BioMed Research International

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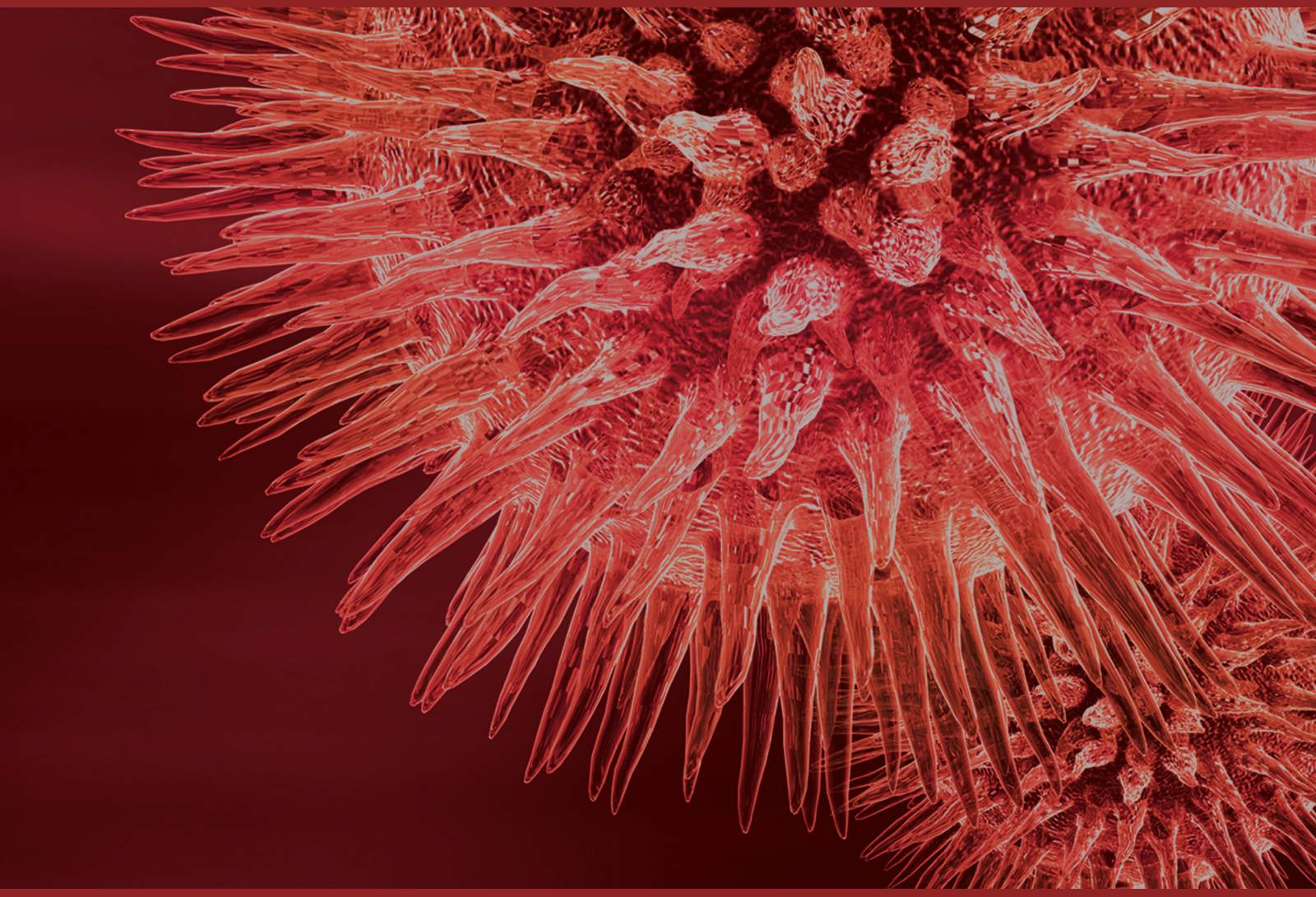
Guest Editors: Ioannis P. Nezis, Maria I. Vaccaro, Rodney J. Devenish, and Gábor Juhász



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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 349623, 2 pages
http://dx.doi.org/10.1155/2014/349623

*Editorial*

# Autophagy in Development, Cell Differentiation, and Homeodynamics: From Molecular Mechanisms to Diseases and Pathophysiology

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Received 21 July 2014; Accepted 21 July 2014; Published 28 August 2014



The focus of this special issue is to highlight the role of autophagy in cellular homeodynamics, cell differentiation, and development with an outlook to diseases. Autophagy is an evolutionarily conserved catabolic process where cytoplasmic components are sequestered into double-membrane vesicles called autophagosomes, which then fuse with lysosomes and their content is degraded. Despite the significant progress observed over recent years in our understanding of the molecular mechanisms of autophagy, the elucidation of its role in developmental processes still remains a challenge for the scientific community. Given the role of autophagy in pathophysiology and diseases, it is essential to uncover how the mechanisms of autophagy function during developmental processes in the context of tissue and organismal physiology.

This special issue contains a collection of four original research papers, four review articles, and one methodology report, covering a broad range of topics.

A. P. Sagona et al. in their paper entitled "*Association of CHMP4B and autophagy with micronuclei: implications for cataract formation*" report that the ESCRT-III subunit CHMP-4B localizes to chromosome bridges and micronuclei in lens epithelial cell cultures. These structures are subject to autophagy as evidenced by the close proximity of autophagosomes and lysosomes. Moreover, based on the observation that CHMP4B can be coimmunoprecipitated with chromatin, the authors propose that CHMP4B contributes to selective autophagy, here leading to the degradation of micronuclei and other extranuclear chromatin. As a CHMP4B mutation associated with an autosomal dominant form of cataract abolishes the ability of CHMP4B to localize to micronuclei, the autophagic degradation of DNA is implicated in the protection of lens cells from cataract development.

