of glutamine repeats. For Huntington's disease (HD) caused by aggregation of Huntingtin (Htt) fragments, a stretch of around 40 glutamines may be sufficient to cause a disease [201]. Polyglutamine-expanded mutant Htt is degraded by autophagy, and autophagy reduces the toxicity associated with mutant Htt expression both in cell culture and in mouse, fly, and zebrafish models of Huntington's disease [46, 175, 185, 215, 216]. Autophagy also has a role in clearance of

other polyglutamine-expanded proteins, including mutant ataxin-3 that is causing the spinocerebellar ataxia type 3 (SCA3) [153]. However, in a fly model of dentatorubral-pallidoluysian atrophy (DRPLA), a disorder caused by mutations in the atrophin-1 protein, autophagy induction was unable to rescue the degenerative phenotype because lysosomal degradation was impaired [217]. There is also evidence that mutant Htt has a negative effect on selective autophagy affecting cargo recognition causing accumulation of "empty" autophagosomes as analyzed by immunoEM [218].

Several neurodegenerative diseases are characterized by inclusions of hyperphosphorylated forms of the microtubule-associated protein tau [219]. The most common and best known disease with tau inclusions is Alzheimer's disease (AD). Tau forms neurofibrillary tangles in AD, but also soluble oligomers of hyperphosphorylated tau contribute to neuronal degeneration [220]. AD is also associated with plaques of amyloid-β ($\alpha\beta$) peptide produced by cleavage of amyloid precursor protein (APP) by β- and γ-secretases. In a mouse model, induction of autophagy delayed the onset of AD although it had no effect at later stages associated with formation of plaques and tangles [221]. Tau binds tubulin, and the normal function of tau is to promote stabilization of microtubules in neuronal axons. This is needed for long-distance transport and for the maintenance of cellular morphology. Hyperphosphorylated tau has a reduced affinity for tubulin, and this is believed to result in destabilization of microtubules [219]. As mentioned above, p62 knock-out mice display an AD-like phenotype as they grow older and their brains contain increased amounts of hyperphosphorylated tau and K63-linked ubiquitinated proteins [154, 155].

In a cell model, transport of tau to the aggresome in response to proteasomal inhibition was inhibited by knockdown of HDAC6, and this inhibited clearance of tau aggregates and resulted in an accumulation of insoluble tau [222]. However, although the level of HDAC6 is elevated in AD brain [223] HDAC6 is not present in neurofibrillary tangles or senile plaque of AD [224]. Tau binds to HDAC6 [223], and is an inhibitor of HDAC6 function [225]. HDAC6 knock-out mice have hyperacetylated tubulin, but they are viable and develop without neurological abnormalities [226]. Consistent with this, increased acetylation of tubulin is also found in brain of AD patients. However, tau also inhibits the role of HDAC6 in aggresome formation [225]. Inhibition of aggresome formation favors the formation of smaller and possibly more toxic aggregates and will also have a negative effect on tau degradation.

IBMPFD caused by mutations in p97/VCP primarily affects muscle, brain, and bone tissue and is characterized by the accumulation of cytoplasmic and nuclear ubiquitinated inclusions [107]. Recently, TDP-43 was shown to play a role in frontotemporal dementia induced by expression of mutants of p97/VCP [227, 228]. In a *Drosophila* model of IBMPFD induced by mutant p97/VCP, an elevated level of TDP-43 is directly responsible for the degeneration [228]. TDP-43-positive inclusions are hallmarks of frontotemporal dementia and amyotrophic lateral sclerosis (ALS), and there seems to be a lack of HDAC6 in these inclusions [223]. This correlates with the recent finding that TDP-43 binds to HDAC6 mRNA and knockdown of TDP-43 destabilizes HDAC6 mRNA and leads to downregulation of HDAC6 expression. This causes reduced aggregate formation and increased cytotoxicity in cells expressing a polyQ-expanded ataxin-3 mutant [229]. A novel surprising finding is that TDP-43 appears to stabilize ATG7 mRNA by binding to it via its RRM1 domain. Depletion of TDP-43 caused reduction of the ATG7 mRNA/protein and inhibition of autophagy leading to accumulation of polyubiquitinated proteins and p62 [230]. Hence, functional TDP-43 is important for efficient autophagy.

The reason why IBMPFD mutants of p97/VCP cause accumulation of TDP-43 is not known, but it suggests a role for p97/VCP in degradation of TDP-43 and/or for the segregation of TDP-43 from the ribonucleoprotein particle complex during translation [228]. Mutations in p97/VCP can also cause familial ALS [231]. Very recently, p62 mutations were reported in familial and sporadic ALS patients with 8-9 missense mutations predicted by in silico analyses as candidate disease mutations [232].

8.3. Serpinopathies with ER Luminal Location. Similar to the nucleus, the ER is a compartment lacking autophagosomes. However, unlike nuclear aggregates that are not efficiently degraded by autophagy [49], ER luminal aggregates can be degraded by autophagy. Serpinopathies are a group of diseases associated with aggregation of serpin family proteins in ER (reviewed in [233]). Serpins are inhibitors of extra- and intracellular proteases, and they act as pseudosubstrates that upon cleavage change conformation resulting in the formation of an inactive serpin-protease complex. Functional serpins are monomeric. In contrast, mutated variants are associated with the formation of long and ordered polymers caused by the insertion of the flexible and reactive centre loop of one molecule into a β-sheet of another. These aggregates cannot be degraded by ERAD and accumulate inside the ER lumen. Aggregation-prone and disease-causing mutant variants are known for several serpin family members, including α1-antitrypsin, neuroserpin, α1-antichymotrypsin, C1-inhibitor, and antithrombin.

The Z variant of α1-antitrypsin forms polymers that accumulate in the ER of hepatocytes, and homozygosity for this mutant allele causes the genetic disease α1-antitrypsin deficiency. Since polymerization of serpin mutants occurs posttranslation and most likely after complete folding of the monomers [234], there is a window when monomers can be degraded by ERAD. Hence, the Z-variant of α1-antitrypsin is degraded by ERAD, but it also accumulates in autophagosomes in liver cells of patients with α1-antitrypsin deficiency and in cell culture [235, 236]. Its degradation is reduced in autophagy deficient cells, and this supports a role for autophagy in degradation of mutated α1-antitrypsin [235].

Another familial dementia is FENIB (Familial Encephalopathy with Neuroserpin Inclusion Bodies) that is caused by polymerization of mutant neuroserpin in ER of neurons. Studies of mammalian cells and a *Drosophila* model of

serpinopathy revealed that ERAD and macroautophagy cooperate also in degradation of mutant neuroserpin [234]. Autophagic degradation of polymeric neuroserpin and other serpinopathies is probably coupled to autophagic degradation of ER itself. In this process, portions of ER are believed to be engulfed along with proteins and protein aggregates. It remains to be shown whether there exist mechanisms for the specific delivery of serpin polymers to those regions of ER that undergo degradation. No selectivity towards mutated neuroserpin was observed for the autophagic degradation of neuroserpin in neuronal-like PC12 cells, suggesting that degradation of neuroserpin by autophagy is mainly a non-selective bulk degradation process [234].

9. Concluding Remarks

Selective autophagy of protein aggregates has emerged as an important protein quality control system in cells, and the last decade has provided some major leaps in our understanding of aggrephagy. The autophagy receptors p62 and NBR1 and the large adaptor protein ALFY play major roles in aggrephagy. The level of ALFY in the brain is high [143], and loss of ALFY or p62 is associated with neurodegeneration [144, 155]. It is anticipated that more autophagy receptors are involved in aggrephagy, and optineurin is one of them. How much can be learned from studies of selective autophagy of intracellular bacteria (xenophagy) that is also relevant for aggrephagy? Novel autophagy receptors like NDP52 and optineurin have emerged from studies of xenophagy, and ubiquitination is heavily involved [26, 134]. Likely, also the selective removal of damaged mitochondria (mitophagy) may provide knowledge applicable to the understanding of aggrephagy. For instance, p62 is involved in clustering of mitochondria during mitophagy [237, 238].

As reflected in this paper, there is recent progress in the understanding of the roles played by chaperones and their cofactors in sorting of misfolded proteins to the different degradation pathways. Chaperones and co-chaperones, particularly BAG3, in addition to p97/VCP, HDAC6, TDP-43, and ubiquilin-1, are important players in the formation of aggregates, and they also affect aggrephagy at several steps. However, the study of autophagic degradation of protein aggregates is still in its infancy in the sense that some fundamental questions remain unanswered. For example, we still do not know what size(s) of aggregates can be degraded by selective autophagy. Is there an upper size limit? Is the most efficient degradation of a large aggregate a combination of UPS-, CMA-, and aggrephagy-mediated degradations? An important role for chaperones and cofactors may then be to orchestrate the different degradative pathways and to help to dissolve the aggregates. There is clearly some confusion in the field as to what are the similarities and differences between different types of protein aggregates described in the literature. How should the different types of aggregates and protein inclusions be classified?

A central question is whether modulation of aggrephagy is a relevant therapeutic strategy for neurodegenerative diseases and other proteinopathies. There is a direct parallel here to cancer where many clinical trials are under way to test effects of inhibiting or boosting autophagy as part of treatment regimens for various cancers. It may very well be that the broad preliminary conclusion is the same for cancer and neurodegenerative diseases; autophagy is generally acting protectively before advanced disease, while it may be harmful to stimulate autophagy in advanced disease states. In cancer, successful tumor cells often depend on autophagy (so inhibition is the best strategy), and in neurodegenerative diseases there is often already a dysfunctional downstream step so that stimulation of autophagosome formation may not be beneficial. The challenge is now to gain more knowledge about the mechanisms involved in aggrephagy and of the particular deficiencies in these mechanisms that are decisive for onset and progression of neurodegenerative diseases.

Acknowledgments

The authors thank members of their lab for critical discussions and suggestions during the writing of this paper. This work was supported by grants from the Norwegian Research Council, the Norwegian Cancer Society, the Tromsø Research Foundation, the Aakre Foundation, and the Blix Foundation to T. Johansen.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 673290, 9 pages
doi:10.1155/2012/673290

Review Article

Receptor Proteins in Selective Autophagy

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Received 17 November 2011; Accepted 17 December 2011

Academic Editor: Masaaki Komatsu

Autophagy has long been thought to be an essential but unselective bulk degradation pathway. However, increasing evidence suggests selective autophagosomal turnover of a broad range of substrates. Bifunctional autophagy receptors play a key role in selective autophagy by tethering cargo to the site of autophagosomal engulfment. While the identity of molecular components involved in selective autophagy has been revealed at least to some extent, we are only beginning to understand how selectivity is achieved in this process. Here, we summarize the mechanistic and structural basis of receptor-mediated selective autophagy.

1. Introduction

Macroautophagy or bulk autophagy (referred to as autophagy in the text) is an evolutionarily highly conserved program for sequestration and transport of macromolecules and organelles to the vacuole or lysosomal compartment where they are degraded [1–4]. This form of autophagy is considered to be a rather unselective process for bulk degradation of cellular constituents that serve to recycle macromolecules to maintain cellular homeostasis and energy balance and to provide new building blocks for anabolic processes under deprivation of nutrition [5]. In addition, autophagy represents a quality control mechanism to clear damaged or surplus organelles and aggregated or misfolded proteins, respectively [6]. Autophagy is engaged by the formation of the isolation membrane or phagophore, a double membrane that enlarges and wraps around cytosolic cargo yielding a closed multilamellar vesicular structure, coined autophagosome. The subsequent fusion of autophagosomes with the vacuole in yeast or with lysosomes in mammalian cells initiates degradation of enclosed cargo by acidic hydrolases (Figure 1(a)). In contrast to bulk autophagy, selective autophagy involves targeted recognition and removal of protein inclusions, organelles or microbes [7]. A set of specific proteins play a pivotal role in both the recognition as well as the delivery of cytoplasmic cargo to the incipient autophagosome for engulfment and ultimately lysosomal degradation [8, 9]. These so-called autophagy receptors mediate simultaneous binding of cytosolic cargo and components of the autophagy machinery (Figure 1(b)). The modular composition of binding domains and motifs in autophagy receptor proteins ensures efficient tethering of cargo to the site of developing and engulfing autophagosomes.

2. Cargo Binding Domains in Autophagy Receptors

Autophagy receptors can be grouped based on their specific cargo-binding domains. Fundamental different principals have been employed for the use of these binding domains in selective autophagy ranging from protein-specific interaction domains via posttranslational modification- (PTM-) binding domains to transmembrane domains (Figure 1(c)). While protein-specific interaction domains yield autophagosomal delivery of only a set of very specialized targets, PTM-specific binding domains, namely, ubiquitin-binding domains, allow for autophagy engagement of a huge variety of proteins. Lastly, by the virtue of membrane embedding, autophagy receptors mediate organelles-specific targeting for selective autophagy.

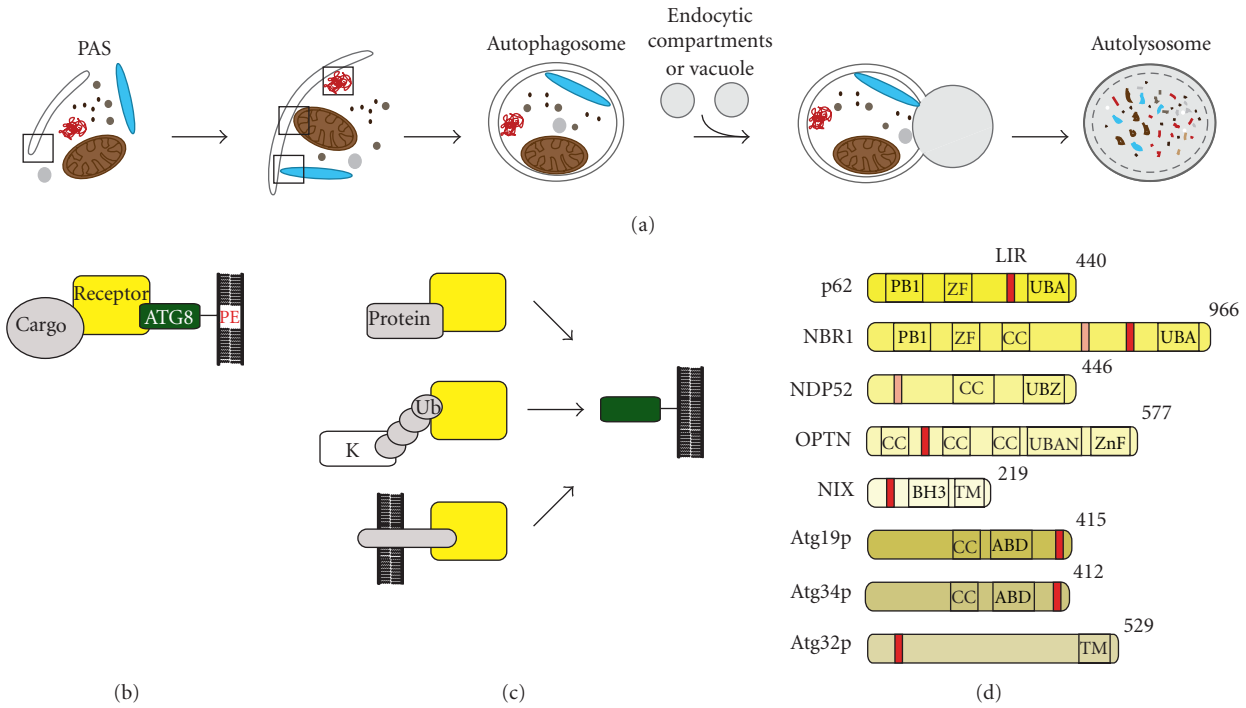

Figure 1: (a) Overview of selective autophagy. Boxes indicate localization of ATG8 and autophagy receptor proteins. (b) Scheme of autophagy receptor function. (c) Different cargo-binding concepts of autophagy receptors. (d) Domain architecture of the known characterized autophagy receptors.

3. Protein-Specific Binding Domains

In yeast, at least two vacuole-resident enzymes, aminopeptidase 1 (Ape1p) and α-mannosidase (Ams1p), are selectively and constitutively transported into the vacuole as part of their biosynthesis via an autophagy-like process called cytoplasm to vacuole targeting (Cvt) pathway [10]. Following translation in the cytosol as proenzyme (prApe1p), Ape1p oligomerizes into a dodecamer and further assembles with Ams1p and the autophagy receptor Atg19p into large so-called Cvt complexes. Atg19p binds to the propeptide of prApe1p and to Ams1p via its central coiled coil and carboxy-terminal Ams1-binding domain (ABD) (Figure 1(d)), respectively, and is essentially required for recruitment of the Cvt complex to the pre-autophagosomal structure (PAS) prior to vacuolar delivery [10]. The autophagy receptor Atg34p, a recently characterized Atg19p homolog, acts cooperatively with Atg19p in the Cvt pathway [11, 12]. Like Atg19p, Atg34p contains a C-terminal ABD (Figure 1(d)). However, since Atg34p lacks the prApe1p-binding specific coiled coil, Atg34p mediates the delivery of Ams1p to the vacuole but not of prApe1p [12]. The structures of ABD in Atg19p and Atg34p have recently been solved and show an eight β-strand-composed immunoglobulin-like fold [12]. Though, the exact Ams1p-binding mechanism by ABD has not been determined in detail yet. Likewise, we do not understand structurally how the coiled coil of Atg19p binds prApe1p. Recently, another biosynthetic enzyme, leucine aminopeptidase III (Lap3p), has been identified as Atg19p-dependent cargo for selective autophagy under starvation conditions [13], indicating that more proteins than previously anticipated might be delivered to the vacuole via forms of selective autophagy. However, whether Atg19p's coiled coil or ABD domain mediates Lap3p binding and whether additional Cvt receptor proteins exist is currently unknown. An intriguing question remains whether mammalian homologues of these enzymes (i.e., LAP3) employ selective autophagy pathways for their lysosome targeting.

4. Ubiquitin-Specific Binding Domains

Covalent attachment of ubiquitin to proteins has emerged as a versatile regulatory signal mediating several forms of selective autophagy targeting aggregated proteins (aggrephagy), bacterial pathogens (xenophagy), and damaged mitochondria (mitophagy) [8, 9]. Ubiquitylation occurs through isopeptide bond formation between the ε-amino group of a lysine residue in a target protein and the C-terminal carboxyl group of ubiquitin [14, 15]. Proteins can be modified by ubiquitin monomers (monoubiquitylation and multi-monoubiquitylation) or by ubiquitin polymers (polyubiquitylation), in which ubiquitin moieties are most often connected via lysine-mediated isopeptide linkages [16]. Different chain linkage types arise from the fact that all 7 lysine residues in ubiquitin (K6, K11, K27, K29, K33, K48, and K63) as well as the N-terminal methionine serve as ubiquitin acceptor [17, 18]. These diverse ubiquitin signals are decoded by distinct classes of ubiquitin-binding domains [19, 20].