K. Zielniok et al. in their paper entitled "*Functional interactions between 17β-estradiol and progesterone regulate autophagy during acini formation by bovine mammary epithelial cells in 3D cultures*" use a 3D culture model of developing mammary epithelial cell acini to elucidate the mechanisms of autophagy regulation by 17$\beta$-estradiol and progesterone. They investigate the genomic effect of both sex steroids on the expression of chosen autophagy-related genes and proteins (ATGs). They show that both hormones induce ATG3, ATG5, BECN1, LC3B, and their protein products during the formation of alveoli-like structures by bovine BME-UV1 mammary epithelial cells. Moreover, the treatment with both hormones slightly increased the level of phosphorylated AMPK but diminished phosphorylated Akt and mTOR on day 9 of 3D culture. These results also suggest that the synergistic actions of the two steroid hormones studied accelerate the development of bovine mammary acini, which may be connected in part with the regulation of the molecular machinery involved in autophagy.

K. Hegedűs et al. in their paper entitled "*The putative HORMA domain protein Atg101 dimerizes and is required for starvation-induced and selective autophagy in Drosophila*" study the Atg1-family kinase complex in *Drosophila* and identify Atg101 as a member of this complex. They show that loss of *Drosophila* Atg101 impairs both starvation-induced and basal autophagy. They also show that Atg101 dimerizes and is predicted to fold into a HORMA domain. In addition Atg101 interacts with Atg1, Atg13, and Ref(2)P. These results suggest an important role of *Drosophila* Atg101 in autophagy and highlight that the Atg1 kinase complex is conserved among the metazoans.

P. Lőrincz et al. in their paper entitled "*Atg6/UVRAG/Vps34-containing lipid kinase complex is required for receptor downregulation through endolysosomal degradation and epithelial polarity during Drosophila wing development*" analyse the role of class III phosphatidyl-inositol 3-kinase (PI3K) complexes during wing development in *Drosophila*. The core complex consists of the lipid kinase Vps34 and its regulatory subunit Vps15, plus Atg6/Beclin1. Distinct PI3K complexes are specified by the two mutually exclusive subunits Atg14 and UVRAG. It is shown that both Atg6 and Atg14, but not UVRAG, are required for autophagy in the wing, whereas both Atg6 and UVRAG, but not Atg14, are involved in the endolysosomal degradation of receptors, such as Notch, and also in the establishment of proper epithelial polarity. There is some controversy regarding the role of UVRAG in autophagy in the published literature. This study supports a series of papers showing the existence of an autophagy-specific Atg14 complex, while the UVRAG-containing complex is found to be necessary for endolysosomal degradation and cell polarity.

The review by N. C. Mulakkal et al. entitled "*Autophagy in Drosophila: from historical studies to current knowledge*" comprehensively summarises the role and regulation of autophagy in the fruit fly, *Drosophila melanogaster*, an established model system for in vivo analysis of autophagy in the context of a developing organism. Autophagy genes and their regulators are conserved in *Drosophila*, and autophagy is induced in response to nutrient starvation and hormones during development. Application of sophisticated genetic tools allows investigation of autophagy in *Drosophila* models of disease. The power of the *Drosophila* model means it has made important contributions to the identification of novel developmental and physiological roles of autophagy.

The review by D. Romanelli et al. entitled "*A molecular view of autophagy in Lepidoptera*" focuses on Lepidopteran insects including the silkmoth *Bombyx mori*, which are classical subjects of autophagy research. Enormous induction of autophagy is seen in the polyploid larval tissues of these animals in response to starvation or during metamorphosis, similar to *Drosophila*. A key advantage of studying Lepidopteran models is their direct economic impact. For example, insects belonging to this order can produce silk or decrease crop yields as pests. Recent advances in developing tools for molecular studies hold the promise that the analysis of autophagy and programmed cell death in Lepidopteran larvae (caterpillars) may shed light onto the role and regulation of these processes in a developmental context.

The review by J. M. I. Barth and K. Köhler entitled "*How to take autophagy and endocytosis up a Notch*" summarizes the interplay and published links between two catabolic pathways: endocytosis and autophagy, both of which culminate in lysosomal degradation. The established role of endocytosis in regulating Notch receptor activity and the availability of its ligands Delta, Serrate, and Lag-2 is also discussed, together with emerging data on autophagy as a modulator of Notch signaling. Vice versa, loss of Notch leads to the activation of autophagy in certain contexts. Considered together these data indicate a complex network of interactions between autophagy, endocytosis, and Notch signaling, which is only beginning to be understood.

The review by M. Lippai and P. Lőw entitled "*The role of the selective adaptor p62 and ubiquitin-like proteins in autophagy*" provides a brief overview of autophagy and the ubiquitin-proteasome system and how these degradation systems coordinate their functions. They highlight the presence of ubiquitin and ubiquitin-like proteins in both systems and discuss the basic mechanisms of their function. Moreover, the authors underscore the selectivity of degradation in both systems and focus extensively on selective autophagy and its associated adaptor proteins including p62/SQSTM1, NBR1, NDP52, and Optineurin. The involvement of ubiquitin and ubiquitin-like proteins of Atg8 family in selective autophagy is also discussed.

A. L. Kovács in his paper entitled "*A simple method to estimate the number of autophagic elements by electron microscopic morphometry in real cellular dimensions*" describes a morphometric method ($S_{sp}$ method) for calculation of surface values and estimation of average diameter and number of autophagic elements in real cellular dimensions using data from electron micrographs. The method is based on morphometric determination of relative surface (surface density) and volume (volume density). Since electron microscopy is still indispensable for autophagy research, the $S_{sp}$ method will be very useful for providing quantitative analysis of electron microscopy data.

In conclusion, the papers presented in this special issue underscore a prominent role of autophagy during cell differentiation and development and highlight its emerging association with diseases. A deeper understanding of the mechanisms of autophagy in the context of developmental processes thus emerges as a critical factor for the development of novel therapeutic approaches.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 902315, 11 pages
http://dx.doi.org/10.1155/2014/902315

*Review Article*

# A Molecular View of Autophagy in Lepidoptera

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Received 24 January 2014; Revised 6 June 2014; Accepted 20 June 2014; Published 16 July 2014

Academic Editor: Gábor Juhász



Metamorphosis represents a critical phase in the development of holometabolous insects, during which the larval body is completely reorganized: in fact, most of the larval organs undergo remodeling or completely degenerate before the final structure of the adult insect is rebuilt. In the past, increasing evidence emerged concerning the intervention of autophagy and apoptosis in the cell death processes that occur in larval organs of Lepidoptera during metamorphosis, but a molecular characterization of these pathways was undertaken only in recent years. In addition to developmentally programmed autophagy, there is growing interest in starvation-induced autophagy. Therefore we are now entering a new era of research on autophagy that foreshadows clarification of the role and regulatory mechanisms underlying this self-digesting process in Lepidoptera. Given that some of the most important lepidopteran species of high economic importance, such as the silkworm, *Bombyx mori*, belong to this insect order, we expect that this information on autophagy will be fully exploited not only in basic research but also for practical applications.

## 1. Introduction

More than one million species of insects have been described in the literature and they are characterized by a great morphological and physiological heterogeneity. Despite the great diversity among insects that belong to different orders, metamorphosis, a biological process by which the adult organism is formed through a series of gradual changes, represents a common feature. In this context, holometabolous insects clearly represent an intriguing model because the overall body organization of the larva changes completely during metamorphosis: most of the organs undergo deep remodeling or even completely degenerate at that stage, and proliferation and differentiation processes are required to form the new body structure typical of the adult insect [1].

Over the years it has been demonstrated that larval organs in holometabolous insects degenerate mainly through apoptosis and autophagy. However, while considerable attention has been paid to the role of apoptosis and to characterizing the signals and mediators that regulate this process, the story of autophagy is more complex and still incomplete, probably because clear markers to identify this process in insects were not available for a long time [2].

In this paper, we deal with aspects of autophagy in Lepidoptera, such as identification of autophagic genes and proteins, dissection of the autophagic pathway, and assessment of the relationship between autophagy and apoptosis, and we discuss specific features of the autophagic process in these insects that are expected to be unraveled in the near future.

## 2. A Glimpse of the Past and a Look into the Future

Due to indisputable advantages such as a short and well-known life cycle, well-characterized developmental genetics, and a wide range of molecular tools for gene manipulation, *Drosophila melanogaster* has played the primary role among insects and has traditionally represented a key model system for studies on autophagy (for a complete review see [3]). It also represents a reference point for other insect species, as outlined in the present paper. Although Lepidoptera cannot offer the advantages of a model organism such as *Drosophila* by far, the larvae of these insects are amenable to endocrinological, physiological, and developmental biology studies and, owing to the increasing repertoire of molecular tools for some lepidopteran species, the study of autophagy in these insects

has been reappraised in recent years. In addition, Lepidoptera have an added value in terms of practical applications. In fact, some of the most important species of high economic importance, such as the silkworm, *Bombyx mori*, which is bred for silk production, or insect pests that reduce crop production or destroy stored food grains, belong to this insect order. Therefore, a deep understanding of the processes that regulate metamorphosis in the larval organs of these organisms, cell death processes in particular, could provide essential information that could be exploited for practical applications.

Studies on autophagy in Lepidoptera date back to a half-century ago, when Locke and Collins [4, 5] provided evidence that autophagy occurs in the larvae of the larger canna leafroller, *Calpodes ethlius*, during metamorphosis. Since then, dozens of studies have reported morphological or biochemical features that can be ascribed to this self-digesting process, and in the last 15–20 years molecular evidence of autophagy in moths and butterflies emerged.

Basically, three main periods can be identified in Lepidoptera autophagy research: the morphological, the biochemical, and the molecular period. During the early period, the examination of several larval organs, including fat body [6], midgut [7], silk gland [8–10], intersegmental muscles (ISMs) [11, 12], and wing epithelium [13], gave evidence of the presence of autophagic features, such as autophagosomes and lysosomes, during metamorphic degeneration (Figure 1). In particular, ISMs were demonstrated to be a powerful model. Some articles published in the sixties by Lockshin and Williams on ISMs of silk moths [14, 15], not only introduced the term "programmed cell death" in relation to insect tissue development, but also led to the classification of this degenerative process as Type 2 cell death that will be detailed below. Subsequent studies demonstrated that the elimination of the muscle cytoplasm was brought about by a combination of lysosomal and proteasomal activity. In fact, the demise of ISMs is accompanied by increased activity of cathepsins and acid phosphatases, and lysosome-like organelles containing mitochondria could be observed during this process [14, 15]. Moreover, ISMs death is mediated by enhanced protein catabolism via the ubiquitin-proteasome pathway: *de novo* expression of several genes associated to proteolysis is required and all the main components of this pathway are increased [16–18]. Thus ISMs are an excellent system for studying the role of autophagy in muscle atrophy and death, which may also provide useful information for clinical disorders.