So far, three different ubiquitin-binding domains have been implicated in specific cargo receptors for selective autophagy: ubiquitin-associated (UBA), ubiquitin binding in A20-binding inhibitor of NF-kappa-B (ABIN) and NF-kappa-B essential modulator (NEMO) (UBAN), and ubiquitin-binding zinc finger (UBZ) domains. While the UBA domain is found in p62/SQSTM1 (referred to as p62) and neighbor of BRCA1 (NBR1), UBAN and UBZ domains are found in optineurin (OPTN) and nuclear dot protein 52 (NDP52), respectively (Figure 1(d)). In contrast to the aforementioned autophagy receptors Atg19p and Atg34p, which bind directly to their cargo, this group of ubiquitin-binding domain-containing receptors binds to cargo in an ubiquitin-dependent manner. Thus, implementation of ubiquitin-binding domains in autophagy cargo receptors provides a flexible signal, which allows a much broader range of proteins to be targeted for autophagosomal degradation. So far, a variety of cargos have been discovered, which depend on its ubiquitylation to be efficiently incorporated into autophagosomes, including protein aggregates, mitochondria (via ubiquitylation of outer mitochondrial membrane proteins), and microbes (via ubiquitylation of bacterial membrane proteins or host binding proteins) [7–9]. Notably, though the ubiquitin E3 ligases CHIP and Parkin have been implicated in ubiquitylation of misfolded proteins and damage mitochondria, respectively [21–23], the machineries, which are responsible for targeted ubiquitylation of these distinct autophagosomal substrates, are not clearly defined yet.

Defining the ubiquitin chain linkage preference of ubiquitin-binding domains employed in the known autophagy receptors will be critical to fully understand the molecular basis of ubiquitin-mediated selective autophagy. While it has been established that p62's UBA domain binds both K63 and K48 polyubiquitin chains but with higher affinities to K63 ubiquitin chains [24, 25], the picture is less clear for NBR1. The isolated UBA domain of NBR1 binds to K63 and K48 polyubiquitin chains with a slight preference for K63 ubiquitin chains *in vitro* [26], whereas the chain type specificity of full-length NBR1 has not been determined conclusively. NDP52 follows a similar trend as p62 and NBR1 by preferentially binding to K63 polyubiquitin chains [27]. Finally, OPTN's UBAN domain binds specifically to linear polyubiquitin chains as paradigmatically shown for NEMO [27, 28].

p62 and NBR1 cooperatively mediate aggrephagy [26, 29, 30]. Ubiquitylated proteins are bound via their respective UBA domain and consequently delivered to autophagosome. Besides the C-terminal UBA domain, p62 and NBR1 share common N-terminal Phox and Bem1 (PB1) domains (Figure 1(d)), which mediate homooligomerization of p62 and that drives multimerization of p62 and NBR1 in complex with ubiquitylated proteins, thereby amplifying the engagement of ubiquitylated proteins [31]. Importantly, formation of ubiquitylated protein aggregates required polymerization and ubiquitin binding by p62 and possible NBR1 mediated by UBA and PB1 domains, respectively [29]. Sequestration of misfolded proteins into aggregated inclusions likely shields aberrantly exposed hydrophobic surfaces from harmful interaction with essential cellular proteins and might serve as a sink fueling subsequent autophagosomal or proteasomal degradation [32]. Clearance of p62-driven aggregates depends on constitutive autophagy, since autophagy deficiency by ATG7 depletion causes accumulation of ubiquitylated protein inclusions, which were substantially reduced in ATG7/p62 double knockout cells [33]. Notably, p62 and NBR1 are themselves autophagy substrates, which are continuously degraded along with their bound substrates [26, 29, 30]. Elevated levels of p62 caused by autophagy inhibition have been shown to compromise degradation of proteasome substrates [34]. Thus, shifting the abundance of p62 (and possibly NBR1) might lead to competition with other ubiquitin-binding proteins such as ubiquitin shuttling factors or proteasomal ubiquitin receptors, ultimately causing a nonproductive partitioning of ubiquitylated substrates from proteasomes to p62 aggregates. Notably, K48 and K63 ubiquitin chains together with monoubiquitin have been implicated in the formation of protein inclusion but only K63 chains contributed to autophagic clearance of these aggregates [35]. Furthermore, p62-positive aggregates are commonly detected in neurodegenerative diseases, which are often accompanied by proteasome dysfunction [36]. Though NBR1 and p62 have partially redundant functions, we do not fully understand their individual contribution and requirement for driving aggregate formation in the context of selective autophagy.

Together with NDP52 and OPTN, p62 participates in the cellular defense mechanism against infection termed xenophagy [27, 37, 38]. Mammalian cells ubiquitylate bacteria that intrude the cytosol or reside in sequestered membrane compartments as part of their protective response thereby marking these microbes for destruction by selective autophagy [3, 4, 38]. Recent studies have shown that clearance of ubiquitylated bacteria is mediated by specific autophagy receptors that facilitate the assembly of an autophagosomal membrane surrounding the bacterial invaders and deliver them to the autophagosomal degradation machinery. This selective removal of invading bacteria by autophagic degradation has been described to protect cells from bacterial colonization [27, 37]. For example, p62 has been implicated in clearance of *Salmonella*. It was reported that p62 is recruited to ubiquitin-decorated *Salmonella* in the cytosol via its UBA domain [38]. Furthermore, NDP52 has recently been described to recognize ubiquitylated *Salmonella* and to restrict their cytosolic growth by destruction via the autophagy pathway [37]. NDP52 binds to ubiquitin-coated bacteria and recruits the TANK-binding kinase 1 (TBK1) via the adaptor proteins Nap1 and Sintbad [37]. In addition, p62 and NDP52 proteins were recently reported to target *Shigella* and *Listeria* to distinct autophagy pathways [39]. Recently, OPTN has been reported to restrict the pathogenic cytosolic growth after bacterial infection with *Salmonella* [27]. As for NDP52, OPTN recruitment to ubiquitylated *Salmonella* required a functional ubiquitin-binding domain. Interestingly, OPTN and NDP52 were reported to localize to common microdomains on ubiquitin-coated bacteria that could be separated from those occupied by p62 [27]. Similarly, NDP52 and p62 were

described to localize to non-overlapping microdomains on the surface of ubiquitylated bacteria to target *Salmonella* to the autophagy pathway [40]. Depletion experiments indicated that all three selective autophagy adaptor proteins, that is, NDP52, p62, and OPTN, act in the same pathway to cooperatively drive efficient autophagic removal of bacteria [27, 40]. However, the specific role of each of these three different ubiquitin-dependent autophagy receptors and their interdependences in mediating selective engulfment of ubiquitylated bacteria, in particular with respect to hierarchical and temporal recruitment of NDP52, p62 and OPTN, remains to be determined.

A growing body of evidence suggests that ubiquitin may serve as a general recognition signal for many targets of selective autophagy and that p62 acts as a universal receptor for this ubiquitylated cargo. Besides misfolded proteins and bacteria, p62 has been implicated in ubiquitin-dependent autophagosomal degradation of soluble proteins, peroxisome, mitochondria, and midbody ring structure [22, 41–43]. Thus, whereas p62 participates as ubiquitin receptor in many autophagic processes, NBR1, NDP52, and OPTN are specialized to function in specific types of selective autophagy. Clearly, the molecular underpinnings of this partitioning need to be mechanistically dissected in more detail. Given the plethora of ubiquitin binding domains, it would not be surprising to see more been involved in selective autophagy. A yet new twist to the autophagy receptors emerged from the identification of an NBR1-fold domain in ATG19p [9], raising the question whether some of these autophagy receptors (or at least NBR1) have ubiquitin-independent roles in targeting substrates.

5. Transmembrane Domain

The targeted removal of damaged mitochondria by the autophagic machinery represents the currently best-studied example of selective autophagy of organelles that is mediated by specific autophagy adaptors. Mitochondria are the powerhouse of the cell that play a crucial role in the regulation of cellular bioenergetics and metabolism. Therefore, the maintenance of a pool of functional mitochondria is vital for the cellular homeostasis. NIP3-like protein X (Nix), which is also known as BCL2/adenovirus E1B 19 kDa interacting protein 3-like (BNIP3L) (Figure 1(d)), was cloned back in 1998 via its homology with Bnip3 from a human placenta cDNA library [44]. Under physiological conditions, Nix localizes to the mitochondrial outer membrane, where it is anchored via its transmembrane domain. Nix functions as a mitophagy receptor in mammalian cells that mediates selective clearance of mitochondria [45]. The phenotype of Nix-deficient mice is characterized by defective erythrocyte differentiation with high reticulocyte count and corresponding anemia. This phenotype is due to impaired removal of mitochondria from reticulocytes by mitophagy due to the failure to deliver damaged mitochondria to autophagosomes. Removal of mitochondria from reticulocytes represents a prototype form of programmed mitophagy in development and is a crucial step during erythropoiesis for the proper differentiation of erythrocytes that normally become devoid of mitochondria once they pass the reticulocyte status [46, 47]. Nix expression becomes markedly upregulated during the terminal differentiation stages of red blood cells [48], in line with its key role in the programmed removal of mitochondria during development.

In addition, Nix has been implied to mediate the ubiquitylation of damaged mitochondria by the E3 ligase Parkin [49]. Upon depolarization of mitochondria, which marks an early step of mitochondrial dysfunction, Nix facilitates the recruitment of Parkin to depolarized mitochondria [49]. In addition, Pink1 is required for Parkin recruitment to mitochondria [50]. Parkin in turn labels mitochondria for the removal by the autophagic machinery through ubiquitylation of mitochondrial proteins such as VDAC1 and mitofusins [22, 51, 52]. However, additional studies are required to determine what the ubiquitylated target molecules are on the mitochondrial membranes that mediate the autophagic clearance of mitochondria during mitophagy. Moreover, Nix may also initiate mitophagy by causing mitochondrial depolarization, as Nix is also an inducer of mitochondrial cell death [53].

Bnip3 was originally identified as interaction partner of Bcl-2 and adenovirus E1B 19 kDa protein in a yeast two-hybrid screen [54]. Based on the homology to Nix, Bnip3 is likely an additional mitophagy receptor. Bnip3 is anchored to mitochondria via its C-terminal transmembrane domain [53]. Bnip3-mediated mitophagy is triggered upon hypoxia as part of an adaptive, HIF1-dependent response [55]. Since Nix is also induced by hypoxia [56], Bnip3 and Nix may have overlapping functions. Also, Bnip3 and Nix may have a broader role in the regulation of hypoxia-triggered autophagy by interfering with the Bcl-2/Beclin-1 interaction via their BH3 domain, which in turn results in the activation of bulk autophagy by stimulating the Beclin-1/class II PI3K complex [56].

A similar control of mitophagy by selective autophagy receptors exists also in the yeast system. There, Atg32 represents the mitophagy receptor that resides in the mitochondrial outer membrane (Figure 1(d)) [57, 58].

6. Autophagosomes Recruitment Motifs in Autophagy Receptors

Once cargo destined for selective autophagy is bound by the respective autophagy receptors, subsequent delivery to the autophagosomal membrane is mediated by interaction between cargo-specific autophagy receptor proteins and members of the ATG8 ubiquitin-like (Ubl) protein family (Figure 1(b)). The evolutionary conserved ATG8 family encompasses Atg8p in yeast and seven members in humans (microtubule-associated protein-1 light chain 3A (MAP1LC3A), MAP1LC3B, MAP1LC3C, γ-aminobutyric acid type A (GABA) receptor-associated protein (GABARAP), GABARAP-like 1 (GABARAPL1), GABARAPL2, and GABARAPL3) [59, 60]. ATG8 is unconventionally conjugated to phosphatidylethanolamine (PE) via its C-terminal Glycine residue through the action of an E1-E2-E3 conjugation cascade involving ATG7, ATG3, and the

oligomeric complex formed by ATG16-ATG5–ATG12 (where – refers to a covalent bond) (Figure 2(a)) [61–64].

Lipidated ATG8 is thereby incorporated into the membrane of the developing autophagosome and serves as docking site for specific autophagy adaptors. The direct interaction between lipidated ATG8 and autophagy adaptors tethers cargo specifically bound by distinct adaptors to the site of autophagosome formation, leading to engulfment and sequestration of ATG8-adaptor-cargo complexes in autophagosomes. The structural basis of adaptor docking to ATG8 has been revealed by several analyses [65–68]. Briefly, ATG8 proteins generally adopt an ubiquitin fold with an N-terminal extension encompassing two α-helices (α1 and α2). An exposed β-strand (β2) within the ubiquitin fold of ATG8 and two adjacent hydrophobic pockets (hp1 and hp2), formed mainly by residues originating from β1, β2, and α3 critically, contributes to adaptor protein binding (Figure 2(b)).

Importantly, this docking site is conserved among different ATG8 family members. Though structurally divert, all known autophagy adaptors (Atg19, Atg34, p62, NBR1, NDP52, OPTN, Nix, and ATG32) harbor a common, short linear peptide motif, which binds to the ATG8-docking site and thereby essentially mediates direct adaptor-ATG8 interaction (Figure 2(c)) [69]. Note that the LIR in NDP52 is only a candidate LIR motif based on bioinformatics studies and has not been confirmed experimentally. Due to its initial identification in the context of MAP1LC3B (LC3) binding [30, 65], this peptide motif was paradigmatically termed LC3-interaction region (LIR). The consensus sequence for the LIR motif is broadly defined as ΘxxΓ wherein Θ and Γ represent aromatic (i.e., tryptophan, tyrosine, and phenylalanine) and hydrophobic (i.e., leucine and Isoleucine) residues, respectively (Figure 2(b) and 2(c)) [68]. Residues at the Θ position bind to ATG8‘s hp1, whereas residues at the Γ position bind hp2. LIR peptides adopt an extended β conformation and form an intermolecular β-sheet with β2 of ATG8. Notably, acidic residues N-terminally preceding the LIR motif have been shown to additionally contribute to the LIR-ATG8 interaction, possibly by interacting with the positively charged α2 [68]. Recent NMR studies revealed that tryptophan in the Γ position has the strongest influence on binding affinities [68]. Remarkably, mutation of a single residue at the Γ position within the LIR motif of p62 (W338A), OPTN (F178A), Nix (W35A) or Atg19p (W412A) abrogated binding to ATG8/LC3/GABARAP proteins [27, 30, 45, 66, 70]. As a functional consequence, these autophagy receptors retain binding to their respective cargo but fail to be recruited into autophagosomes. For example, OPTN carrying the LIR mutant F178A was detected on *Salmonella* but was unable to restrict bacterial growth upon gene complementation in cells, underlining the functional role of OPTN as autophagy receptor in recruiting *Salmonella* into autophagosomes for their degradation [27].

Lastly, LIR motifs are not restricted to autophagy receptors but emerge as a general surface for interaction with ATG8 family proteins. For example, functional LIR motifs have been identified in adaptor proteins regulating movement of autophagosomes along microtubule and autophagosome maturation such as the Rab7 effector FYCO1 and TBC domain-containing GTPase-activating protein TBC1D25, respectively, as well as components of the ATG8 conjugation system such as ATG3 [71–73]. A recent proteomic approach coupled to *in vitro* binding studies identified numerous proteins as novel ATG8-binding proteins [74]. Despite challenging due to the shortness of LIR motifs, a systematical bioinformatics-based identification of candidate LIR motifs followed by their experimental validation will be critical to assess the cellular repertoire of autophagy receptors and other regulatory ATG8-interacting proteins.

7. Regulation of Cargo-Receptor-ATG8 Complex Assembly

Until lately, the spatiotemporal regulation of cargo binding by autophagy receptors and the subsequent recruitment of cargo-receptor complexes to autophagic membranes for selective engulfment remained elusive. Recent reports by two different groups have now shed light on possible mechanisms controlling dynamics of cargo-receptor and receptor-ATG8 interactions, respectively. First, p62 is specifically phosphorylated at serine 403 (S403), which resides within its UBA domain [75]. S403 phosphorylation increases the affinity between UBA and polyubiquitin chain. Intriguingly, upon binding of phosphorylated UBA to a polyubiquitin chain, phospho-S403 is not accessible for dephosphorylation anymore, indicating a possible mechanism for capturing ubiquitylated proteins for formation of aggregates and autophagosomal engulfment, respectively. These findings raise the questions whether other ubiquitin-binding domains are similarly regulated by phosphorylation. Casein kinase 2 (CK2) has been demonstrated to phosphorylate S403 of p62 directly *in vitro* and in cells. However, determining the kinase network responsible for ubiquitin-binding inducing phosphorylation events will be critical for understanding the signaling circuits underlying cargo binding via ubiquitin-dependent autophagy receptors in particular and ubiquitin-binding proteins in general.

Second, recognition of bacterial pathogens in the cytosol through specific pattern-recognition receptors as part of the innate immune response eventually leads to activation of TANK-binding kinase 1 (TBK1), which in turn binds and phosphorylates OPTN at a serine residue (S177) that precedes the hydrophobic core sequence of the LIR motif in OPTN [27]. S177 phosphorylation causes an increase in the affinity of OPTN for MAP1LC3B. Mechanistically, the increase in binding affinity due to the presence of the phosphoserine preceding OPTN's LIR motif might result in altered hydrogen bond formation, which could potentially counterbalance the suboptimal binding affinity of the unmodified LIR sequence context due to the presence of phenylalanine instead of tryptophan at the Γ position within the LIR motif of OPTN. Remarkably, a phospho-mimicking version of OPTN bound to MAP1LC3B with a higher affinity than its wild-type counterpart, while a nonphosphorylatable version of the protein was strongly impaired in its MAP1LC3B-binding ability. Thus, recruitment of TBK1 and OPTN to the surface of ubiquitylated Salmonella leads

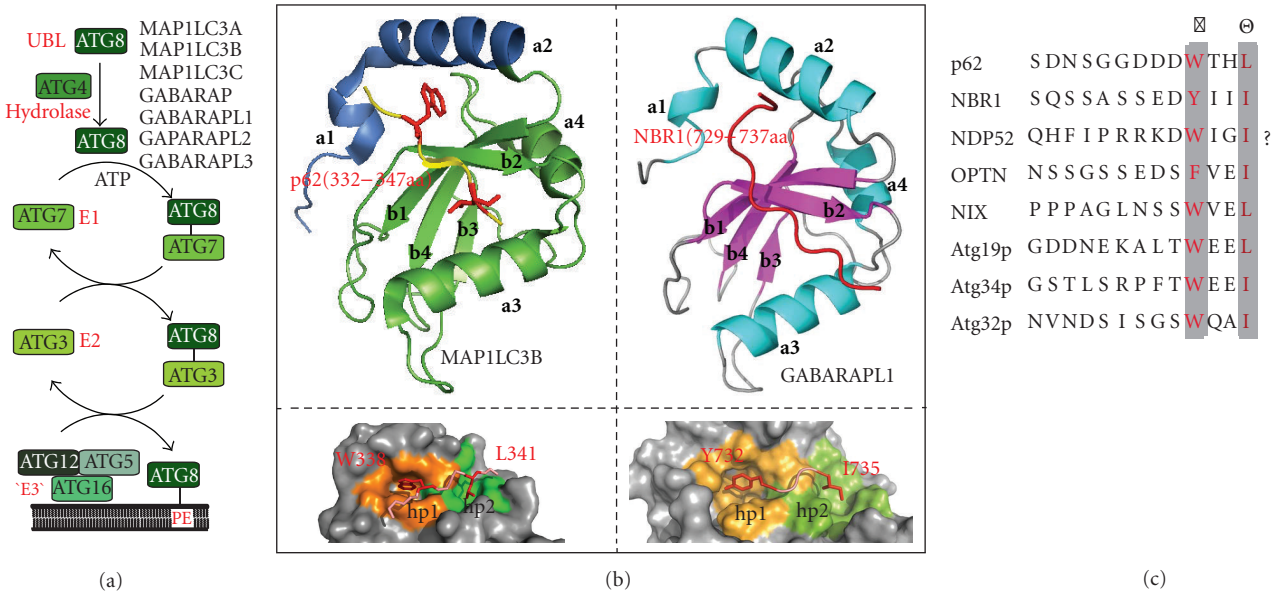

Figure 2: (a) ATG8 conjugation cascade. (b) Structures of MAP1LC3B/p62-LIR (upper left; pdb code: 2K6Q) and GABARAPL1/NBR1-LIR (upper right; pdb code: 2ZJD) complexes. LIR binding sites of MAP1LC3B (lower left) and GABARAPL1 (lower right). (c) Sequence alignments of functional LIR motifs in autophagy receptors.

to spatial activation of TBK1 to enable timely recruitment of MAP1LC3 by OPTN. As mentioned above, TBK1 recruitment is mediated by NDP52, placing the autophagy receptor NDP52 potentially upstream of TBK1 and OPTN. However, the hierarchical nature of signaling events leading to autophagosomal engulfment of ubiquitylated bacteria is still poorly understood. Furthermore, whether conserved serine residues preceding the LIR motifs of Nix and NBR1 are phosphorylated to control autophagosomal engulfment similarly to OPTN remains to be addressed.