Some of the pioneering studies hypothesized that the rough endoplasmic reticulum, Golgi, and mitochondria might be a source for the membrane that formed the autophagosome. Lysosomes are critical for autophagy and several authors directed their attention to this organelle, showing an accumulation of lysosomes in several larval tissues undergoing autophagy and a parallel increase in acid phosphatase levels [6, 9, 11, 19]. Matsuura and colleagues [19] also suggested a model in which acid phosphatase activity occurs in a biphasic pattern during degeneration of the silk gland, which supports a dual role of autophagy during the demise of this organ. The importance of lysosomal

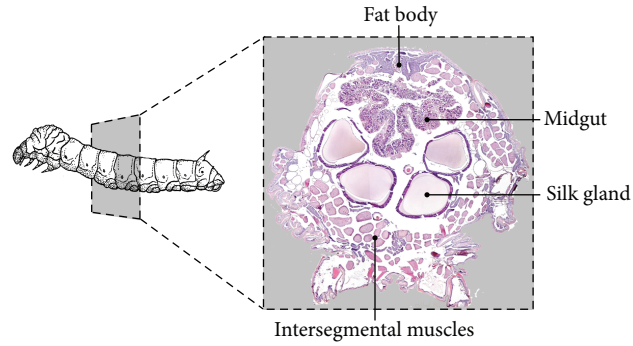


Figure 1: Schematic representation of the main larval organs that undergo programmed autophagy during metamorphosis in *Bombyx mori*.

enzymes in the degeneration of larval organs reported in these early papers has been definitively demonstrated in two recent studies in the silkworm [20, 21], by cloning cathepsins B and D, two enzymes whose expression is induced by 20-hydroxyecdysone (20E) and that work in a fashion similar to acid phosphatase: in fact, silencing these two genes through RNA interference (RNAi) negatively impacts pupal development.

Alongside this morphological approach to the study of autophagy, a biochemical-physiological approach rapidly evolved. This approach was essentially devoted to identifying the activating and regulatory signals of autophagy in lepidopteran organs and tissues. Evidence obtained in the fat body demonstrated that autophagy is triggered in the larva by a pulse of 20E and the onset of this process can be prevented by applying ligatures behind the brain-ring gland complex [22, 23]. Moreover, experiments performed *in vitro* not only confirmed that this hormone is able to switch on autophagy in fat body cells, but also showed that, once the cells are committed to autophagy and the process is activated, ecdysone is no longer required for completion since autophagy can continue in a hormone-free medium [24].

The new period that has emerged in the last ten years is primarily centered on a molecular view of autophagy and is mainly based on the significant advances that were made after genome sequencing of *B. mori* [25, 26]. In general, the increasing use of microarrays and RNA-Seq has deepened our knowledge of gene expression patterns in multiple tissues or in different conditions such as metamorphosis and immune response, and facilitated the identification of miRNAs [27]. Major results have also been gained from extensive proteomic analyses performed on more than ten different tissues and organs in the silkworm. In addition, several genetic tools with which larvae can be genetically manipulated are now available for *B. mori*. Silkworm is, in fact, amenable to systemic RNAi and stable germline transformation, thus providing an opportunity to gain insight into the function of autophagic proteins through gene knockdown or overexpression. As an example, the expression of several autophagy-related (*ATG*) genes has been efficiently silenced in the fat body of this lepidopteran [28].

Additional effort has been invested in identifying and characterizing genes involved in the autophagic process and

Table 1: List of *ATG* genes and proteins identified in Lepidoptera.

| Gene | Nucleotide sequence | Gene expression | Protein expression | Protein structure |
|---|---|---|---|---|
| *ATG1* | [29, 31, 32] | [28, 32] | | [28, 32] |
| *ATG2* | [28] | [28] | | [28] |
| *ATG3* | [29] | [28] | | [28] |
| *ATG4* | [29] | [28, 105] | | [28] |
| *ATG5* | [29, 31] | [28, 31, 33, 34] | [34] | [28] |
| *ATG6* | [29, 31] | [28, 31, 33–35, 88] | [78, 106] | [28] |
| *ATG7* | [29] | [28] | | [28] |
| *ATG8* | [29, 31, 75, 78, 106] | [28, 29, 31, 33–35, 75] | [28, 33, 34, 40, 75, 78, 106] | [28, 39, 40] |
| *ATG9* | [29] | [28] | | [28] |
| *ATG11* | [28] | [28] | | [28] |
| *ATG12* | [29] | [28, 29, 34] | | [28] |
| *ATG13* | [28] | [28] | | [28] |
| *ATG16* | [29] | | | [28] |
| *ATG18* | [29] | [28] | | [28] |

in evaluating their expression in larval tissues of different Lepidoptera. From the results obtained in these studies, and in particular thanks to the bioinformatics analyses performed by Zhang and colleagues [29], it became rapidly clear that homologs of most of the genes that belong to the autophagic pathway are present in the silkworm genome. These include multiple *ATG* genes (Table 1), originally identified in yeast and subsequently in higher eukaryotes, and genes involved in the phosphatidylinositol-3-kinase (PI3K) signal transduction pathway and in the formation of autophagosomes [29, 30].

*ATG* genes constituted the primary target of these investigations. They were identified in silkworm starting from yeast, other insects (mainly *Drosophila*), and human sequences available in public databases [29, 31]. Expression of *ATG* genes has been described in different silkworm tissues. In particular, *BmATG1*, *BmATG5*, *BmATG6*, and *BmATG8* were expressed in peritracheal athrocytes and gonads [31, 32]. A striking upregulation of various *ATG* genes during fifth larval instar and metamorphosis of this insect has also been observed in the larval midgut epithelium [32, 33], silk gland [29, 34, 35], and fat body [28, 32]. In fat body it has also been demonstrated that 20E levels affect the expression of these genes *in vivo*, confirming that the ecdysone titer in the hemolymph is linked to the induction of the autophagic program in the larval organs [28]. It is important to underscore that, although monitoring of *ATG* gene transcription is not recommended as a general readout for autophagy [36], the results obtained by using several markers to monitor activation of autophagy in these tissues confirm that, at least in midgut, silk gland, and fat body of *B. mori*, these changes in gene expression just prior to cell death of these organs correlate with autophagic activity.