Finally, several adaptor proteins facilitating cargo-receptor-ATG8 assembly on incipient autophagic membranes are implicated in selective autophagy, though their specific functions are not well characterized yet. In yeast, Atg11p acts as an adaptor protein for Atg19p, Atg34p, and Atg32p [10–12, 57, 58]. Atg11p binds directly to Atg19p, Atg34p, and Atg32p and is responsible for recruitment of receptor-cargo complexes to the PAS for autophagosomal engulfment via interaction with Atg1p and Atg17p. In mammals, the 400 kDa scaffold autophagy-linked FYVE protein (ALFY) has been implicated in selective autophagy [76]. ALFY translocates from the nucleus or nuclear envelope to autophagic structures in the cytosol in response to amino acid starvation and binds p62 via a C-terminal BEACH domain. Additionally, ALFY binds ATG5 via a WD40 repeat region and PtdIns(3)P through a FYVE domain [76, 77]. Similar to p62 and NBR1, ALFY is required to recruit ubiquitylated proteins into aggregates prior to their autophagosomal degradation [78]. Intriguingly, deletion of the ALFY homologue in flies led to accumulation of ubiquitylated protein aggregates and manifestation of a neurodegenerative phenotype [79]. A common feature of these two structurally diverse adaptor proteins seems to be their ability to tether cargo-receptor complexes to autophagic membranes, thereby mediating recruitment to the site of autophagosomal engulfment. As a functional consequence, adaptor proteins might ensure that cargo-receptor complexes only bind to ATG8 proteins lipidated to autophagic membranes and prevent presumably unproductive interactions with cytosolic, free forms of ATG8 proteins.

8. Concluding Remarks

The work described here underscores the mechanistic and architectural complexities employed in selective autophagy to control autophagosomal turnover of a broad range of selective substrates ranging from proteins, via organelles to whole organisms. However, many questions remain concerning identities of additional cargo and receptor pairs as well as signaling cascades leading to efficient cargo binding and recruitment to autophagic membranes under different physiological and pathophysiological conditions.

Acknowledgments

The authors would like to thank Alexis Rozenknop and Volker Dötsch (Goethe University Frankfurt, Germany) for providing coordinates for the structural models of MAP1LC3B and GABARAPL1. C. Behrends is supported by grants from the Emmy-Noether Program of the Deutsche Forschungsgemeinschaft (DFG) and the European Research Council (ERC). They apologize to authors for not citing all original publications due to reasons of space.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 354914, 8 pages
doi:10.1155/2012/354914

Review Article

The Physiological Role of Mitophagy: New Insights into Phosphorylation Events

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Received 14 October 2011; Revised 16 December 2011; Accepted 19 December 2011

Academic Editor: Fulvio Reggiori

Mitochondria play an essential role in oxidative phosphorylation, fatty acid oxidation, and the regulation of apoptosis. However, this organelle also produces reactive oxygen species (ROS) that continually inflict oxidative damage on mitochondrial DNA, proteins, and lipids, which causes further production of ROS. To oppose this oxidative stress, mitochondria possess quality control systems that include antioxidant enzymes and the repair or degradation of damaged mitochondrial DNA and proteins. If the oxidative stress exceeds the capacity of the mitochondrial quality control system, it seems that autophagy degrades the damaged mitochondria to maintain cellular homeostasis. Indeed, recent evidence from yeast to mammals indicates that the autophagy-dependent degradation of mitochondria (mitophagy) contributes to eliminate dysfunctional, aged, or excess mitochondria. In this paper, we describe the molecular processes and regulatory mechanisms of mitophagy in yeast and mammalian cells.

1. Selective Degradation of Mitochondria by Autophagy

Autophagy is a catabolic process that degrades cytoplasmic components and organelles and is conserved in almost all eukaryotes. Autophagy is initiated in response to cellular stresses such as nutrient starvation, oxidative stress, infection, or inflammatory stimuli. Upon its induction, a cup-shaped double-membrane structure, called an isolation membrane (or phagophore), emerges in the cytoplasm, then the isolation membrane elongates with curvature and finally becomes enclosed, forming an autophagosome containing cytoplasmic components. Subsequently, autophagosomes fuse with lysosomes/vacuoles, and lysosomal hydrolases degrade the sequestered material [1–5]. This process facilitates physiological processes such as survival during starvation, clearance of dysfunctional or aggregated proteins and organelles, development, differentiation, and aging [6–8]. In addition to the nonselective degradation of cytoplasmic components, autophagy can selectively degrade specific organelles or proteins. These include peroxisomes, endoplasmic reticulum, ribosomes, the nucleus, intracellular pathogens, protein aggregates, lipid droplets, and secretory granules. These catabolic processes are termed pexophagy, reticulophagy (ERphagy), ribophagy, nucleophagy, xenophagy, aggrephagy, lipophagy, and zymophagy, respectively. Similarly, the yeast Cvt complex (a protein complex comprising aminopeptidase I (Ape1) and alpha-mannosidase (Ams1)) is delivered to vacuoles *via* an autophagy-like process; Ape1 and Ams1 are processed and activated in the vacuoles, and this autophagic process is called the Cvt pathway. It has been known for some time that mitochondria are also degraded by autophagy in mammalian cells (first described by Clark in 1957 [9]) and in yeast (first described by Takeshige and colleagues in 1992 [10]), but this selective autophagic process has recently been described in more detail. Daughter mitochondria with reduced membrane potential after a fission event are preferentially removed by autophagy in mammalian cells [11]. Photoirradiation-damaged mitochondria are selectively degraded by autophagy in hepatocytes [12, 13]. During the maturation of erythroid cells, mitochondria are preferentially degraded by autophagy in a manner dependent on the mitochondrial outer membrane protein Nix [14, 15]. Recently, it has been reported that there are two types of autophagy in mammalian cells: autophagy related protein 5

(Atg5) and Atg7-dependent (conventional) autophagy and Atg5/Atg7-independent (alternative) autophagy [16]. Both conventional and alternative autophagic processes are implicated in the autophagic degradation of mitochondria during erythroid cell maturation [16, 17]. Similarly, during white adipose tissue differentiation, mitochondria are preferentially degraded by autophagy [18]. When yeast cells were cultured in lactate-containing medium as the sole carbon source and were subjected to nitrogen starvation, the mitochondria were exclusively taken into microautophagic structures [19]. These findings support the idea that mitochondria are selectively recognized and degraded by autophagy. The identification of the yeast mitophagy-specific protein Atg32, which plays a key role in the recognition of mitochondria by the autophagic machineries, confirmed the existence of selective degradation of mitochondria by autophagy [20, 21].

2. The Mechanisms of Selective Autophagy of Mitochondria in Yeast

Atg11 is a cytosolic adaptor protein that is required for selective cargo recognition by autophagy. For example, during the Cvt pathway, the cargo proteins Ape1 and Ams1 generate a complex with the receptor protein Atg19 that is recognized and bound by Atg11. Similarly, during pexophagy, *Pichia pastoris* Atg30 (PpAtg30) binds peroxisomal proteins PpPex3 and PpPex14 and is recognized and bound by Atg11. Finally, in both cases, Atg11 transports the cargo to the pre-autophagosomal structure/phagophore assembly site (PAS), where the isolation membrane emerges, and the cargo is surrounded by the autophagosome [22, 23]. Atg11 is also essential for mitophagy, suggesting the presence of a receptor protein for mitophagy that corresponds to Atg19 or PpAtg30 in the Cvt pathway and pexophagy, respectively, [24]. A genetic screen for yeast mutants defective in mitophagy identified such a receptor protein, which is now known as Atg32 [20, 21, 25]. Atg32 is a mitophagy-specific protein that is not required for nonselective autophagy or other types of selective autophagy [20, 21]. Atg32 consists of 529 amino acids and localizes in the mitochondrial outer membrane with its N-terminal domain towards the cytoplasm. Similarly to the Cvt pathway and pexophagy, when mitophagy is induced, Atg32 is bound by Atg11 and the Atg11–Atg32 complex recruits mitochondria to the PAS [20, 21]. During this recruitment step, Atg32 interacts with Atg8 *via* its WxxI motif. This Atg32–Atg8 interaction is thought to increase the efficiency of mitochondrial sequestration by the isolation membrane [20].

3. Regulation of Mitophagy in Yeast

Although the molecular processes by which the autophagic machinery selects and degrades mitochondria have been revealed, little is known about the upstream signaling pathways. Recently, it was reported that the related signaling pathways of two mitogen-activated protein kinases (MAPKs), Slt2 and Hog1, are involved in the induction of mitophagy [26, 27]. In the Slt2 signaling pathway, all of protein kinase C (Pkc1), MAPKKK (Bck1), MAPKK (Mkk1/Mkk2), Slt2, and the upstream cell surface stress sensor Wsc1 are required for mitophagy [26]. In the Hog1 signaling pathway, Pbs2-Hog1 and the upstream stress sensor Sln1 are required for mitophagy [26]. The downstream proteins in both pathways have not been identified. The role of Slt2 is, however, controversial: in the above-mentioned study, nitrogen starvation-induced mitophagy was deficient in *slt2*-deleted cells [26], whereas another study reported normal mitophagy in *slt2*-deleted cells cultured to the post-log phase [28]. The Slt2-related signaling pathway might be associated with starvation-induced mitophagy only.

Recently, we found that, when mitophagy is induced, Ser114 and Ser119 on Atg32 are phosphorylated and that the phosphorylation of Atg32, especially on Ser114, mediates the Atg32–Atg11 interaction and mitophagy [27]. Similarly it has been noted that phosphorylation of Ser112 on PpAtg30 is required for PpAtg30–PpAtg11 interaction and pexophagy in *Pichia pastoris* [21]. These findings suggest that both mitophagy and pexophagy are regulated by kinase activity and/or the localization of the kinases that phosphorylate Atg32 and/or PpAtg30. The kinase(s) that directly phosphorylate Atg32 or PpAtg30 have not been identified. Although the MAPK Hog1 is required for Atg32 phosphorylation, the direct phosphorylation of Atg32 by Hog1 was not observed in an *in vitro* phosphorylation assay [27]. Presumably, the unidentified kinase that phosphorylates Atg32 is downstream of Hog1 and Slt2.

Atg33 is a mitophagy-related protein that was identified by a genetic screen for yeast mutants defective in mitophagy [25]. Atg33 is located in the mitochondrial outer membrane and functions in mitophagy but not in nonselective autophagy, the Cvt pathway, or pexophagy. Interestingly, in an *atg33*-knockout strain, although mitophagy was partially inhibited when induced by starvation, it was blocked almost completely when induced during the stationary phase. Although the function of Atg33 in mitophagy is unknown, it might be a factor for the selection or detection of damaged or aged mitochondria when cells have reached the stationary phase [25, 29]. Further studies are required to reveal the function of Atg33 in mitophagy.

In addition to Atg33, Whi2, Uth1, and Aup1 have also been reported as related to mitophagy [30–32]. Whi2 is a stress response protein that predominantly influences mitophagy and, to a lesser extent, autophagy [31]. Müller and Reichert speculated that Whi2 and the Ras/PKA (protein kinase A) signaling pathway are linked to the regulation of mitophagy [33]. Uth1 is a mitochondrial outer membrane protein and is reported to be required for mitophagy induced by rapamycin or nitrogen starvation [32]. Aup1 was identified by a screen for protein phosphatase homologs that interact with the serine/threonine kinase Atg1 that is required for autophagy and is suggested to be needed for efficient mitophagy to survive in prolonged stationary phase culture in a medium containing lactate as the carbon source [30]. Interestingly, it was shown that deletion of *RTG3*, a transcription factor that mediates the retrograde signaling pathway, causes a defect in stationary phase mitophagy and that deletion of *AUP1* leads to alterations in the patterns of Rtg3 phosphorylation under these conditions, implying that the function

of Aup1 in mitophagy may be regulation of Rtg3-dependent transcription [34]. Inconsistently, both Uth1 and Aup1 have also been reported to be not required for mitophagy [21] and were not identified in genome-wide mitophagy screens [20, 29]. Further studies are required to clarify these discrepancies, which could be due to differences in the condition used to assess autophagy.

Cellular oxidative status is one factor that contributes to the induction of mitophagy. Deffieu et al. reported that N-acetylcysteine, which increases cellular levels of reduced glutathione, prevents mitophagy [35]. Okamoto et al. reported that the expression of Atg32 is suppressed by N-acetylcysteine treatment, and, as a result, mitophagy is inhibited [20]. These findings suggest that Atg32 expression and mitophagy are affected by cellular oxidative conditions. Because mitophagy is thought to preferentially eliminate damaged mitochondria, it is reasonable that cellular oxidative status, which is compromised by reactive oxygen species (ROS) generated by damaged mitochondria, is related to the induction of mitophagy.

We have summarized the above-described molecular processes and regulatory mechanisms in Figure 1.

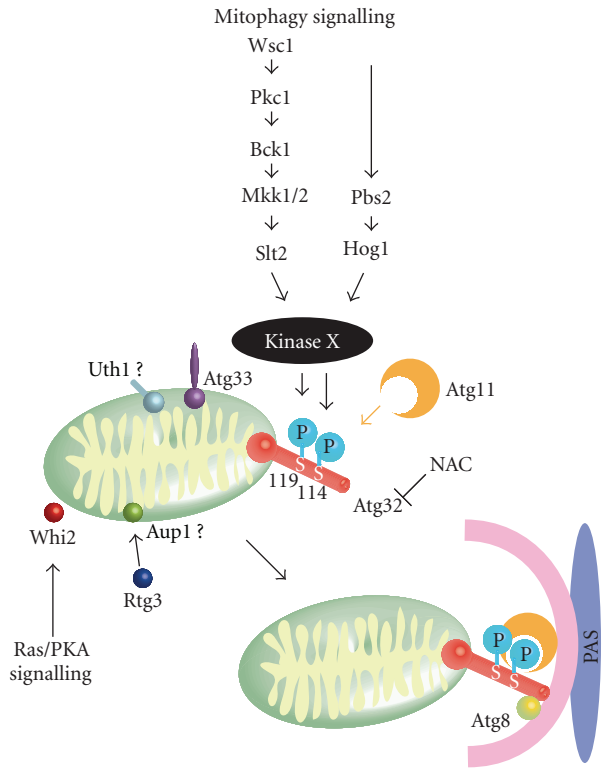

Figure 1: Mitophagy in yeast. Environmental or intracellular factors trigger the mitophagy-signaling pathways that include two MAPKs (Slt2 and Hog1), finally reaching and activating an unidentified kinase X. This kinase phosphorylates Ser114 and Ser119 on Atg32. Phosphorylation of Atg32, particularly at Ser114, mediates the Atg11–Atg32 interaction. Atg11 recruits mitochondria to the phagophore assembly site (PAS) where the autophagosome is generated to enclose the mitochondria. The antioxidant compound N-acetylcysteine (NAC) inhibits mitophagy, presumably by suppressing Atg32 expression. The Atg32–Atg8 interaction increases the efficiency of mitochondrial sequestration by the isolation membrane. Atg33, Whi2, Uth1, and Aup1 have been reported to be required for mitophagy. However, the function of these proteins in mitophagy has not been identified.

4. The Physiological Role of Mitophagy in Yeast

It has been suggested that mitophagy eliminates damaged or aged mitochondria, thereby maintaining mitochondrial quality. There are several lines of evidence demonstrating that damaged mitochondria are eliminated by mitophagy in yeast. Priault et al. suggested that conditional knockout of *fmc1*, a gene encoding the Fmc1 protein that is concerned with the folding of the F_1F_o-ATPase, induces mitophagy under anaerobic conditions [36]. Nowikovsky et al. suggested that interference with the mitochondrial K^+/H^+ exchanger Mdm38 causes the swelling of mitochondria and the degradation of those mitochondria by mitophagy [37]. Zhang et al. blocked mitochondrial DNA (mtDNA) replication using ethidium bromide or a mtDNA polymerase temperature-sensitive mutant and observed rapid degradation of mitochondria *via* autophagy [38]. These results indicate that mitochondrial damage is related to the induction of mitophagy, but are not direct evidence that autophagy selectively eliminates damaged mitochondria. Accordingly, it is still unknown whether mitophagy contributes to mitochondrial quality control in yeast. In fact, it has been difficult to identify the physiological role of mitophagy in yeast, because mitophagy-deficient *atg32*-deleted cells do not show any phenotype, including phenotypes of mitochondrial dysfunction [21].

Our latest studies have partly revealed the physiological role of mitophagy in yeast. When mitophagy-deficient *atg32*-deleted cells were precultured in nonfermentable medium (for instance, lactate-containing medium as the sole carbon source) and were then shifted to nitrogen starvation for long-term culture (~5 days), the *atg32*-deleted cells grown on nutrient-rich plates generated small colonies, while wild-type cells did not. Further analysis revealed that, when wild-type cells encounter nitrogen starvation, they induce mitophagy and quickly eliminate mitochondria that have proliferated during respiratory growth. As a result, cellular ROS production, which occurs mainly in mitochondria, is suppressed. On the other hand, in mitophagy-deficient *atg32*-deleted cells, undegraded mitochondria produce excess ROS during nitrogen starvation. ROS damage mitochondria, and damaged mitochondria produce further ROS, finally leading to mtDNA deletion. Ultimately, cells with mtDNA deletion generate small colonies even in fermentable medium; this phenotype is called "petite" [39]. This suggests that mitophagy is required to regulate the number of mitochondria to minimize ROS production and, as a result, maintains the quality of mitochondria.

There have been several studies suggesting the interplay between mitochondria and autophagy. Bulk autophagy-deficient yeast strains exhibited reduced mitochondrial membrane potential, reduced activities of the electron transport chain, and higher levels of ROS and oxidative stress, resulting in the loss of mtDNA [38, 40]. In bulk autophagy-deficient

cells, cellular ROS accumulate during nitrogen starvation because the cellular amino acid pool is reduced and the expression of the ROS scavenger proteins is suppressed [40]. This finding suggests that autophagy, including mitophagy, contributes to the quality control of mitochondria. In a contrasting situation, Graef and Nunnari demonstrated that healthy mitochondria are required for efficient induction of autophagy under amino acid starvation [41]. Autophagic flux is regulated by Atg1, target of rapamycin (TOR) kinase complex I, and cAMP-dependent protein kinase A (PKA), whereas *ATG8* induction is solely dependent on PKA. Defects in mitochondrial respiration induce PKA activity, resulting in the suppression of both *ATG8* induction and autophagic flux. Therefore, mitochondrial dysfunction directly affects and regulates autophagy. The data presented by Graef and Nunnari indicate that defects in mitochondrial respiration inhibit autophagy including mitophagy during amino acid starvation. They suggest that the effect of mitochondrial dysfunction on the regulation of autophagy varies according to the severity of the defect. Furthermore, these authors also suggest that inordinate accumulation of mitochondria that are defective in respiration beyond a certain level decreases the capacity for autophagy and mitophagy in these cells and evokes a negative feedback that results in cellular aging or death [41].

5. Studies of Mitophagy in Higher Eukaryotes

As described above, the molecular processes and regulatory mechanisms of mitophagy in yeast have been slowly but surely identified. Since the 2008 report that a defect in mitophagy might be involved in the pathogenesis of Parkinson's disease [42], there has been much interest in mitophagy in higher eukaryotes and, in particular, mammalian cells. We will now summarize these studies.

5.1. Parkin/PINK1 and Mitophagy. Most mitophagy studies in mammalian cells have focused on PTEN-induced putative kinase protein 1 (PINK1)/Parkin-dependent mitochondrial degradation by autophagy. Parkin and PINK1 are encoded by the *PARK2* and *PARK6* genes, respectively; both are responsible for familial Parkinson's disease and have been reported to be associated with mitophagy [42–44]. PINK1 is expressed in the cytoplasm and constitutively translocates into the mitochondrial inner membrane where it is promptly degraded by the mitochondrial inner membrane rhomboid protease presenilin-associated rhomboid-like protein (PARL) [43, 45–47]. When mitochondria lose their membrane potential, PINK1 can target to the mitochondria, but cannot translocate across the mitochondrial outer membrane; therefore, it accumulates there. Accordingly, only depolarized mitochondria are marked by PINK1 accumulation. Parkin translocates to mitochondria in a PINK1-dependent manner [42–44, 48, 49]. Parkin triggers the ubiquitination of many mitochondrial proteins such as mitochondrial assembly regulatory factor (MARF) in flies or mitofusin 1, mitofusin 2, and voltage-dependent anion channel 1 (VDAC1) in mammalian cells [49–54]. The ubiquitinated proteins on mitochondria are bound by the autophagy substrate p62/SQSTM1, which

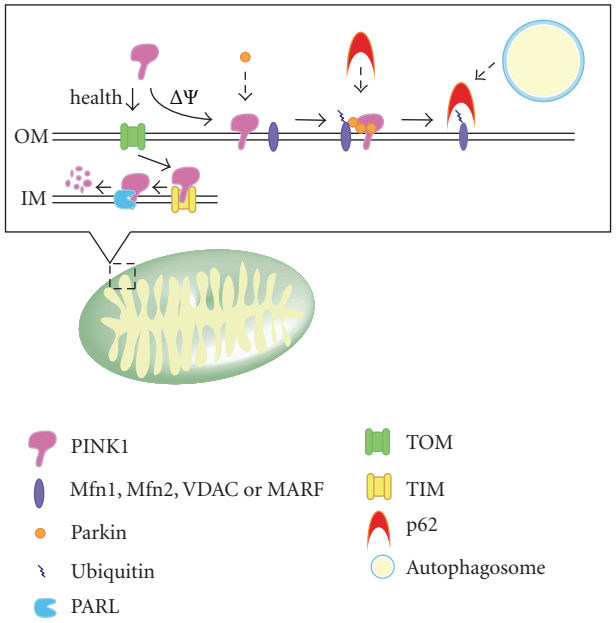

Figure 2: Parkin/PINK1 and mitophagy in higher eukaryotes. PINK1 is constitutively targeted and imported into the inner membrane *via* the mitochondrial import machinery, the TOM and TIM complexes, and degraded by presenilin-associated rhomboid-like protein (PARL). When the mitochondrial membrane potential is depolarized, PINK1 cannot translocate across the mitochondrial outer membrane and instead accumulates on it. PINK1 on the outer membrane causes the translocation of Parkin to mitochondria which triggers the ubiquitination of mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and voltage-dependent anion channel 1 (VDAC) in mammals, and mitochondrial assembly regulatory factor (MARF) in flies. The ubiquitinated proteins on mitochondria are captured by p62, a substrate of autophagy that can bind ubiquitinated proteins, resulting in the sequestration of mitochondria into autophagosomes. OM: outer membrane; IM: inner membrane.

contains a ubiquitin-associated domain, and the p62-associated mitochondria aggregate near the nucleus [49, 54, 55]. Because p62 is a substrate of autophagy, it is thought that p62-associated mitochondria are eventually degraded by autophagy [49, 54, 55]. We have summarized Parkin/PINK1-dependent mitophagy in Figure 2. Although it is accepted that p62 associates with mitochondrial proteins ubiquitinated by Parkin and mediates the aggregation of mitochondria, there have been conflicting reports showing that p62 is not indispensable for mitophagy [56, 57]. The histone deacetylase HDAC6, which can bind ubiquitinated proteins and facilitates the clearance of protein aggregates, is also reported to accumulate on mitochondria after Parkin translocation from the cytosol and mediate mitophagy [54]. Further studies are required to clarify the precise roles of p62 and HDAC6 in mitophagy.

5.2. Other Factors Related to Mitophagy in Higher Eukaryotes. The study of Parkin/PINK1-dependent mitophagy has dominated the field in recent years. However, other studies have focused on Parkin/PINK1-independent mechanisms of mitophagy in higher eukaryotes.

In mammalian cells, ULK1, a homolog of yeast Atg1, is known to be associated with the control of autophagy by the TOR signaling network. ULK1 activity is suppressed under nutrient-rich conditions by TOR complex 1 (TORC1) [58]. Recently, it has been suggested that phosphorylation of ULK1 by adenosine monophosphate-activated protein kinase (AMPK) is concerned with autophagy. Loss of AMPK or ULK1 resulted in deficient mitophagy and aggrephagy during starvation in mouse embryonic fibroblasts and hepatocytes, resulting in increases in the overall mitochondrial number and aberrant morphology [59]. This finding suggests that AMPK-mediated phosphorylation of ULK1 is required for mitochondrial homeostasis in nutrient-poor conditions.

Tectonin domain-containing protein 1 (Tecpr1) has been identified as an Atg5-binding protein. This protein forms a complex with Atg12-Atg5-Atg16L1 and binds to WIPI-2, which is capable of association with phosphatidylinositol 3-phosphate at an isolation membrane. Interestingly, Tecpr1 is required for xenophagy, which selectively recognizes and eliminates bacterial pathogens such as *Shigella*, *Salmonella*, and Group A *Streptococcus*. Tecpr1 is also required for the autophagic degradation of misfolded protein aggregates and depolarized mitochondria but not for nonselective autophagy [60]. These findings suggest that Tecpr1 is an essential factor for specific cargo recognition in selective autophagy.

It has been reported that mitophagy is induced under several conditions such as mitochondrial permeability transition, during cellular development or during hypoxia. These three examples will be discussed in turn. First, nutrient starvation and photodamage, which both lead to mitophagy [12], cause mitochondrial permeability transition (MPT) [61], in which the opening of the MPT pores causes mitochondria to become permeable to all solutes up to a molecular mass of approximately 1500 Da, leading to mitochondrial depolarization and outer membrane rupture [62, 63]. Cyclosporin A, an inhibitor of MPT through interaction with cyclophilin D, blocks mitophagy during MPT [12, 64]. These findings suggest that MPT is a trigger for mitophagy that arises from mitochondria themselves.

Second, recent studies have revealed that mitophagy plays an important role in cellular differentiation. During reticulocyte maturation (as with erythroid cell maturation mentioned in Section 1), mitochondria are eliminated *via* autophagy in a Nix-dependent manner [14, 17, 65]. Nix, in both *in vivo* and *in vitro* assays, interacts with LC3/GABARAP, which anchors to the isolation membrane and is involved in isolation membrane extension, and this Nix-LC3/GABARAP interaction is thought to mediate efficient targeting of mitochondria to autophagosomes [66, 67]. Similarly, when autophagy was inactivated by targeted deletion of the autophagy-essential gene *Atg7*, post-differentiated white adipocytes exhibited large numbers of mitochondria compared with the relatively few mitochondria observed in wild-type white adipocytes. This suggests that mitochondria are preferentially eliminated by autophagy during adipogenesis [18, 68–71].

Third, mitophagy is induced by hypoxia in a Bcl-2/adenovirus E1B 19 kDa interacting protein 3-(BNIP3-) dependent manner; the expression of *BNIP3* is regulated by hypoxia-inducible factor [72–74]. This indicates that mitophagy might be a survival mechanism to regulate the production of ROS from mitochondria during hypoxia. As shown here, mitophagy plays a role in several aspects of cellular physiology, not just eliminating depolarized mitochondria in a Parkin/PINK1-dependent manner.

5.3. Unanswered Questions on Mitophagy in Mammalian Cells. Although there are at present more than 50 publications regarding Parkin/PINK1-dependent mitophagy, the precise mechanisms are still unknown. Recently, it was reported that Parkin induces rupture of the outer membrane of depolarized mitochondria, depending on proteasomal activity, and then the ruptured mitochondria are eliminated by mitophagy [75]. This finding implies that Parkin and PINK1 are not the primary factors required for mitophagy but rather that they present depolarized mitochondria to the autophagic machineries by disrupting the mitochondrial outer membrane.

Most of the autophagy-related genes identified in yeast are also present in mammals, suggesting that the molecular processes of autophagy are conserved throughout evolution. It is surprising then that the molecular processes of mitophagy and the essential factors identified to date are completely different between yeast and mammals. For example, in mammals, the mitochondrial receptor protein corresponding to Atg32 in yeast has not been identified.

6. Conclusion

In recent years, there has been significant progress in studies of mitophagy in both yeast and mammals. In particular, the molecular processes and regulatory mechanisms of mitophagy in yeast have been well described, such as the specific Atg32–Atg11 interaction and the requirement for signaling by the two MAPKs Slt2 and Hog1. Meanwhile, the physiological role of mitophagy in mammalian cells has been well understood. Because mitophagy is evolutionarily conserved, it is reasonable to speculate that there will be similar molecular processes, regulatory mechanisms, and physiological roles in both yeast and mammals. The interplay of yeast and mammalian mitophagy studies will consolidate our understanding of this cellular process.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 182834, 9 pages
doi:10.1155/2012/182834

Review Article

Reticulophagy and Ribophagy: Regulated Degradation of Protein Production Factories

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Received 21 October 2011; Accepted 19 December 2011

Academic Editor: Anne Simonsen

During autophagy, cytosol, protein aggregates, and organelles are sequestered into double-membrane vesicles called autophagosomes and delivered to the lysosome/vacuole for breakdown and recycling of their basic components. In all eukaryotes this pathway is important for adaptation to stress conditions such as nutrient deprivation, as well as to regulate intracellular homeostasis by adjusting organelle number and clearing damaged structures. For a long time, starvation-induced autophagy has been viewed as a nonselective transport pathway; however, recent studies have revealed that autophagy is able to selectively engulf specific structures, ranging from proteins to entire organelles. In this paper, we discuss recent findings on the mechanisms and physiological implications of two selective types of autophagy: ribophagy, the specific degradation of ribosomes, and reticulophagy, the selective elimination of portions of the ER.

1. Introduction

Autophagy is a degradative process that allows cells to maintain their homeostasis in numerous physiological situations. It is required, for example, to face prolonged starvation periods and nutritional fluctuations in the environment, developmental tissue remodeling, organelle quality control, and immune responses [1, 2]. In addition, this pathway has been implicated in the physiopathology of multiple diseases [3, 4]. Autophagosomes are the hallmark of autophagy. These double-membrane vesicles are generated in the cytosol and during their formation they engulf the cargo to be delivered into the mammalian lysosomes or yeast and plant vacuoles for degradation [5]. Two types of autophagy have been described: selective and non-selective autophagy. During non-selective autophagy bulk cytosol, including organelles, is randomly sequestered into autophagosomes. On the other hand, during selective autophagy, a specific cargo is exclusively enwrapped by double-membrane vesicles, which contain little cytoplasm with their size corresponding to that of their cargo [6].

Autophagy progression relies on the function of the autophagy-related (Atg) proteins that mediate autophagosome biogenesis, selective cargo recognition, fusion with the lysosome/vacuole, or vesicle breakdown [5, 7, 8]. Upon nutritional stresses, fractions of the cytoplasm are consumed via autophagy and the resulting catabolic products are used as sources of energy or as building blocks for the synthesis of new macromolecules. In these situations autophagy is mainly considered as a non-selective process. Nonetheless an increasing number of selective types of autophagy are being described [6, 9] and these findings challenge the concept whether autophagosomes in fact sequester their cargo randomly.

2. Short Overview of Selective Types of Autophagy

One of the best-studied examples of selective autophagy is the biosynthetic cytoplasm to vacuole targeting (Cvt) pathway in the yeast *Saccharomyces cerevisiae*. During the Cvt pathway a protein oligomer composed of the vacuolar hydrolases aminopeptidase 1 (Ape1), α-mannosidase 1 (Ams1), and aspartyl aminopeptidase (Ape4), is delivered to the vacuolar lumen by small double-membrane vesicles [10–13]. Interestingly, this oligomer is also a specific cargo of autophagosomes under starvation conditions [14]. In higher eukaryotes autophagy also supports the selective destruction of intracellular pathogens, called xenophagy, and protein aggregates, named aggrephagy. In addition, metabolically dispensable or dysfunctional organelles can be specifically degraded by autophagy in both yeast and mammals. Examples of the latter include the exclusive elimination of superfluous or damaged mitochondria, termed mitophagy, and the selective consumption of excessive peroxisomes, called pexophagy [15, 16].

The underlying mechanisms of each of these pathways remain to be characterized in detail but some common principles are emerging. First, a receptor-like recognition of the cargo directing it to the autophagosome or alternatively recruiting the Atg machinery is required for all the selective types of autophagy. Second, the involvement of ubiquitin as a signaling molecule has been described for several selective types of autophagy in higher eukaryotes [17]. Several of the autophagosomal cargos can be degraded in a selective manner under specific conditions or in a random manner during bulk autophagy. It remains to be investigated in more detail how certain autophagy pathways can choose specific cargo in time and space. As the subject of selective autophagy pathways is covered in other reports in this special issue of the *International Journal of Cell Science*, in this review we will discuss the molecular principles and mechanisms underlying two selective types of autophagy that remain less well understood: ribophagy and reticulophagy.

3. Ribophagy: Mechanisms and Physiological Implications

Since the discovery of autophagy, ribosomes have been detected in the interior of autophagosomes by electron microscopy [18, 19]. For a long time these large multiprotein complexes were viewed as a marker for bulk degradation of cytoplasm. However, it has recently been shown that ribosomes are turned over through a selective type of autophagy [20]. Accurate examination of ribosome fate under nutrient starvation conditions in yeast *S. cerevisiae* has revealed that these structures are more rapidly degraded compared to other cytoplasmic components, supporting the notion of a selective degradation process [20]. The involvement of autophagy in this event was demonstrated by uncovering that the transport of ribosomes to the vacuole relies on core autophagy components such as Atg1 and Atg7. A genetic screen in yeast designed to isolate mutant strains with a defect in ribosome turnover revealed that the ubiquitin protease Ubp3 and its cofactor Bre5 are required for this selective type of autophagy, however, not for bulk autophagy [20]. Importantly, a catalytically inactive mutant of Ubp3 also displayed a defect in the autophagy-mediated degradation of ribosomes indicating that ubiquitination plays a key role in this process. This selective autophagic turnover of ribosomes is now termed ribophagy [20] (Figure 1(a)).

4. Ribophagy and Ubiquitination

It remains to be investigated whether ubiquitination is important for either the regulation of signaling pathways triggering ribophagy or in dictating the specificity in the cargo selection. This latter possibility is evoked by the fact that ubiquitin-based modifications are a common theme in the selective elimination of specific structures in higher eukaryotes [17]. As Ubp3 interacts with and influences the ubiquitination status of Atg19 [21], a receptor protein of the Cvt pathway [22], it is plausible that Ubp3 could contribute to other selective types of autophagy in a similar manner. Further evidence for the involvement of ubiquitination in ribophagy comes from the finding that a decrease of the cytoplasmic levels of the ubiquitin ligase Rsp5 together with the deletion of *UBP3* results in a defect in the turnover of ribosomes higher than in the *ubp3*Δ cells [23]. Importantly, cytoplasmic proteins are normally degraded by autophagy in this strain. These findings imply that both ubiquitination and deubiquitination are crucial for the regulation of ribophagy. A reciprocal control mechanism has also been found to be important for the specific removal of midbody rings by autophagy during cytokinesis [24]. To understand the regulation and mechanism of ribophagy, it will be important to identify the targets of Ubp3/Bre5 and Rsp5 during this process.

5. Putative Physiological Roles of Ribophagy

What could be the physiological role of ribophagy? The deletion of *UBP3* results in the inhibition of starvation-induced ribophagy and leads to cell death, without affecting general bulk autophagy [20]. These findings support the notion that not only bulk autophagy, but also ribosomal turnover is important for cell survival during nutrient limiting conditions. This does not come as a surprise as ribosomes constitute half of the cell's protein mass [25], and consequently, represent a major source of amino acids during times of nutrient deprivation. In addition, or alternatively, the importance of ribosomal degradation during starvation might be its contribution to the rapid and simultaneous downregulation of protein translation, a process that consumes large amounts of energy and amino acids.