Among the *ATG* genes, *ATG1* and *ATG8* are particularly interesting because they play a pivotal role in the autophagic process. *ATG1* is necessary and sufficient itself to induce the autophagic process in *Drosophila*: overexpression of this gene triggers downstream pathways and stimulates autophagy in a kinase-dependent manner [37]. In silkworm, *BmATG1* expression is significantly enhanced during the first day of the spinning phase both in the larval fat body and midgut and can be quickly induced in the fat body by complete food withdrawal [32] and 20E injection [28]. *BmATG1* cloning revealed the expression of two full-length coding sequences (*BmATG1* transcript variants A and B), closely related to orthologs of other insects [32]. An ecdysone response element (EcRE) is located within the *BmATG1* promoter, confirming its role as a 20E primary-response gene [28]. The encoded BmAtg1 proteins share extensive homology with orthologs from yeast to mammals, showing high conservation at the N-terminal region where the catalytic domain and ATP- and Mg-binding sites are located, as revealed by sequence analysis and *in silico* prediction of its three-dimensional structure [32]. On the other hand, Atg8 is a key factor in autophagosome formation and can be used as an undisputable marker for autophagy given its localization on the autophagosome membrane [38]. *BmATG8* expression peaks in several silkworm tissues at the onset of metamorphosis as well as after injecting 20E [28, 29, 33]. Determination of the crystal structure of BmAtg8 showed that not only the sequence but also structural domains such as the ubiquitin fold and some essential amino acid residues are conserved [39]. Recently, Zhang et al. [40], by expressing Atg8 protein fused with different tags in *Spodoptera litura* cells, demonstrated that Atg8 has both a nuclear and cytoplasmic localization when expressed at high levels and that the protein moves to the cytoplasm when autophagy is activated. This study not only provides evidence on localization and shuttling of Atg8 between cytoplasm and nucleus, but also gives interesting information for the interpretation of autophagic assays based on Atg8-fusion proteins.

The evidence collected from the aforementioned proteomic and molecular studies set the stage for functional analyses of the molecular pathways and signals that regulate autophagy in Lepidoptera.

## 3. Developmentally Programmed Autophagy: A Lesson from *Drosophila*?

Programmed cell death (PCD) plays an important role in animals in the removal of superfluous or damaged cells and is thus a key process that sculpts tissues and organs during morphogenesis. Three major forms of cell death have been described based on morphological criteria. Type 1 PCD (apoptosis) shows nuclear condensation and fragmentation and membrane blebbing and formation of apoptotic bodies that are engulfed by phagocytes and depends on caspase activation. Type 2 PCD (autophagic cell death) is characterized by an accumulation of autophagosomes and autolysosomes in the cytoplasm that self-digest the cell and thus it is less dependent on phagocytes that clear up cellular debris. Type 3 PCD (necrotic cell death) involves

cell swelling, membrane rupture, and release of cytoplasmic content in the extracellular environment [41]. The occurrence of autophagic cell death has been postulated in different organisms, but since these descriptions are mainly based on morphological features, only in limited cases autophagy has been shown to have a causative role in cell death. For this reason, it is possible to outline two main scenarios: (i) "cell death by autophagy," where autophagy actively contributes to the cell death process, and inhibiting autophagy rescues the cell from the death stimulus, keeping it alive; and (ii) "cell death with autophagy," where autophagy simply accompanies the cell death process and does not have an active role in it [42]. Thus autophagy does not seem to have a universal role in executing PCD but rather is required in a context-specific manner. The demise of larval organs in holometabolous insects, which requires both autophagy and apoptosis, represents an ideal model for tackling such questions: in particular, one of the most intriguing and controversial problems is the understanding of the function of autophagy and its regulatory mechanisms in this context. Studies in *Drosophila* have demonstrated that autophagy actively intervenes during the removal of salivary gland. In fact, mutations in several *ATG* genes or knockdown of *ATG* genes specifically in salivary gland cells are associated with incomplete degradation of this organ during metamorphosis. Moreover, overexpression of *ATG1* in salivary gland induces premature degradation in a caspase-independent manner [43]. Interestingly, the combined inhibition of autophagy and caspases enhances the impairment of the degradation process, thus suggesting that removal of the gland requires a cooperative action of autophagy and caspases [43]. Concerning midgut, an early paper suggested that histolysis of this organ can be inhibited by ectopically expressing the caspase inhibitor p35 [44], and Yin and Thummel showed that midgut deficient in the proapoptotic genes *Rpr* and *Hid* fails to undergo apoptosis during metamorphosis [45]. Interestingly, subsequent work has demonstrated that autophagy plays a role in the PCD of larval midgut, too, although in a distinctive manner. Similarly to salivary gland, loss-of-function *ATG* mutants or knockdown of *ATG1* and *ATG18* severely delays midgut removal [46], and overexpression of *ATG1* is sufficient to induce autophagy and premature removal of midgut cells [47]. In contrast to salivary gland, even though caspases are active, they are not necessary for midgut removal, so that the combined inhibition of autophagy and caspases does not increase the delay of this process compared to inhibition of autophagy alone [46]. In fat body, induction of autophagy by *ATG1* overexpression is sufficient to induce caspase-dependent cell death: in this case, cells show apoptotic features corroborating the hypothesis that autophagy can induce apoptosis [37]. An additional example of a context-dependent relationship between autophagy and apoptosis in the fly comes from oogenesis. Indeed, during oogenesis, cell death requires autophagy and components of the apoptotic machinery: Nezis et al. [48] have demonstrated that the autophagic degradation of the inhibitor of apoptosis protein (IAP) dBruce is required to induce DNA fragmentation, thus postulating a role for autophagy in caspase activation and occurrence of apoptosis. All of the aforementioned studies support the conclusion that the involvement of caspases during developmentally programmed autophagy is tissue-specific in *Drosophila*.

In Lepidoptera, the coexistence of autophagic and apoptotic features has been frequently described in many organs that die during metamorphosis: DNA fragmentation, apoptotic nuclei, and caspase activation have been detected in silk gland [49], fat body [50–53], midgut [33, 54–58], and other tissues [59–62] in which autophagic features have been found as well. Even though most of these studies are based on morphological observations—and functional studies will be necessary to demonstrate a role of autophagy in tissue degradation—this copresence of autophagic and apoptotic characteristics within the same organ does not seem to represent mere redundancy and adds evidence to the hypothesis that there is an overlap in the regulatory pathways of autophagy and apoptosis in Lepidoptera as well.

In insects, the intertwining between autophagy and apoptosis extends to the activation phase, which is triggered by 20E [1, 63]. The signaling pathways of these two mechanisms in Lepidoptera have been studied in silk gland and fat body, indicating both peculiarities and overlaps. Although some evidence suggests that apoptosis can be triggered by a single injection of 20E in silkworm fat body [52], Sakurai and coworkers [64–67] showed that, in the anterior silk gland, cell death is regulated by a double pulse of this hormone, which peaks twice during larval-pupal transition. The first peak (commitment peak) is able to activate the so-called genomic response mediated by the EcR/USP receptor complex, upregulating the expression of apoptosis-related genes. Moreover, at the commitment peak, the larvae stop feeding and silk spinning is induced. The second peak, called the metamorphic peak, which occurs during the pupal stage and is higher than the previous one, may activate the so-called nongenomic response via a putative membrane ecdysone receptor, probably a G protein-coupled receptor. This nongenomic response activates the apoptotic machinery and the effector caspase-3-like protease (Figure 2). Interestingly, Tian and colleagues [28] proposed a similar model specifically based on autophagy for a genomic/nongenomic response to 20E. They clearly showed that, in *B. mori* fat body, the increase in 20E titer upregulates most of the *ATG* genes during molting and pupation. By injecting 20E in larvae on the second day of the fifth instar, *ATG* genes are transcriptionally upregulated, target of rapamycin complex 1 (Torc1) is inhibited (as confirmed by a decreased phosphorylation of Eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1)), and autophagic compartments are increased. In contrast, autophagy is reduced by RNAi of *ATG1* and *USP* genes and in *EcR* dominant-negative mutants during larval-pupal transition. All this information produced a working model in which 20E is able to increase autophagy in two different ways: (i) by acting through the receptor complex EcR/USP to activate transcription of *ATG* genes, both directly (this happens for *ATG1* thanks to the presence of an EcRE in the promoter) and indirectly (through the action of Br-C and downstream proteins) and (ii) by inhibiting the PI3K/Torc1 pathway, allowing activation of the downstream Atg1/Atg13 complex and initiating autophagosome formation (Figure 2).

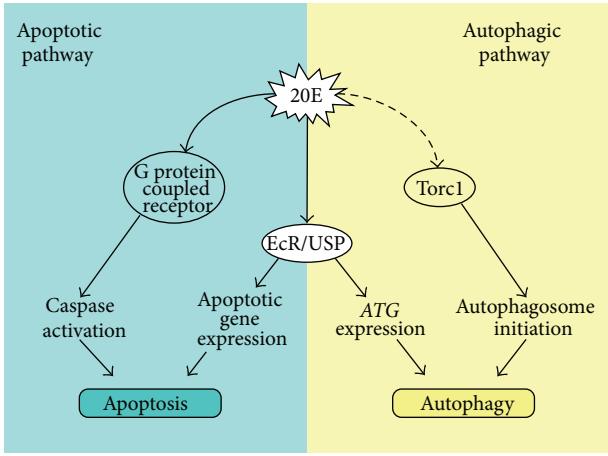


Figure 2: Activation of autophagy and apoptosis by 20E. The model is based on results obtained in the silk gland [64] and fat body [28].

This is in line with what has been reported for *Drosophila* fat body, where inhibition of the PI3K/Tor signaling by 20E can activate autophagy [68]. Another similarity with *Drosophila* is that autophagy can also be stimulated by injecting rapamycin, a Tor inhibitor, into feeding larvae [28]. However, the effect elicited in the fat body of the silkworm is weaker than that for treatment with 20E, probably reflecting the inability of this drug to increase the expression level of all critical *ATG* genes [28].

It is now becoming progressively clear that, at least in Lepidoptera, the induction of autophagy and apoptosis by 20E is probably more complex. Indeed, although both autophagy and apoptosis can be activated by 20E, it must be emphasized that, while both 20E commitment and metamorphic peak are required to trigger apoptosis, which produces an increase in apoptosis-related gene expression and activation of the apoptotic pathway, respectively, autophagy might be controlled exclusively by the first 20E peak. In fact, a single pulse of 20E injected in the hemocoel is sufficient to activate the autophagic pathway in silkworm fat body [28]. In addition, during larval-pupal transition, autophagy is activated simultaneously with the 20E commitment peak in silkworm larval midgut [33]. A few more words must be devoted to juvenile hormone (JH), the second player involved in regulating insect development and metamorphosis. The common view appears to be that JH inhibits autophagy prior metamorphosis. Accordingly, in *Manduca sexta* it has been demonstrated that topical administration of JH at early phase of last larval instar inhibits autophagy in the fat body, while treatments at later stages results to be ineffective, thus suggesting that autophagy can be activated only when 20E concentration increases, provided that JH is absent or present in a low amount [22, 69]. In Lepidoptera, there is limited evidence on the signaling pathway that links JH to autophagy: in particular, Guo et al. [70], by investigating the role of the putative JH receptor MET during metamorphosis of *B. mori*, reported that RNAi of this gene had a marked effect on the autophagic response at the early wandering stage.