Interestingly, a ribophagy-like process has also been proposed in plants. The endoribonuclease Rns2, a conserved member of the RNAse T2 protein family, is required for ribosomal RNA decay in plants [26]. Mutant cells lacking Rns2 activity fail to degrade ribosomal RNA. If this results in a failure of disassembling and/or degrading entire ribosomes

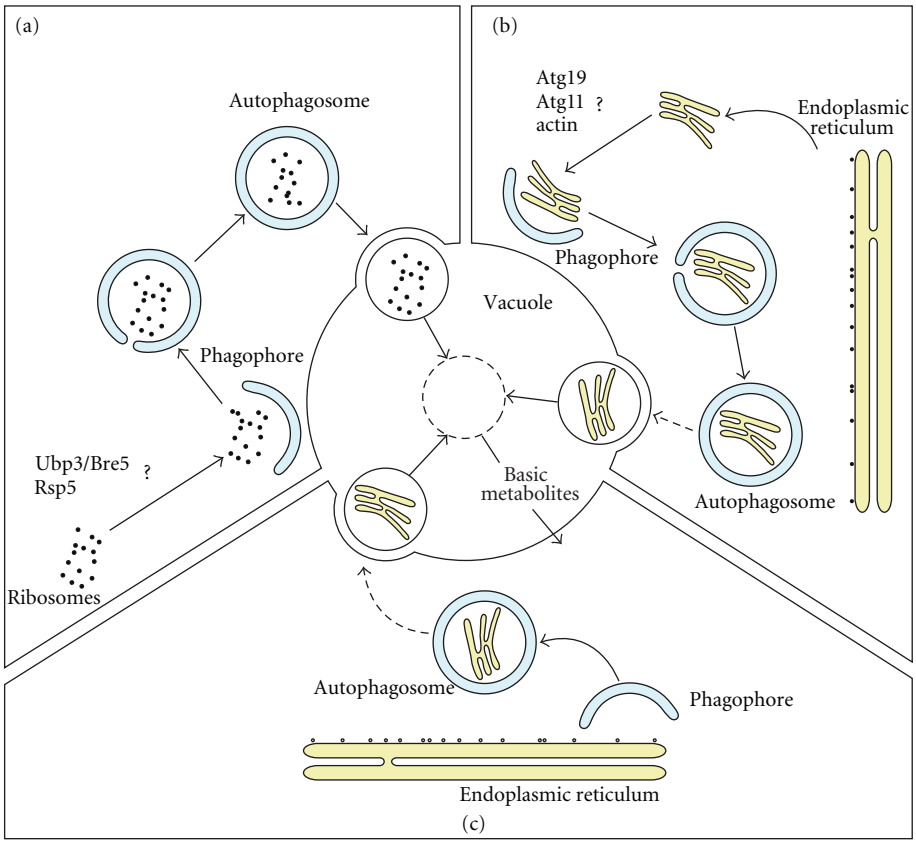

Figure 1: Mechanisms of ribophagy and reticulophagy in yeast. (a) A model for ribophagy. Under ribophagy inducing conditions, ribosomes are selectively engulfed into autophagosomes and subsequently degraded in the vacuole. The generated basic metabolites (amino acids, sugars, fatty acids etc.) are then recycled back to the cytoplasm for reuse or as a source of energy. ((b) and (c)) Models for reticulophagy. Under stress conditions, due to an accumulation of unfolded proteins and/or protein aggregates, a partial scission of the ER occurs and the formed fragments are specifically transported to the sites where autophagosome biogenesis takes place (b). ER stress triggers the recruitment of the Atg proteins onto or close to this organelle. There, possibly by utilizing the ER membranes, the Atg proteins mediate the formation of autophagosomes, which expand around the ER sections that have to be removed (c). The dashed arrows indicate that under specific ER stress conditions, autophagosomes do not fuse with the vacuole. Question marks highlight proteins that have been implicated in the transport and selection of the cargo in which the mechanism of action remains to be elucidated.

has not yet been determined. Nevertheless, the defect in the turnover of ribosomal RNA suggests that Rns2 is a component of a ribophagy-like process in plants. The plant *rns2* mutants also exhibit this phenotype in nutrient rich conditions. This suggests that ribophagy might also serve a housekeeping function by recycling some of the ribosomal components such as amino acids and nucleotides. To date, only the degradation of ribosomal RNA has been studied. Consequently, the fate of ribosomal proteins as well as the existence of ribophagy in plants will require more detailed investigations.

6. Ribophagy in Higher Eukaryotes

Ribosomes have also been observed in the interior of autophagosomes in mammalian cells [18]. In particular, the relative abundance of proteins in MCF7 cells during amino acid starvation has been measured using quantitative mass spectrometry [27]. This approach has revealed that in mammalian cells ribosome degradation by autophagy occurs with different kinetics than that of other cytoplasmic proteins and organelles [27]. It has yet to be explored, however, whether a selective type of autophagy is responsible for the different turnover rates of ribosomes and cytoplasmic proteins. Additional evidence for the possible existence of ribophagy in higher eukaryotes comes from a murine study on neurodegeneration in Purkinje cells, where the disassembly of actively translating polysomes to nontranslational monosomes was observed among other changes [28]. Interestingly, a fraction of the free monosomes was specifically sequestered into autophagosomes suggesting that an autophagy-related pathway is involved in the selective degradation of ribosomes in these cells [28]. Thus, these neuronal cells appear to be an optimal model to study ribophagy and possibly gain additional insight into the involvement of both ubiquitination and the mammalian Ubp3 homologue Usp10 in the turnover of ribosomes in higher eukaryotes.

Autophagy of ribosomal proteins has also been demonstrated to serve an antimicrobial function. Several bacteria

are directly captured in the cytosol by the autophagy adapter p62 or NDP52, and subsequently sequestered into autophagosomes to be delivered and degraded in lysosomes [29]. In the case of *Mycobacterium tuberculosis*, autophagy can also be used for its removal from the cell, however, through a different mechanism. Mycobacteria are phagocytosed by macrophages whereupon they delay phagosome maturation, thereby preventing their destruction in the lysosome. In the phagosomes, they persist and replicate often leading to lethal infections. Recently it has been shown that upon autophagy induction the cytosolic ribosomal protein rpS30 precursor FAU and ubiquitin are sequestered into autophagosomes in a p62-dependent manner [30]. In the mature autophagosome, these proteins are processed into peptides possessing antimicrobial properties that direct the killing of *Mycobacterium* [30]. Because of the involvement of p62, this antimicrobial turnover of ribosomal protein precursors appears to have all the characteristics of a selective type of autophagy.

An alternative role for ribophagy in cell homeostasis arises from the possibility that this pathway could also target defective ribosomes under normal growth conditions. In this scenario, by specifically eliminating nonfunctional, incorrectly assembled, and/or damaged ribosomes, ribophagy would have a quality control function. Avoiding the translation of incorrect and potentially harmful proteins might be crucial for cell homeostasis. Along this line, it is important to note that several diseases have been associated with specific mutations in ribosomal subunits [31]. The identification of such a quality control function, as well as the mechanism underlying it will be important directions for future analyses.

7. Protein Folding and ER Stress

While ribosomes located in the cytosol mainly translate cytoplasmic proteins, the synthesis of proteins that are secreted or reside in one of the organelles of the endomembrane system is mediated by ribosomes associated with the ER. As these newly synthesized proteins are cotranslationally translocated into the ER, a conspicuous amount of these molecules remains localized to this compartment. In order to prevent the accumulation of misfolded polypeptides, the ER counts on a specialized group of proteins, the so-called chaperones, which assist the folding of the nascent polypeptides or recognize misfolded proteins and mediate their refolding [32]. Under certain circumstances, this quality control function of the ER can be overcome by the natural occurrence of mutations or peculiar environmental conditions that affect general protein folding. This scenario can also be mimicked by expression of specific mutant proteins or treatment with particular chemical agents [33–37]. These situations may result in the accumulation of unfolded proteins and aggregates in the ER. Two interconnected safeguard mechanisms, the unfolded protein response (UPR) and the ER-associated degradation (ERAD), are in place to cope with misfolded protein buildups [38–40]. The UPR is an intracellular signaling cascade triggered by ER stress. This signal is transduced into cytoplasmic and nuclear actions aimed at increasing the inherent folding capacity of the ER and eliminating the misfolded proteins accumulated in this organelle. Among the responses initiated by the UPR are inhibition of general translation and upregulation of genes encoding ER chaperones and components of the ERAD machinery. The ERAD in turn, recognizes misfolded proteins and retrotranslocates these proteins into the cytoplasm where they are degraded by the ubiquitin-proteasome system. This elimination is mediated by the retrotranslocon complex, a multiprotein system seated in the ER membrane that facilitates the transport of unfolded proteins across the ER, catalyzes the polyubiquitination of the exported proteins, and mediates their delivery to the proteasome. Autophagy might serve a third cellular mechanism complementing the UPR and ERAD systems in coping with the harmful accumulation of unfolded or aberrant proteins in the ER.

8. Autophagy in ER Stress

Molecular events occurring upon autophagy induction are the association of Atg8/LC3 with autophagosomal membranes through its conjugation to the lipid phosphatidylethanolamine (PE) [41, 42] and the formation of autophagosomes. Yeast and mammalian cells subjected to different ER stresses exhibit levels of lipidated Atg8/LC3 similar to those displayed by starved cells [33, 37, 43, 44]. Additionally, light microscopy studies have revealed that ER stress induces the formation of autophagosomes in all eukaryotes analyzed [33, 44, 45]. Both this lipidation event and the formation of autophagosomes during ER stress can be blocked by chemical inhibitors of autophagy or Atg protein depletion [33, 37, 43, 44]. This, in combination with ultrastructural analyses of both yeast and mammalian cells following ER stress, which showed the induction of autophagosomes and autophagolysosomes, confirms the induction of an autophagy response upon ER stress [36, 43, 45, 46]. A detailed scrutiny of the luminal contents of these carriers has revealed that autophagosomes enclose portions of the ER [45, 46]. The amount of ER sequestered in their interior, however, depends on the nature and strength of the stimuli triggering the reticulophagy response. For example, when yeast are treated with the reducing agent dithiothreitol (DTT), which inhibits disulfide bond formation and thus prevents correct protein folding, autophagosomes are mostly filled with tightly stacked ER membrane cisternae [45]. In contrast, when ER stress is initiated by glucose deprivation, which leads to a defect in the N-glycosylation important for the proper folding of glycoproteins, each autophagosome carries a single ER fragment [46]. The existence of ER-containing autophagosomes is supported by the juxtaposition of Atg8 and the ER marker protein Sec61 in fluorescence microscopy analyses in yeast [45]. Additionally, *in vitro* and *in vivo* studies in mammals on the Z mutant form of α_1-antitrypsin (α_1-ATZ), which aggregates and accumulates in the ER [47], have shown that the cytoplasmic α_1-ATZ aggregates colocalize with GFP-LC3 and ER resident KDEL-containing proteins [48]. Ultrastructural analyses have confirmed that these structures are indeed ER-containing autophagosomes [36].

Several evidences suggest that the sequestration of ER portions by autophagosomes might be a selective process. In yeast, induction of reticulophagy by DTT results in autophagosomes that contain tightly packed ER fragments that are devoid of cytoplasm [45]. Importantly, immunoelectron microscopy analysis in these cells using anti-GFP antibodies directed against GFP-HDEL, an ER marker protein, has demonstrated that the density of the gold particles is higher inside autophagosomes than in the total cell area [46]. This result is in agreement with the concept of a selective type of autophagy, since in a non-selective scenario the label would have been equally distributed outside and inside of the sequestering vesicles [49]. It cannot be excluded, however, that the increased density of the gold particles is the result of a longer half-life of ER components in the interior of autophagosomes. Further support for a selective nature of this pathway emerges from the notion that the actin cytoskeleton and the selectivity adaptor proteins Atg11 and Atg19 are required for the progression of reticulophagy in yeast (see below).

9. Models for the Selective Sorting of ER into Autophagosomes

How is ER targeted for degradation specifically sequestered into autophagosomes? One possibility is that fragments of ER containing unfolded proteins or aggregates are pinching off from the main ER body and are directly transported to the site where autophagosomes arise (Figure 1(b)). During the yeast Cvt pathway, for example, the selective sorting of the cargo oligomer requires the receptor Atg19, the adaptor protein Atg11, and the actin cytoskeleton. Interestingly, these three components have been linked to ER degradation under both stress conditions and nutrient deprivation in yeast [45, 46, 50]. A second possibility is that the selection and enwrapping by autophagosomes occurs in very close proximity to the ER (Figure 1(c)). In contrast to the previous model, this situation does not require a specific machinery to direct the cargo, but rather a system to recruit the Atg proteins to the location where the cargo resides. In both scenarios it remains a mystery how the ER fragments are generated, which factors regulate this scission, and how the ER is selectively sequestered. Interestingly, a recent study in *S. cerevisiae* has shown that Atg8 and Cvt pathway components are recruited onto the ER and negatively regulate the extraction and proteasomal degradation of the misfolded Hmg2 transmembrane protein [51]. Cells could potentially exploit a similar mechanism to recruit the Atg proteins to the ER during reticulophagy. At the ER, the Atg machinery could catalyze the expansion of new membranes destined to sequester an ER fragment or alternatively rearrange a preexisting ER cisterna to constitute the limiting membrane of the sequestering autophagosome. The latter possibility is supported by studies in yeast showing the presence of autophagosomes with ribosomes attached to the membrane surface [45]. In addition, electron tomography analyses in mammalian cells have shown that autophagosomes can be physically connected to the ER, suggesting that these carriers might directly emerge from the ER [52, 53].

10. Regulation of Reticulophagy by ER Quality Control Signaling

The yeast UPR consists of a main signaling pathway initiated by the ER transmembrane kinase inositol requiring enzyme 1 (Ire1). The luminal domain of Ire1 senses the accumulation of unfolded proteins, while the cytoplasmic extension transduces the signal into the nucleus initiating a cellular response at the transcriptional level [54]. Activated Ire1 initiates the nonconventional splicing of *HAC1* mRNA, leading to the production of the transcription factor Hac1, which in turn upregulates the expression of UPR target genes (Figure 2) [54]. Together with the Ire1 counterparts, mammals have two additional ER-stress sensors to induce the UPR: the RNA-dependent protein kinase-like ER kinase (PERK) and the activating transcription factor 6 (ATF6) (Figure 2) [54].

Increasing Atg8 protein levels upon ER stress has been shown to depend on functional Hac1 in yeast (Figure 2) [45]. Additional signaling cascades, however, might be involved in triggering reticulophagy, as the expression of constitutively active spliced Hac1 is not sufficient to stimulate the formation of autophagosomes [45]. Accordingly, cells lacking Hac1 or Ire1 remain capable of inducing the transcription of *ATG8*. This suggests that redundancies or crosstalk among the signaling events regulating autophagy in response to ER stress exist [45].

The lipidation of Atg8/LC3-I also depends on the formation of a large protein complex composed of Atg16 and the conjugate Atg12-Atg5, which is thought to act as an E3-like enzyme conjugating Atg8/LC3-I to PE on autophagosomal membranes [55, 56]. Upregulation of *ATG12*, and the concomitant conversion of Atg8/LC3-I into Atg8-PE/LC3-II, relies on the phosphorylated eIF2α, which itself depends on PERK activation after ER stress in mammalian cells (Figure 2) [37, 44].

Atg1/ULK kinase activity is required to coordinate the action of the Atg proteins during the early events of autophagosome biogenesis [7]. Numerous signaling cascades regulating autophagy such as the mTOR, the AMPK, and the PKA pathways modulate the Atg1/ULK function [7]. Interestingly, Atg1 kinase activity is also enhanced upon ER stress in yeast (Figure 2) [33]. It remains to be established how ER stress acts on this kinase, whether through the above-mentioned cascades or via alternative signaling pathways. For example, depletion of sphingosine-1-phosphate (S1P) phosphatases in mammalian cells leads to an increase of endogenous S1P levels, which cause an ER stress that triggers autophagy [57]. This induction is mTor-independent and PERK-, Ire1-, and ATF6-dependent. Moreover, ER stress causes a release of Ca^{2+} from the ER into the cytosol initiating various signaling cascades, some of which are likely to be involved in autophagy induction [58, 59]. While future research is required to understand the signaling networks regulating autophagy in response to ER stress, it is conceivable that reticulophagy could be induced differently depending on the type and intensity of the ER stress.

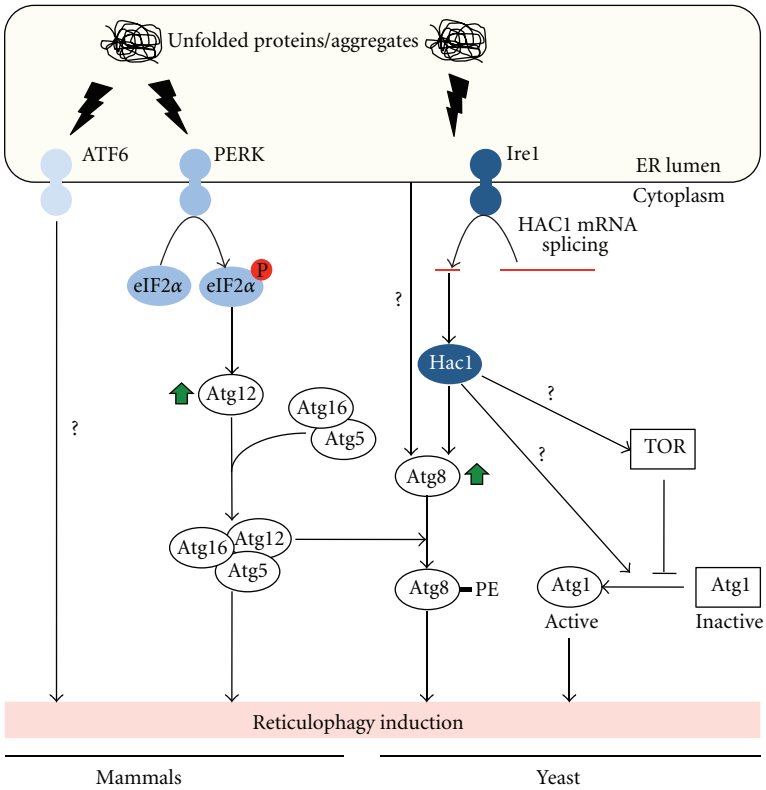

Figure 2: Signalling cascades inducing reticulophagy upon ER stress. The transmembrane protein Ire1 (yeast and mammals), ATF6, and PERK (mammals) sense the accumulation of unfolded proteins and/or aggregates, and trigger a general transcriptional response that affect the levels of proteins involved in autophagy. These include Atg8 (signal mediated through Ire1/Hac1 and unidentified alternative pathways in yeast) and Atg12 (mediated by the PERK/eIF2α signalling cascade in mammals). The Atg12-Atg5 (Atg16) complex facilitates the lipidation of Atg8 and autophagy induction. Unknown signalling events in yeast, dependent or independent of the inhibition of the Tor kinase, promote Atg1 activation. Green arrows indicate an increase in protein levels. Question marks indicate signalling cascades that may exist but have not yet been characterized.

11. Putative Physiological Roles of Reticulophagy

Cells subjected to ER stress contain massively expanded ER with increased total length, distance between the lipid bilayers limiting the cisternae and membrane continuity [45]. These morphological changes are not likely caused by the accumulation of unfolded proteins but rather serve as an adaptive response in order to efficiently buffer the ER stress. This might serve to reduce the concentration of unfolded proteins by increasing the space dedicated to protein folding. This idea is supported by the observation that either yeast expressing the constitutively active Hac1, or mammalian cells with the ectopic expression of its metazoan orthologue Xbp1, two proteins capable of inducing a UPR in the absence of unfolded proteins, exhibit an expanded ER [45, 60]. In addition, mammalian cells in which autophagy has been inhibited or genetically ablated display an extended ER upon stress [43]. Conversely, yeast cells accumulating ER-containing autophagosomes do not contain expanded ER [45]. Together, these observations suggest that autophagy could be important to maintain ER homeostasis during UPR by segregating and/or degrading part of the ER. Thus reticulophagy, through the selective turnover of aggregate-containing and/or damaged ER fragments, could operate in parallel to the ERAD system. This may provide an additional mechanism to dispose unfolded proteins and a way to eliminate damaged membranes. This putative role has been evidenced in yeast expressing pathological mutant versions of human proteins such as the fibrinogen Aguadilla mutation and α_1-ATZ, which accumulate as unfolded aggregates in the ER [34–36]. Knockout strains lacking *ATG* genes expressing these pathological proteins more rapidly amass large amounts of protein aggregates compared to wild-type cells. This suggests that autophagy is important during conditions where the ERAD system is overwhelmed [34, 35]. A similar phenotype was observed in mouse cells lacking Atg5 and expressing expanded polyglutamine repeats [44, 61]. These proteins form cytoplasmic aggregates that trigger ER stress, possibly by impairing ERAD and thus causing an accumulation of unfolded proteins in the ER. Therefore, basal autophagy could serve a similar protective role by preventing the accumulation of misfolded proteins in nonstressed cells. This idea is supported by the observation that an autophagy block caused by the deletion of *ATG6* also induces a UPR in non-stressed cells [35]. The direct

implication of autophagy as an ER housekeeping pathway, however, needs to be analyzed in more detail as Atg6 is also required for endosomal trafficking [62, 63].

Paradoxically, autophagy displays a double role in cell viability. It is able to increase the lifespan by protecting against cellular damage; however, in specific pathological situations or when cells have undergone irreversible stress or injuries, autophagy can also contribute to cell death [64]. How reticulophagy contributes to cell fate is not clear and current available data are in part contradictory. Ogata and coworkers concluded that autophagy has a protective role against ER stress-induced cell death as autophagy-deficient cells show higher vulnerability to ER stress and conversely, pretreatment with rapamycin makes cells more resistant to this damage [65]. In contrast, Ding and collaborators proposed a dual role for autophagy according to the status of the cells; autophagy promotes cell survival in cancer cells displaying ER stress, and induces cell death in nononcogenic cells [43]. In yeast, an intact autophagy machinery is essential for cell growth under strong UPR-inducing conditions [45]. Interestingly, it has been proposed that the engulfment of the ER by autophagosomes, without the degradation of the sequestered cargo, is sufficient for autophagy to mitigate ER stress [45]. This hypothesis has been underscored by the finding that in the presence of high concentrations of tunicamycin, an inhibitor of protein glycosylation, Atg proteins are necessary for cell survival while vacuolar proteases are dispensable [45]. Under the same circumstances, ER-containing autophagosomes do not fuse with vacuoles when ER stress is maintained for longer periods [45]. In contrast, when ER stress is initiated by glucose depletion, ER fragments are transported to the lumen of the vacuole indicating that a complete autophagy process occurs [46]. Additional studies are necessary to understand the exact contribution of autophagy as an ER stress response mechanism. A possible scenario is that reticulophagy could have been adapted to differentially modulate its response according to the nature of the stress, and the status of the cell and/or the tissue.

12. Conclusions

Despite their potential relevance in physiological and pathological contexts, the regulation and mechanisms of ribophagy and reticulophagy remain largely unknown. It remains to be determined which of the known or if novel Atg proteins mediate the recognition and selective sequestration of ribosomes and ER fragments into autophagosomes. Moreover, how the cell regulates the segregation of the unwanted parts of the ER and how this breaks away from the organelle need to be further analyzed. A vast field is waiting to be explored.

Acknowledgments

The authors thank Jason Mercer for critical reading of the paper; René Scriwanek for assistance with the preparation of the figures. F. Reggiori is supported by the Netherlands Organization for Health Research and Development (ZonMW-VIDI-917.76.329), by the Netherlands Organization for Scientific Research (Chemical Sciences, ECHO Grant 700.59.003, and Earth and Life Sciences, Open Program Grant 821.02.017). C. Kraft is supported by a WWTF (Wiener-, Wissenschafts-, Forschungs- und Technologiefonds) "Vienna Research Groups for Young Investigators" Grant. E. Cebollero is supported by an Earth and Life Sciences (ALW-817.02.023) open program grant given to F. Reggiori and Bernd Helms (Department of Biochemistry and Cell Biology, Utrecht University).

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 389562, 6 pages
doi:10.1155/2012/389562

Review Article

Three-Axis Model for Atg Recruitment in Autophagy against *Salmonella*

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Received 14 November 2011; Accepted 22 December 2011

Academic Editor: Fulvio Reggiori

Salmonella enterica serovar Typhimurium enter epithelial cells and take up residence there. Within epithelial cells, a portion of the bacteria are surrounded by an autophagosome-like double-membrane structure, and they are still residing within the *Salmonella*-containing vacuole (SCV). In this paper, we will discuss how the autophagy machinery is recruited in proximity to *Salmonella*. The formation of this double membrane requires Atg9L1 and FIP200; these proteins are important for autophagy-specific recruitment of the PI3-kinase complex. In the absence of Atg9L1, FIP200, and PI3-kinase activity, LC3 is still recruited to the vicinity of *Salmonella*. We propose a novel model in which the mechanism of LC3 recruitment is separate from the generation of the isolation membrane. There exist at least three axes in Atg recruitment: ULK1 complex, Atg9L1, and Atg16L complex.

1. Introduction

Autophagy is primarily a process that delivers cytoplasmic component to lysosomes for degradation. In its original definition, autophagy was conceptually paired with phagocytosis; the former is also termed "autophagocytosis" and the latter "heterophagocytosis," although these terms are less frequently used in modern parlance. The two processes can be distinguished according to what the cell "eats," namely, itself in the case of autophagy or foreign bodies such as bacteria in the case of phagocytosis. We now know, however, that these processes are not entirely distinct from one another. The molecular apparatus identified during early studies in autophagy turns out also to be involved in the processes associated with the infecting bacteria from the cells of mammals and other organisms such as insects. For example, once *Salmonella enterica* serovar Typhimurium invades nonphagocytic cells such as epithelial cells, a subpopulation of the bacteria becomes decorated with autophagic marker proteins. Many studies have been performed on these phenomena, which some have termed "xenophagy" [1–3]. In this paper, we will discuss the mechanism by which the autophagic machinery is recruited, focusing especially on the case of *Salmonella*.

Salmonella enterica serovar Typhimurium, a gram-negative bacterium, infects small intestinal epithelial cells and develops as an intracellular bacterium within this niche, where it causes gastroenteritis [4, 5]. These events allow this bacterium to cause widespread infection. Therefore, in order to better control *Salmonella* infection, it is important to understand the mechanisms by which *Salmonella* develops into an intracellular bacterium in host cells. *Salmonella* possesses a type III secretion system (TTSS), which employs a needle-like structure to inject effector proteins into the host cell's cytosol [4, 6]. By injecting a number of effector molecules into the host cytosol, *Salmonella* can invade epithelial cells via a type of endocytic pathway. Following invasion, the bacteria form a specialized single membrane organelle, the Salmonella-containing vacuole (SCV), by modifying endosomal structures. In the early phase, the SCV temporarily displays early endosome markers, such as Rab5, and EEA1; subsequently, these makers are replaced by late endosomal proteins, such as LAMP1 [7–9]. SCV succsessively develops into a variety of long tubular structure,

termed spacious vacuole-associated tubules (SVATs), SNX3 tubules, and the *Salmonella*-induced filaments (Sifs), after a few hours following infection; thereafter, the bacteria can slowly propagate in SCV and the related structures [7–10]. Meanwhile, a subpopulation of *Salmonella* is targeted for xenophagy, beginning with the recruitment of autophagic machinery to the vicinity of the infecting bacteria. In an experimental system using mouse embryonic fibroblasts, 40% of infected *Salmonella* is decorated by autophagic marker proteins one hour after infection [11, 12]. In the absence of autophagic capacity in host cells, the *Salmonella* replicates more extensively [11, 12]. Xenophagy may serve as a backup system to limit the growth of infections in situations in which the SCV is somehow malformed. It was suggested that SCV is damaged by the action of TTSS, resulting in the induction of autophagy toward it [11].

2. Role of LC3 in *Salmonella* Xenophagy

The first identified specific marker of the mammalian autophagosome, microtubule-binding protein light chain 3 (LC3) is localized on the autophagosome, or its immediate precursor structure, the isolation membrane. LC3 is also distributed throughout the cytosol. Therefore, cells expressing GFP-tagged LC3 exhibit a punctate fluorescence pattern when autophagy is induced [13]. Although the exact function of LC3 in autophagy remains to be precisely understood, it has been proposed to play a role in selective autophagy. In contrast to nonselective autophagy, which targets general cytosolic materials, the targets of selective autophagy range from organelles such as mitochondria and peroxisomes to large protein complexes. It is generally understood that each target possesses a specific tag, such as Atg32 for yeast mitochondria [14, 15]. Adaptor proteins such as p62/SQSTM, Alfy and Nbr1 recognize the target-specific tags [16–19]. Because it can bind both p62/SQSTM and Nbr1, LC3 has been proposed to be involved in the recruitment of autophagic machinery at the target, [16–20]. However, recent studies of xenophagy against *Salmonella* have led to another interpretation regarding this recruitment mechanism.

LC3 and its paralogues are orthologs of yeast Atg8 [13]; they are ubiquitin-like proteins, but are modified at the carboxyl terminus with phosphatidylethanolamine (PE) instead of proteins [21, 22]. In yeast, Atg7 and Atg3 serve in these process as E1 and E2 enzymes; in their absence, the PE modification does not occur and starvation-induced autophagy is defective [21]. This is also the case in *Salmonella* xenophagy. In Atg7- and Atg3-knockout MEF cells, GFP-LC3 is not recruited to the vicinity of *Salmonella enterica* serovar Typhimurium; as a result, the bacteria replicate overwhelmingly, leading to host cell death [12]. When mouse embryonic fibroblast cells expressing GFP-Atg5 are challenged with *Salmonella*, GFP signal can be observed around some of the intracellular bacteria, in a pattern reminiscent of LC3 [12]. In the Atg3 knockout MEF, the efficiency of GFP-Atg5 recruitment is not significantly different [12]. Therefore, even in the absence of LC3 recruitment, *Salmonella* can be recognized by the autophagic apparatus. A number of adaptor proteins, including p62/SQSTM, NDP52, and optineurin, bind to LC3 and are involved in xenophagy against a variety of bacteria [23–25]. These interactions with LC3 may be more important for functions rather than recruitment of other Atg proteins.

This notion is further strengthened from other observations. Two reports, including ours, have revealed detailed phenotypes by depleting the function of all LC3 paralogues involved in starvation-induced autophagy. One group knocked out Atg3 genes in mouse embryonic fibroblasts [26]; another exogenously expressed mutant Atg4B [27]. Atg4 is protease involved in the cleavage of the carboxyl termini of nascent Atg8/LC3 family members and their PE conjugates [22]. Overexpression of Atg4B containing a point mutation at its catalytic center aminoacid, cysteine, titrates out LC3 and its homologues by binding them strongly and preventing the PE conjugation reaction [28]. Both approaches yielded essentially the same results: accumulation of incomplete and unsealed autophagosomes in the cytosol [26, 28]. In these experiments, significant proportions of the autophagosome membranes were mostly, but not completely, closed [26, 28]. Yeast Atg8 can catalyze hemifusion of the vesicles with which it associates *in vitro* [29]. Based on these results, we proposed a "reverse-fusion" model in which LC3 functions in the closing process by directly catalyzing membrane hemifusionmembran- like process [30]. Beyond this role in closing, it remains controversial whether LC3 is involved in the elongation of the autophagosomal membrane [26, 28, 31, 32]. There is also a report that atypical autophagy, which does not require this LC3 system, exists [33]. In mouse embryonic fibroblast cells expressing GFP-LC3, a punctate GFP signal appears in the proximity of infecting *Salmonella* cells and elongates along the surface of the bacterium, just as the isolation membrane elongates to become the autophagosome [12]. Using fluorescence microscopy and electron microscopy correlation, the membrane structure corresponding to GFP-positive *Salmonella* was observed [12]; a double-membrane structure resembling the canonical autophagosome surrounded the *Salmonella* cells. Inside the double-membrane structure, another single membrane thought to be the SCV could also be observed. Therefore, at least in the case of xenophagy in this system, *Salmonella* is surrounded by autophagosome in addition to the SCV [12, 34]. In MEFs lacking Atg7, the E1 enzyme for the LC3 lipidation reaction, a double membrane surrounds the SCV, though it may not be completely closed [12]. Similar images were observed in the MEFs lacking Atg5 [23]. Atg5 is a subunit of the E3 involved in LC3 lipidation, and the mutant is defective in this process [35, 36]. These observations clearly indicated that LC3 function is required neither for membrane elongation nor for recruitment of the autophagic membrane to the target. Therefore, another LC3-independent targeting mechanism must exist.

3. Atg9-Independent Recruitment of Atg16L

The next question arising is the identity of the alterative factors that actually do recruit the autophagic machinery. Good candidates for these factors are found among other

Atg proteins that function in starvation-induced autophagic process [37]. Atg9 is a six-transmembrane protein, essential for autophagy [38, 39], whose precise role remains to be determined. Mammalian cells have two Atg9 homologues, Atg9L1 and Atg9L2, but the latter is expressed only in placenta and pituitary [40]. In mammalian cells, Atg9 travels around the Golgi and endosome and potentially the autophagosome [39]. Knockout of only Atg9L1 brings about severe defects not only in canonical autophagy but also in *Salmonella* xenophagy, evidenced by observations that *Salmonella* replication dramatically increases within infected Atg9L1- knock out cells, just as in Atg7-knock out cells [12, 41]. Even in Atg9L1-knockout MEF cells, GFP-LC3 is efficiently recruited to *Salmonella enterica* serovar Typhimurium at levels comparable to those observed in wild-type cells [12]; however, in these mutant cells, GFP-LC3-positive *Salmonella* is not surrounded by an autophagosome-like double membrane [12]. Thus, Atg9L1 is required for membrane formation in autophagy, but indispensable for LC3 recruitment. This finding was not anticipated based on results from previous studies. The Atg16L complex consists of two sets of Atg16L1 and Atg12–Atg5 conjugate, bound by a ubiquitination-like reaction [42]. Atg12 binds to Atg3, the E2 enzyme of the LC3 lipidation reaction, and the lipidation reaction occurs where Atg16L is localized [36]. Based on these observations, Atg16L complex serves an E3-like role by linking E2 to the target (PE in membrane) in the LC3 lipidation reaction [36, 43]. The Atg16L complex is exclusively localized on forming autophagosome, the isolation membrane in starvation-induced autophagy [35, 44]. In the case of *Salmonella* xenophagy, however, even in the absence of an autophagosome-like double membrane, Atg16L complex can localize to the vicinity of infecting *Salmonella* [12]. This implies that the Atg16L complex can be recruited to SCV independent of the existence of a double-membrane structure [12]. It remains to be determined whether the same mechanism is also applicable to the wild-type cell, but it is highly likely that some targeting mechanism exists that is independent of both the double membrane and LC3.

4. ULK1 Complex Functions in Parallel to Atg9L1

Ulk1 is a mammalian orthologue of yeast Atg1 protein kinase, which is essential for autophagy [45, 46]. Ulk1 forms a protein complex with FIP200, Atg13, and Atg101 [47]. In MEF cells lacking FIP200, *Salmonella* xenophagy is defective, as is starvation-induced autophagy [12]. In the FIP200 knockout, phenotypes pertaining to GFP-LC3 localization and autophagosome-like double-membrane generation were quite similar to those of Atg9L1-knockout cells: GFP-LC3 is efficiently recruited around *Salmonella enterica* serovar Typhimurium, and the double membrane is not observed [12]. One plausible explanation for this result is that one of the proteins is responsible for the recruitment of the other to the vicinity of *Salmonella*, but this is not the case. In FIP200 knock out cells, Atg9 is recruited to *Salmonella*, whereas in Atg9L-knock out cells, Ulk1 is recruited [12]. Thus, localizations of the two proteins are independent of each other. On the contrary, Atg9L1 accumulates to a greater extent near *Salmonella* in FIP200-knock out cells; likewise, Ulk1 accumulates in Atg9L1-knock out cells [12]. Ulk1 complex and Atg9L1 are potentially recycled between the vicinity of *Salmonella* and other cytosolic pools; detachment of either protein from *Salmonella* appears to be dependent on the other. These two players seem to play quite important roles in membrane biogenesis, and it is likely that their functions are tightly coupled with their recycling. There are both similarities and differences between these models and what has been observed in yeast autophagy. In yeast, Atg1 (Ulk1 homologue) is also required for recycling of Atg9 from the PAS, the site of autophagosome formation, to other pools [48]. However, targeting of Atg9 to the PAS is dependent on Atg17, a potential counterpart of FIP200, through direct binding [49]. This FIP200-independent Atg9l1 localization may be explained by the fact that a part of Atg9L is transiting early endosome, which is closely associated with SCV at steady state, even in the absence *Salmonella* infection [12].