Current data support at least two different potential scenarios to describe the autophagic process in Lepidoptera: caspase-dependent versus caspase-independent cell death involving autophagy. In fact, the expression of active caspases has been detected in the larval midgut of *Heliothis virescens* [54], *B. mori* [33] and *Spodoptera littoralis* [55] at pupal stage, by using antibodies specific for cleaved caspase-3. Moreover, in larval motoneurons of the tobacco hornworm, in which the death process is accompanied by autophagy, administration of a chemical inhibitor suppresses caspase activity and impairs the completion of the cell death process in these cells [60]. These data seem to be in contrast with fat body, where no evidence of executor caspase activity has been reported [50, 52]. A further level of complexity was added some years ago by a study performed in the labial glands of *M. sexta*, where an increase in lysosomal proteolytic activity was observed during the demise of this organ. According to the authors these enzymes could substitute for caspases and activate the apoptotic cascade, given that no caspase activity was detected [71]. In conclusion, as seen above for *Drosophila*, the involvement of caspases in PCD could be context-dependent in Lepidoptera, too.

Although studies performed in silk gland and fat body, as described above, have shed light on the activating pathways of apoptosis and autophagy during PCD in larval organs, uncertainty exists about the true role of autophagy in this setting. In *B. mori* fat body, 20E induces the expression of both apoptotic and *ATG* genes at the onset of metamorphosis. This event is accompanied by DNA fragmentation, appearance of autophagosomes/autolysosomes and increased lysosomal activity [28, 52]. In this case it has been proposed that autophagy functions to exploit nutrients in this adipose tissue in order to support the growth and differentiation of the new adult structures [28]. In the larval midgut of silkworm, autophagy is activated at wandering stage: Franzetti et al. [33] not only showed a large number of autophagic compartments in the midgut epithelium and a huge increase in lysosomes and acid phosphatase activity, but also detected BmAtg8 processing to BmAtg8-PE in these cells. Autophagy is set in motion once the larva stops feeding: it reduces the protein concentration in midgut tissue and induces a striking increase in ATP levels, to cope with starvation. On the other hand, apoptosis is activated later and promotes the demise of larval midgut cells. Thus, in this model, autophagy and apoptosis appear to have different functions: the former plays a prosurvival role for midgut cells deprived of nutrients, while the latter is strictly related to cell death [33]. Although molecular and functional analyses are in progress to unravel the true nature of the autophagic process in dying midgut and fat body, according to the preliminary evidence collected so far, it seems that apoptosis plays the key role in the disappearance of the larval organ, similar to early evidence reported for *Drosophila* [44, 45].

Given that a hierarchical relationship has been demonstrated between autophagy and apoptosis in *Drosophila* nurse cells [48] and fat body [37], it will be interesting to determine whether such a regulation can be found also in the aforementioned settings for Lepidoptera (i.e., midgut, fat body and other organs) and to verify whether the two processes work through interconnected regulatory pathway. If so, the molecular mediators of such cross-talk would need

to be identified. Taking the work done on *Drosophila* as a reference, in our opinion two molecular factors deserve particular attention: (1) IAP: in *Drosophila*, dBruce has been shown to provide a mechanistic link between autophagy and cell death [48]. Interestingly, IAP expression changes during midgut remodeling in several Lepidoptera, a situation where autophagy has been shown to occur [55, 58, 72]. Moreover, IAP transcription increases under starvation and decreases after refeeding in *Galleria mellonella*, thus suggesting a link between IAP and autophagy [58]. (2) Atg1: overexpression of Atg1 in *Drosophila* has been demonstrated to induce autophagy and subsequent caspase-dependent cell death with most of Atg1-overexpressing cells removed within 36 hours. In *B. mori* midgut an increase in Atg1 expression is observed during larval-pupal transition [32], concomitantly with autophagy and preceding apoptosis by two days [33]. Therefore, a possible regulation of apoptotic cell death by Atg1 cannot be excluded in this insect model either, an aspect that surely deserves further investigation.

## 4. A Prosurvival Role for Autophagy during Starvation

Although autophagy has been widely described during metamorphosis in holometabolous insects, it must be underlined that this self-eating process probably represents an adaptation of cells to starvation: in yeast, when the cell is subjected to nutrient deprivation, autophagy is activated to break down part of its reserves in order to stay alive until the situation improves [73]. This primary function of autophagy has been conserved and maintained up to pluricellular organisms and, in several insects, an autophagic response has been observed in cells and tissues deprived of nutrients [74–76].

In *Drosophila*, two seminal papers showed that, in the fat body, autophagy is regulated by the PI3K/Tor signaling pathway in response to ecdysone [68] and nutritional stress [76], thus demonstrating that developmentally and nutritionally triggered autophagy are coordinated. The key mediator is Tor, a negative regulator of autophagy activated by the Class I PI3K signaling pathway that affects phosphorylation and repression of Atg1.

In Lepidoptera, too, besides developmentally programmed autophagy, increasing attention is being devoted to the activation and regulation of autophagy following starvation or in relation to nutritional cues, such as lipid metabolism. Several key genes involved in the insulin and PI3K/Akt signaling pathway have been cloned and characterized in the silkworm [77]. In addition, two paralogous *TOR* genes with high sequence similarity, *BmTOR1* and *BmTOR2*, have been identified by Zhou and colleagues [30]. The genomic analysis revealed that BmTor1 is the ortholog, while BmTor2 is then derived after a duplication event. The two *BmTOR* genes have similar expression patterns and tissue distribution in fat body, midgut, silk glands, and other organs, levels being highest during molting and pupation. Both BmTor isoforms can be transcriptionally regulated by starvation and injection of 20E, although BmTor2 seems to respond better to both of these stimuli [30]. The possibility of modifying Tor pathway activation is very useful because the induction and regulation of starvation-induced autophagy can be investigated in Lepidoptera and previous evidence obtained in larvae subjected to food deprivation can be reappraised [32, 75].