5. PI3P Involvements in *Salmonella* Xenophagy

PI3P plays critical roles in canonical starvation-induced autophagy [50].When cells are treated with wortmannin, a potent inhibitor of PI3-kinase, LC3 localization to autophagosome is completely defective [13]. In the case of *Salmonella* xenophagy, however, wortmannin treatment does not affect LC3 targeting to the vicinity of *Salmonella enterica* serovar Typhimurium [12], although another study showed some reduction in the efficiency [51]. This does not necessarily mean, however, that PI3P is indispensable for *Salmonella* xenophagy. For starvation-induced autophagy, there exists a specific PI3-kinase protein complex, consisting of Vps34, Vps15, Beclin-1, and Atg14L [52–54]. The knockdown of Atg14L, the sole complex-specific subunit, leads to *Salmonella* overgrowth in infected cells [53]. The localization of Atg14L is also observed in proximity to infected *Salmonella* [12]. WIPI-1, a PI3P-binding protein involved in autophagy, is also observed there, and this localization is sensitive to wortmannin treatment [12]. Thus, similar to the case of Atg9L1 and Ulk1 complexes, autophagy-specific PI3-kinase activity is involved in *Salmonella* xenophagy, but is dispensable for LC3 targeting [12]. This is easily understandable in light of the fact that localization of Atg14L to *Salmonella* becomes defective in cells lacking either Atg9L1 or FIP200 [12]. This implies that both Atg9L1 and Ulk1 complexes are upstream determinants of autophagy-specific PI3-kinase localization. In the case of starvation-induced autophagy, autophagy-specific PI3-kinase is targeted to the endoplasmic reticulum, where it forms foci (the "omegasome") in order to form the autophagosome [54]. This omegasome is marked by DFCP-1 through its PI3p-binding capacity, whose function in autophagy is still unclear [55]. DFCP-1 is closely associated with *Salmonella* xenophagy, so this may take place in close proximity to the ER [51] (see Figure 1).

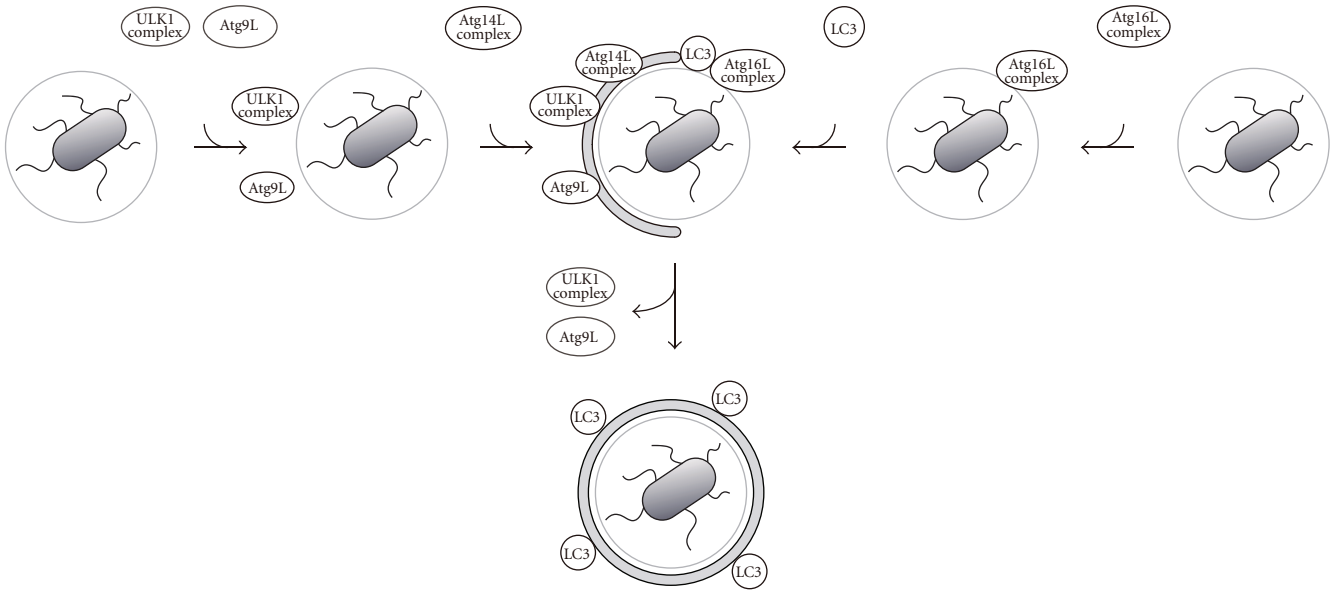

Figure 1: Schematic representation of the three-axis model for Atg recruitment in *Salmonella* xenophagy *Salmonella enterica* serovar Typhimurium is inside the SCV, but some bacterial cells are captured by the autophagic machinery. LC3 is recruited by Atg16L complex, but this recruitment is not dependent on the other factors depicted here. Even in the absence of these factors, an autophagosome-related membrane is observed. Ulk1 complex (including FIP200) and Atg9L1 recycle between the vicinity of *Salmonella* and the other cellular pools. Both are recruited to *Salmonella* independent of one another, but their detachment from *Salmonella* proximity is interdependent. Atg14L-containing PI3-kinase complex recruitment is dependent on both Ulk1 complex and Atg9L1.

6. Conclusion

It is now clear that there exist at least three independent axes for the recruitment of autophagic machinery to the vicinity of *Salmonella enterica* serovar Typhimuriuma*:* Atg16L complex, Atg9L1, and Ulk1 complex. In the case of yeast autophagy, Atg17, a subunit of Atg1 complex, is proposed to be a fundamental determinant of the recruitment of other Atg proteins to the PAS [56]. In the case of mammalian starvation-induced autophagy, a similar role has been proposed for FIP200 [57]. However, both cases of starvation inducue autophagy lack the existence corresponding to SCV, which can become an alternative membrane target of Atg16L complex (i.e., instead of the autophagosome). Therefore, the possibility that an Ulk1 complex-independent Atg16L recruitment mechanism is also involved in starvation-induced autophagy cannot be eliminated.

In that case, what factors exist upstream of Atg16L and Ulk1 complexes? Involvement of adaptor proteins is highly likely, although their direct binding to LC3 is indispensable. Ubiquitin and several adaptor proteins are recruited to the vicinity of *Salmonella*, so they must play critical roles [23–25]. It is possible that these adaptor proteins also bind other Atg proteins, such as Ulk1 and Atg16L complexes. In this regard, it is noteworthy that Tecpr1, a novel adaptor protein involved in xenophagy, binds to Atg5 [58]. Combining with other important players such as diacylglycerol [59], understanding the direct trigger for *Salmonella* xenophagy represents the next important step for this field.

Abbreviations

MEF:	Mouse embryonic fibroblast
S. Typhimurium:	Salmonella enterica serovar Typhimurium
PI3K:	Phosphatidylinositol 3-kinase
ULK:	Uncoordinated 51-like kinase
FIP200:	FAK family: interacting protein of 200 kD
WIPI:	WD-repeat protein interacting with phosphoinositides
PAS:	Preautophagosomal structure
SCV:	Salmonella-containing vacuole.

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Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 142634, 8 pages
doi:10.1155/2012/142634

Review Article

The Cytoplasm-to-Vacuole Targeting Pathway: A Historical Perspective

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Received 12 October 2011; Accepted 8 December 2011

Academic Editor: Fulvio Reggiori

From today's perspective, it is obvious that macroautophagy (hereafter autophagy) is an important pathway that is connected to a range of developmental and physiological processes. This viewpoint, however, is relatively recent, coinciding with the molecular identification of autophagy-related (Atg) components that function as the protein machinery that drives the dynamic membrane events of autophagy. It may be difficult, especially for scientists new to this area of research, to appreciate that the field of autophagy long existed as a "backwater" topic that attracted little interest or attention. Paralleling the development of the autophagy field was the identification and analysis of the cytoplasm-to-vacuole targeting (Cvt) pathway, the only characterized biosynthetic route that utilizes the Atg proteins. Here, we relate some of the initial history, including some never-before-revealed facts, of the analysis of the Cvt pathway and the convergence of those studies with autophagy.

1. The Background

To understand the origin of the studies that led to the identification of the Cvt pathway, we need to briefly step back into the early days of yeast molecular genetics. Randy Schekman's group was studying the secretory pathway and isolating mutants defective in various steps including endoplasmic reticulum (ER)-to-Golgi transport as well as secretion to the cell surface. Two former postdocs from the Schekman lab, Scott Emr and Tom Stevens, decided to pursue a similar direction, but to avoid a direct overlap with Randy Schekman by focusing on a pathway that branches off from the secretory pathway, the delivery of proteins to the vacuole. The Emr and Stevens labs isolated a new set of mutants initially named *vpt* (vacuolar protein targeting) [1] and *vpl* (vacuolar protein localization) [2], and subsequently *vps* (vacuolar protein sorting), which are defective in the delivery of resident proteins to the vacuole. Being interested in protein sorting, one of us (D.J.K.) went to Scott Emr's lab to learn about yeast.

While in the Emr lab, I characterized the vacuolar delivery of proteinase A (Pep4) and vacuolar alkaline phosphatase (Pho8). Around that time, the sequence of the gene encoding another vacuolar hydrolase, aminopeptidase I (Ape1) was published [3, 4]. It is important to keep in mind that this was the late 1980s, quite some time before the *Saccharomyces cerevisiae* genome was sequenced in its entirety. In fact, automated sequencing was relatively new, so it was still a major accomplishment when a gene was sequenced. Until then, only the sequences of Pep4 [5, 6], Prc1 (carboxypeptidase Y) [7], Pho8 [8], Prb1 (proteinase B) [9], and Ams1 (α-mannosidase) [10] were known among the vacuolar hydrolases. Thus, it was quite exciting to those of us studying vacuolar protein targeting when a new protein sequence became available. One of my main goals in the Emr lab was to identify the vacuolar-targeting motif and determine a consensus sequence (mapping consensus targeting or retention signals was very popular in those days), a task that was all the more difficult due to the limited number of proteins available for comparison. Hence, I was particularly interested in having a new protein that I could analyze.

Ape1 was known to be a vacuolar hydrolase, and it was characterized as being a glycoprotein [11]. The latter finding fit with the fact that all of the characterized vacuolar hydrolases traffic through the secretory pathway to the Golgi complex and from there are diverted to the vacuole. One

interesting feature of the protein sequence for the precursor form of Ape1 (prApe1), however, was that it lacked a standard signal sequence. Accordingly, I assumed that it entered the ER by a unique mechanism. This seemed to add some additional interest to the analysis, as the idea of analyzing yet one more vacuolar hydrolase was getting somewhat tedious. When I discussed the idea of analyzing the targeting of prApe1 with Scott Emr, however, he was not interested. After all, even if the details of the process were slightly unusual, we were still talking about the characterization of another vacuolar hydrolase that transits through a portion of the secretory pathway. Indeed, at the time, there seemed to be more interesting projects to pursue, so the analysis of prApe1 was left on the "back burner".

Shortly after that time, I started an independent position at the University of California, Davis. To stay clear of the Emr lab (which, for a new assistant professor, loomed like an 800-pound gorilla), I pursued an analysis of the vacuolar H^+-translocating ATPase and vacuolar acid trehalase. At that time, Scott forwarded to me a letter (this was just before email became widely used) from a postdoc applicant that he was not able to invite to his lab. That postdoc, Nieves Garcia Alvarez, was from one of the labs, that of Paz Suarez-Rendueles, which was involved in characterizing yeast vacuolar hydrolases, and I agreed to offer her a position. Nieves initially worked on the vacuolar ATPase project. I knew, however, that her lab in Spain was one of two that had essentially simultaneously sequenced the *APE1/LAP4* gene encoding prApe1 [4]. During Nieves' time in my lab, I wrote to Beth Jones who had published one paper on Ape1 [12] and asked if she intended to pursue this topic; I did not want to compete with her, but she indicated that she was not going to be working on it, and I was welcome to it. Thus, I obtained the gene from the Suarez-Rendueles lab and a new postdoc from that lab, Rosaria Cueva Noval, along with my postdoc Debbie Yaver and me, began to examine the vacuolar targeting of prApe1.

The initial experiments on prApe1 were confusing, because I could not find any evidence for glycosylation or for the existence of the protein within the compartments of the secretory pathway [13]. (As a side note, our first paper on Ape1 was published back-to-back with the first paper from Yoshinori Ohsumi's lab on the characterization of autophagy in yeast [14]. This was coincidental, and, to be honest, I paid no attention to the Ohsumi paper at that time, because it was on the topic of autophagy; I was studying protein targeting, not some presumed "garbage" pathway that was only used for protein degradation.) Eventually, it dawned on me that the published data were incorrect and that Ape1 was not a glycoprotein. At this time, Fred Dice was making headlines with his analysis of the KFERQ—(KFERQ being the consensus sequence for the recognized substrates) or pentapeptide-dependent pathway for the transport of proteins into the lysosome (the current name for this pathway, "chaperone-mediated autophagy," had not been coined yet) [15]. Considering that Ape1 was not a glycoprotein, and that it did not enter the endoplasmic reticulum, I reasoned that it entered the vacuole by translocating directly across the limiting membrane. Accordingly, I further assumed that there must be protein machinery, similar to the as yet uncharacterized components involved in the KFERQ pathway, in the vacuolar membrane just waiting for me to come along and identify them.

Therefore, in order to identify the vacuolar membrane translocation components, we generated a chimera of prApe1 fused to the *HIS3* gene. Our initial screen was based on the idea that a *his3* mutant strain of yeast would not be able to grow in the absence of histidine if the chimera was efficiently delivered to the vacuole. Accordingly, we could isolate mutants that were able to grow without histidine, and they would have defects in the various components of the translocation machinery. It became clear early on that the screen was not working, although we did not know why; we could not easily follow the localization of the chimera because the green fluorescent protein was not yet being used for cell biology studies. Randy Schekman was giving a seminar on campus at that time, and I told him about our project. He suggested that we generate antibodies that only recognized prApe1 and carry out a screen looking for mutants that accumulate the precursor form of the protein. We did attempt that approach, using colony blots after transferring cells to nitrocellulose, but it was very difficult to score positive colonies. However, we also noticed that wild-type cells analyzed by western blot, when grown appropriately, had essentially no prApe1; all of the protein was in the mature form. We also determined (using a *pep*4Δ mutant as the control) that we could easily detect the precursor that accumulated when one out of ten colonies was defective for prApe1 maturation. Accordingly, even though it was laborious, Tanya Harding, and later Ann Hefner-Gravink, in my lab began to analyze random mutants in batches of ten for the accumulation of prApe1.

We isolated a series of such mutants and placed them into complementation groups [16]. This was quite exciting as we were finally about to identify the long-awaited translocation machinery for the vacuole. To be sure that we were not going to waste our time analyzing mutants that were already known, we began to compare our mutants with all other previously identified mutants that affected vacuolar protein delivery. Of course this included the *vps* mutants from Tom Stevens and Scott Emr, but also endocytosis mutants and vacuolar morphology (*vam*) mutants. Even though we did not expect overlaps from the latter, we wanted to be thorough. In fact, we were so careful that we even requested protein extracts from Yoshinori Ohsumi and Michael Thumm, who had isolated *apg* [17] and *aut* [18] mutants, respectively, that are defective in autophagy. Obviously (or so we thought at the time), there was not going to be an overlap; autophagy is a degradative pathway, and our mutants (then named *cvt*) were defective in a biosynthetic pathway. Imagine our surprise, and disappointment, when we found an essentially complete overlap among these three sets of genes [19, 20]. The disappointment was for two reasons. First, instead of having a unique set of mutants that we could study on our own, we knew we immediately had competitors. Second, we were being dragged against our will into the field of autophagy.

Nonetheless, we continued with our studies of prApe1 targeting and began to clone the *CVT/APG/AUT* genes and

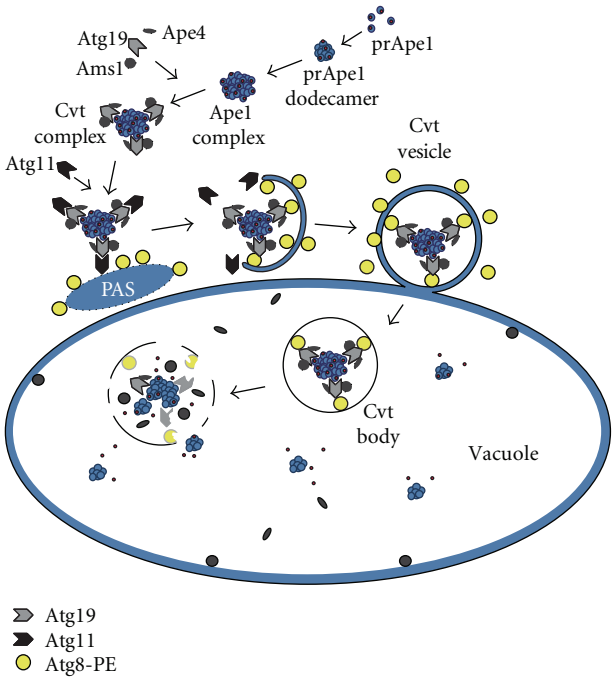

Figure 1: Overview of the Cvt pathway. (1) Formation of the Cvt complex: Precursor Ape1 forms a dodecamer. Multiple dodecamers assemble into an Ape1 complex. The Ape1 complex binds Atg19 via the prApe1 propeptide to form the Cvt complex. Other Cvt cargo, including Ams1 and Ape4, bind Atg19 at distinct domains. (2) Movement to the PAS: Atg19 binds the scaffold protein Atg11, and the Cvt complex moves to the PAS. (3) Formation of the Cvt vesicle: Atg19 binds Atg8–PE, which drives the sequestration of the Cvt complex by the double-membrane phagophore. (4) Fusion of the Cvt vesicle with the vacuole: After completion of the Cvt vesicle, the outer membrane fuses with the vacuole, releasing the single membrane Cvt body into the lumen. The Cvt body is broken down by the Atg15 lipase, allowing access to vacuolar hydrolases. Atg19 and Atg8 are degraded. The propeptide of prApe1 is removed and the enzyme becomes active.

analyze the gene products. After discovering the overlap with the *APG* genes, we sent purified antisera against Ape1 to the Ohsumi lab to be used in an electron microscopy analysis by Misuzu Baba. I can still remember Yoshinori Ohsumi cryptically telling me about some striking and exciting results that "could not be described" over the phone, but that had to be seen in person. This resulted in a visit to Japan, and the viewing of images that were indeed striking, revealing that prApe1 import was morphologically similar to autophagy (Figure 1) [21]. Much of the initial work on the characterization of the Atg proteins was done in collaboration with the Ohsumi lab [20, 22–27] and also with the lab of Bill Dunn [22, 28–32], who was studying peroxisome degradation in *Pichia pastoris*. Having established the historical perspective, we now present some of the details of those initial studies of the Cvt pathway, starting with the characterization of aminopeptidase I import by a mechanism that is independent of the secretory pathway, identification of the vacuolar targeting domain, the isolation of mutants defective in prApe1 delivery to the vacuole, and concluding with the genetic and morphological studies that revealed the overlap with autophagy.

2. The Transport of prApe1 to the Vacuole Is Mediated by the Cvt Pathway

Ape1 was initially characterized as a vacuolar enzyme that hydrolyzes leucine peptides (hence the original nomenclature leucine aminopeptidase, or LAP, which is unfortunately confusing because LAPI is encoded by the *LAP4* gene, whereas LAPIV is encoded by *LAP2*, etc.) [33]. The hydrolase is synthesized as an inactive zymogen containing a propeptide that may sterically block its active site; it is processed to its mature form in the vacuole by proteinase B in a *PEP4*-dependent manner [34]. As mentioned above, published data suggested that the Ape1 precursor was transported through part of the secretory pathway, because it was characterized as a glycoprotein [11]. However, a detailed characterization of prApe1 biosynthesis suggested that its delivery to the vacuole was independent of the secretory pathway: (1) prApe1 lacks a signal sequence for transport into the ER, and it is not glycosylated; (2) the half-life of processing (i.e., removal of the propeptide in the vacuole) of prApe1 is substantially longer (~30 min) than that of Prc1 or Pep4 (~6 min), both of which are transported to the vacuole via part of the secretory pathway; (3) vacuolar import of prApe1 is relatively unaffected by *sec* mutants [13].

The obvious question then became, how does prApe1 target to and enter the vacuole? A series of biochemical analyses were performed to address this issue. After it is synthesized as a 61-kDa protein in the cytosol, prApe1 is proteolytically processed to a mature 50-kDa form in the vacuole. The prApe1 propeptide plays an essential role in the transport process [35]. A detailed mutagenesis analysis carried out by Mike Oda revealed that the first amphipathic α-helix in the propeptide is critical for the vacuolar targeting

of the enzyme. Deletion of the precursor region or mutations that affect the first α-helical region inhibit its binding to the membrane fraction and prevent subsequent vacuolar delivery and processing. Further analysis by John Kim revealed that prApe1 is assembled as a dodecamer (~669 kDa) in the cytoplasm prior to vacuolar delivery, which argued against direct translocation across the vacuole limiting membrane [36]. The propeptide of prApe1 is not required for its oligomerization. A pulse chase analysis showed that the oligomeric assembly and the subsequent membrane association are very rapid events with a half-life of ~3 min. These results suggested that the long half-life of prApe1 transport may be due to the rate limiting step of the import of the dodecameric enzyme into the vacuole lumen after its binding to membrane.

3. The Cvt and Autophagy Pathways Share the Same Machinery

The oligomerization of prApe1 and the slow kinetics of import into the vacuole argued against transport through the secretory pathway. To understand the mechanism of vacuolar delivery, a detailed biochemical and genetic analysis was carried out in *S. cerevisiae*, which revealed that autophagy and the Cvt pathway largely share the same machinery for double-membrane vesicle formation [16, 19, 20, 27]. A genetic screen to analyze the Cvt pathway was carried out by monitoring the accumulation of prApe1 as described in Section 1. From the initial screen, five *cvt* mutants (*cvt2/atg7, cvt3, cvt5/atg8, cvt6 and cvt7/atg9*) were isolated, which showed a complete block in prApe1 processing, but were not defective in the maturation of the precursor form of Prc1 or Pep4 [16]. Most of these mutants also showed a defect in nonselective autophagy [19, 20]. Just prior to the isolation of the *cvt* mutants, Michael Thumm in Dieter Wolf's lab isolated a series of *aut* mutants, based on defects in the degradation of the fatty acid synthase. The *aut* mutants including *aut3* (*cvt10/atg1*), *aut5* (*cvt17/atg15*), *aut7* (*cvt5/atg8)*, and *aut9* (*cvt7/atg9*) also displayed a significant block in the maturation of prApe1, providing genetic evidence for a role of these proteins in both the Cvt pathway and autophagy [19]. A similar analysis of the *apg* mutants from Yoshinori Ohsumi's lab also revealed an extensive overlap [20]. Subsequently, all of the *ATG* genes, except *ATG11*, *ATG17*, *ATG19*, *ATG22*, *ATG29*, and *ATG31* were found to be required for both pathways. In 2003, the nomenclature for these *CVT* and *APG/AUT* genes was unified as "*ATG*" for "autophagy related" [37].

4. Precursor Aminopeptidase I Is Imported by a Vesicular Mechanism

The genetic overlap between the *cvt* and *apg/aut* mutants gave rise to the idea of a vesicle-mediated mechanism for prApe1 import. Indeed, electron microscopy analyses performed by Misuzu Baba revealed that the prApe1 dodecamers further assembled into a large complex composed of multiple dodecamers (called an Ape1 complex), and that in the cytoplasm this complex is surrounded by a double membrane-bound structure, followed by fusion with the vacuolar membrane [21], similar to what was observed in bulk autophagy [38]. This result demonstrated the use of an autophagy-like mechanism for the Cvt pathway. However, the double membrane structure enwrapping the Ape1 complex (termed a Cvt vesicle) is ~150-nm in diameter, in contrast with that of the autophagosome, which is 300–900 nm. In addition, the Cvt vesicle, in contrast to the autophagosome, excludes bulk cytoplasm. Furthermore, while autophagy is induced under starvation conditions, the Cvt pathway occurs constitutively in growing conditions. Finally, as we mentioned above, the Cvt pathway is a selective, biosynthetic pathway, whereas autophagy is generally nonselective and is degradative. How then could we explain the apparent overlap in the import machinery? Importantly, when cells are subjected to starvation, the Cvt complex is sequestered within a larger autophagosome [38], although the kinetics for import are essentially the same as during vegetative growth. Thus, while the biosynthetic Cvt pathway can be distinguished from autophagy, the Ape1 complex can be taken up by autophagosomes under starvation conditions, again suggesting that the Cvt pathway and autophagy utilize much of the same machinery.

In *S. cerevisiae*, the biogenesis and the vacuolar transport of both autophagosomes and Cvt vesicles include the following steps: (1) membrane from various sources generates vesicles containing Atg9 (see below) as a critical integral membrane protein, and these vesicles form into tubulovesicular clusters in a SNARE-dependent manner; (2) one or more clusters contribute to the formation of a perivacuolar phagophore assembly site (PAS), which is considered to be a foundation/nucleation site that (3) leads to formation of the phagophore, the initial sequestering compartment; (4) two ubiquitin-like protein conjugation systems including Atg8 and its conjugation to phosphatidylethanolamine (PE) contribute to the formation and elongation of the phagophore to generate the double-membrane Cvt vesicle and autophagosome; (5) the completed vesicles dock and fuse with the vacuole, releasing the inner vesicle into the lumen where the single-membrane structures are referred to as Cvt or autophagic bodies.

Both autophagosomes and Cvt vesicles are said to be formed *de novo*, to emphasize the fact that their generation occurs by a mechanism that is distinct from that used in the budding of transient transport vesicles in the secretory pathway. Although the details of sequestering vesicle biogenesis are still not clear, almost all of the Atg proteins are localized at least transiently to the PAS [39]. Atg9, which is the sole integral membrane protein in yeast that is essential for Cvt vesicle and autophagosome formation, is relatively unique in that it is localized at multiple sites including the PAS. The population of Atg9 at the non-PAS sites (Atg9 reservoirs) corresponds to the tubulovesicular clusters and is proposed to traffic between these sites and the PAS, providing membrane for phagophore expansion. The function of most of the Atg proteins is still not known. For example, Atg8–PE participates in cargo recognition during selective types of autophagy and is also involved in determining the size of the

autophagosome [40], but the details of these mechanisms are not known.

5. Discovery of the Cvt-Specific Genes

As mentioned above, not all of the *cvt* and *apg/aut* mutants displayed an overlap; some mutants were defective only in autophagy or the Cvt pathway, but not both. For example, the *atg11* mutant shows a complete block in the maturation of prApe1, but is essentially normal for autophagy [19, 23]. These results suggested that the Cvt pathway and autophagy share most of the same machinery, but that they also need some molecules that are specific for each pathway. One of the fundamental differences between the Cvt pathway and autophagy concerns their temporal and physiological activity. The Cvt pathway is active during vegetative growth, consistent with its role as a biosynthetic trafficking route. In contrast, autophagy is induced under starvation conditions, where it can break down cellular macromolecules to supply building blocks and energy. A complex of proteins including Atg13, which is required both for the Cvt pathway and autophagy, appears to be partly responsible for switching these pathways in response to changes in the environment. In starvation conditions, Atg13 interacts with the Atg1 complex including Atg17, Atg29, and Atg31 to induce autophagy [22, 41–43]. Under vegetative conditions, Atg13 may have a lower affinity for Atg1, a condition that may promote the Cvt pathway. Atg13 is regulated by its phosphorylation status in a TORC1-dependent manner; Atg13 is highly phosphorylated in growing conditions but dephosphorylated in starvation conditions [41, 44].

Another characteristic of the Cvt pathway is the specificity for its cargo, whereas macroautophagy is a nonselective process, suggesting that the Cvt pathway requires a receptor, which recognizes the substrate. In this case, the substrate corresponds to the cargo of the Cvt vesicles, which is comprised primarily of the Ape1 complex. A systematic yeast two-hybrid screen in *S. cerevisiae* was performed and the gene product of *YOL082W* was found as a potential interacting protein with prApe1 [45]. Biochemical analysis demonstrated that *YOL082W* encodes a protein that functions as a receptor for the targeting of prApe1 by the Cvt pathway, and the gene was renamed *CVT19* [46, 47] and later *ATG19* [37]. In *atg19*Δ cells, the precursor form of Ape1 accumulates in the cytoplasm in both nutrient rich and starvation conditions, suggesting that Atg19 is necessary for the targeting of prApe1 both by the Cvt pathway and autophagy. An important point in this regard is that import of prApe1 by autophagy is still a selective process that utilizes a receptor protein; this explains why the kinetics of import are the same as for the Cvt pathway and are much faster than would be expected for bulk uptake of cytoplasm.

An immunoprecipitation analysis showed that Atg19 physically interacts with the propeptide of prApe1, and the coiled-coil domain of Atg19 mediates this interaction [48]. Atg19 localizes at the PAS with the Ape1 complex [49]; the combination of the Ape1 complex bound to Atg19 is referred to as the Cvt complex. In *atg19*Δ cells, GFP-Ape1 forms a dodecamer, but it does not localize at the PAS. The kinetics of the maturation of prApe1 and the degradation of Atg19 are quite similar. Together with the localization data, these findings suggest that Atg19 is delivered to the vacuole by the Cvt pathway along with the precursor Ape1 dodecamer. Interestingly, deletion of *APE1* results in a dispersed Atg19 distribution, and Atg19 does not localize to the PAS in *ape1*Δ cells, suggesting that the Ape1 complex itself is required for concentrating its soluble receptor at this site. Further analyses revealed that Atg19-prApe1 movement to the PAS is dependent on Atg11, which we now know acts as an adaptor or scaffold protein for selective autophagy pathways, such as the Cvt pathway, and the selective autophagic degradation of peroxisomes and mitochondria (termed pexophagy and mitophagy, resp.) [22, 50]. Atg11 may mediate the transport of Atg9 to the PAS for selective autophagy during vegetative growth [51], whereas Atg17 may carry out this role for bulk autophagy during starvation. Atg11 has certain characteristics of a scaffold protein in that it interacts with several Atg proteins, including Atg1, Atg9, Atg17, Atg19, Atg20, and itself [51, 52].

In the Cvt pathway, Atg19 binds the prApe1 propeptide independent of any other Atg proteins. Atg11 can then interact with Atg19, allowing movement of the cargo to the PAS. Once at the PAS, Atg19 also interacts with Atg8–PE; it is not known if both Atg8 and Atg11 bind Atg19 at the same time, as their binding sites are distinct, but very close to each other. Thus, Atg19 is a receptor that is responsible for recognizing the prApe1 dodecamer to target it to the PAS due to its interaction with Atg11. Furthermore, Atg19 leads to the incorporation of the Cvt complex into a double-membrane vesicle (i.e., a Cvt vesicle or autophagosome) via its interaction with Atg8 [48]. In the absence of other Atg proteins such as Atg1, Cvt vesicles, and autophagosomes do not form; however, the Cvt complex is still targeted to the PAS, suggesting that Atg19 transport of prApe1 to the PAS occurs independent of the vesicle formation steps. Atg19 is both ubiquitinated and deubiquitinated *in vivo*, and these modifications of Atg19 are required for the efficient trafficking of prApe1 via the Cvt pathway [53]. Atg19 interacts with the deubiquitinating enzyme Ubp3, and the deletion of *UBP3* leads to decreased targeting of prApe1. Furthermore, the mutation on the ubiquitin acceptor site, Lys213 and Lys216 of Atg19, reduces the interaction of Atg19 with prApe1. Thus, the ubiquitination and deubiquitination of Atg19 are likely to play a structural or mechanistic role in the normal progression of the Cvt pathway, instead of serving as a degradation signal for the proteasome.

As described above, many of the yeast Atg proteins responsible for the Cvt pathway and autophagy have been identified, and the general mechanism involved in these processes has been explored through genetic and biochemical approaches. Nevertheless, the molecular mechanism underlying nucleation of the sequestering phagophore remains largely unknown. Many processes involving membrane rearrangement and movement, such as endocytosis or membrane ruffling, require the cytoskeleton. The actin cytoskeleton is required for the selective Cvt pathway, but not for nonselective autophagy in yeast [54]. Actin plays a role in trafficking of Atg9 to the PAS and recruitment of the Cvt

cargo in growing conditions. Further studies identified actin-related proteins, including components of the Arp2/3 complex, as playing a role in the transport of Atg9 for specific types of autophagy [55]. The Arp2 protein itself interacts with Atg9 and regulates the dynamics of Atg9 movement. Thus, the Arp2/3 complex may allow Atg9, along with its associated membrane, to move in a directed fashion to the PAS along actin cables. The specific autophagy factors such as Atg19 and Atg11, and perhaps other molecular components, may serve as adaptors between the Cvt cargo and the actin cytoskeleton.

6. Discovery of Other Cvt Cargo, Ams1 and Ape4

Prior to the analysis of the Cvt pathway, Ams1 was shown to enter the vacuole independent of the secretory pathway [56], although the mechanism of import was unclear. We found that Ams1 is another hydrolase targeted to the vacuole by the Cvt pathway [57], as its delivery is blocked in *cvt* (*atg*) mutants. Similar to prApe1, Ams1 forms oligomers composed of 4 to 6 of the 122-kDa species in the cytosol, and the oligomeric state is maintained during the import process. Ams1 transport is also mediated by Atg19 [47] and its binding site is distinct from that used by prApe1 [48]. Thus, Ams1 is part of a prApe1-Atg19-Ams1 Cvt complex. In *ape1*Δ cells, the Ams1-Atg19 interaction still occurs, but this complex is dispersed in the cytosol, whereas deletion of *AMS1* does not affect the transport of the prApe1-Atg19 complex. These results indicate that Ams1, which is synthesized at a level that is substantially lower than prApe1, might exploit the prApe1-Atg19 import system to achieve its own efficient transport to the vacuole.

Recently, it was shown that Ams1 is delivered to the vacuole in an Atg19-independent manner under starvation conditions [58]. During autophagy, Atg34 (Yol083w), a homolog of Atg19, functions as a receptor for Ams1. In *atg19*Δ cells, Ams1 targeting is disrupted in nutrient-rich conditions [47, 48]. However, Ams1 is efficiently transported into the vacuole under starvation conditions by autophagy even in *atg19*Δ cells. A genome-wide yeast two-hybrid screen suggested that Yol083w is an Ams1 interacting protein [45], and Atg34 indeed physically interacts with Ams1 [58]. Similar to Atg19, Atg34 binds Atg8 and Atg11 using distinct domains, and these interactions are essential for its function in targeting Ams1 into an autophagosome; an Atg34 mutant that lacks its Atg8 interacting motif forms a complex with Ams1, but shows a defect in sequestration into autophagosomes. Importantly, the transport of Ams1 mediated by Atg34 in starvation conditions is prApe1 independent, unlike that mediated by Atg19 in growing conditions.

Also recently, aspartyl aminopeptidase (Yhr113w/Ape4) was found to be a third Cvt cargo protein [59]. Yeast two-hybrid analyses suggested that Ape4 can associate with Atg19 and prApe1 [60]. Unlike prApe1, Ape4 does not possess a propeptide region and it does not self-assemble into aggregates [59]; however, it still binds to Atg19. An immunoprecipitation analysis with truncated versions of Atg19 revealed that the three identified Cvt cargo components, prApe1, Ams1, and Ape4, associate with Atg19 by binding to distinct sites. GFP-Ape4 colocalizes with RFP-Ape1 at the PAS in growing conditions, and this localization is dependent on Atg19. Notably, Ape4 transport to the vacuole by the Cvt pathway is significantly decreased in *ape1*Δ cells, suggesting that Ape4 relies on the prApe1-Atg19 complex for its targeting, similar to Ams1 in vegetative conditions. In *atg11*Δ cells, Ape4 can colocalize with prApe1, but it does not localize at the PAS.

7. Conclusions

An intriguing question has been why yeast cells have utilized the Cvt pathway to import a resident vacuolar hydrolase. In higher eukaryotes, there is no evidence for a Cvt pathway, and the *ATG* genes specifically involved in this pathway are not conserved; in contrast, those genes that are also needed for autophagy are highly conserved [61]. However, selective types of autophagy clearly take place in higher eukaryotes, including mitophagy and pexophagy. The molecular machinery involved in these processes in mammalian cells has not been completely elucidated, but it is likely that the general mechanism is conserved. For example, receptors such as BNIP3L and BNIP3 function as receptors in mammalian mitophagy, whereas Atg32 carries out this function in yeast; BNIP3L and BNIP3 are not homologs of Atg32, but they are functional counterparts, supporting the concept of mechanistic conservation. Furthermore, most of the machinery for the Cvt pathway is also used for pexophagy and mitophagy, which, as noted above, take place in higher eukaryotes. This means that with regard to the Atg proteins, the apparent absence of the Cvt pathway in mammals may be viewed as a deficiency in the specific receptor Atg19, rather than a major difference between yeast and other eukaryotes.

Returning to the initial question regarding the origin of the Cvt pathway, one possibility is that the oligomeric structure of prApe1 or Ams1 is critical for stability and/or function. The size of the oligomeric form of these hydrolases would prevent translocation through the ER translocon, necessitating a vesicle-mediated import process. Also, Ams1 does not appear to be synthesized as a zymogen. Thus, it would be problematic for this hydrolase to traverse the secretory pathway along with other newly synthesized glycosylated proteins. These vacuolar hydrolases are likely required in large amounts when the cell is starved or when aggregated proteins or damaged organelles accumulate, and the synthesis of most vacuolar hydrolases increases substantially during starvation. Under these conditions, the efficient transport of these hydrolases as oligomers by means of a vesicle-mediated mechanism such as autophagy would be extremely efficient. It would seem reasonable for the cell to modify the autophagy pathway very slightly with the addition of a small number of specificity components to take advantage of the existing autophagy machinery and allow it to be used for various types of selective sequestration processes.

Acknowledgment

This work was supported by NIH public health service Grant GM53396 to D. J. Klionsky.

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