In *G. mellonella*, Khoa and Takeda demonstrated that *GmATG8* transcription is upregulated and the expression of both GmAtg8 and GmAtg8-PE proteins increases in larval midgut after 5 days of starvation. After refeeding the larvae, *GmATG8* transcription and protein expression return to physiological levels [75]. This observation suggests that starvation alone is sufficient to activate autophagy in the absence of 20E stimulation, at least in this experimental model. Additional information on the turnover of Atg8 under starvation conditions comes from a study performed in a *S. litura* cell line [78]. In Sl-HP cells, amino acid deprivation causes a striking change in the expression of the autophagic marker Atg8. Interestingly, although a slight increase in GFP-Atg8 spots is observed 1-2 hours after the beginning of starvation, the total levels of Atg8 and Atg8-PE protein expression decrease. This apparently contradictory result can be explained by an enhanced conversion of Atg8 to Atg8-PE and a consequent acceleration of Atg8-PE degradation in the autolysosome. Accordingly, by treating cells with bafilomycin A1, an autophagosome-lysosome fusion inhibitor, this degradation process is slowed down. Based on these results, the authors suggest that, in Lepidoptera, starvation appears to induce autophagosome-autolysosome maturation rather than autophagosome formation [78].

In silkworm the impact of starvation on the onset of autophagy has also been monitored by evaluating the expression of *BmATG1* in the midgut and fat body. After food deprivation, the expression of this gene is increased in fifth instar larvae and this effect is stronger and faster in the fat body than in the midgut [32]. In fact, at least four days of starvation are needed to significantly increase *BmATG1* expression in the midgut. In contrast, autophagy is induced rapidly in the fat body, probably due to the energy storage function of this organ. This evidence is in accordance with a growing body of evidence suggesting that autophagy also has a role in regulating lipid metabolism and intracellular lipid stores during starvation and in response to 20E in Lepidoptera: (i) in *Drosophila*, Wang and colleagues [79] proposed that Rab32, a member of the Ras GTPase subfamily involved in lipid storage, may execute its function by affecting autophagy, thus supporting the notion that the autophagic process is involved in lipid metabolism. Rab32 is highly conserved among insects and its expression in the moth *Helicoverpa armigera* is induced by 20E in epidermis and midgut during metamorphosis, a developmental stage during which the animal does not feed and must rely only on its energy stocks, thus identifying Rab32 as a possible player in this response [80]; (ii) the transcriptional factor FOXO has been proven to be a key regulator of autophagy both in mammals [81, 82] and *Drosophila* [83]. Hossain et al. [84] found that activation of the transcriptional factor FOXO by 20E promotes lipolysis in the fat body. In particular, they cloned the *B. mori* homolog of FOXO and demonstrated that

its expression increased, and protein localization changed from cell membrane to the nucleus during fourth instar molting and larval-pupal transition as well as after exposure to 20E both in cell cultures and *in vivo* [84]. According to these data, an involvement of autophagy in lipid metabolism in the silkworm fat body seems plausible, as also suggested by Tian et al. [28]; (iii) in the IPLB-LdFB cell line, established from *Lymantria dispar* fat body, the administration of oligomycin A can induce a starvation-induced autophagic response, as detailed below. A concomitant shift towards lipid metabolism has been observed in these cells during exposure to oligomycin A, supporting a link between autophagy and lipid metabolism [85].

A large array of cell lines has been derived from the larval organs of *B. mori* and other lepidopterans. Along with the undisputed advantages of experimentation in cell lines, concomitant activation of autophagy and apoptosis as well as of other forms of cell death can be induced in some of them. Thus, this *in vitro* model proves to be a powerful and promising system to specifically dissect the autophagic response to starvation and to evaluate its cytoprotective role and/or its relationship with apoptosis or other forms of cell death [86].

In Sl-1 cells (from *S. litura*), glucose starvation increases the number of autophagic vacuoles. If starvation persists for more than 48 hours, these compartments gradually decrease, while apoptosis-related phenotypes appear [87]. In *S. litura* SL-ZSU-1 cell line, inhibition of starvation-induced autophagy with 3-methyladenine promotes a quick apoptotic response. A similar effect is observed in *B. mori* Bm36 cells, in which inhibition of starvation-induced autophagy by the same drug causes necrotic cell death. These observations can be interpreted by hypothesizing a role for autophagy in preventing the onset of cell death under nutrient deprivation conditions [88].

In the IPLB-LdFB cell line, the effect of nutrient starvation on autophagy was investigated by using oligomycin A, an inhibitor of the mitochondrial ATP synthase [85, 89]. Treatment of cells with this drug rapidly decreases the ATP content within 30 minutes and mimics the condition of nutrient scarcity [89, 90]. In a small percentage of cells, this drug promotes the production of a high quantity of reactive oxygen species and subsequent cell damage, leading to apoptotic, oncotic, and necrotic cell death within 48 hours after the treatment. In contrast, in the majority of the cells, administering oligomycin A induces the onset of autophagy (targeting mainly mitochondria) and actin reorganization [89, 91]. This autophagic response precedes cell death and the authors suggested a correlation between autophagy and cell demise. Thus in this model, autophagy seems to be necessary for the cells to completely recover from ATP depletion and cellular damage, promoting cell protection; however, this action is rapidly overtaken and autophagy becomes associated with cell death mechanisms. Interestingly, a proteomic screening, aimed to identify mediators of this autophagy-mediated response to oligomycin A, found a correlation between the activation of autophagy and a drastic reduction in the levels of imaginal disk growth factor (IDGF)-like protein, a mitogenic factor that regulates growth processes in insects [85]. These data suggest that lack of this prosurvival factor in the cell medium may activate signaling that leads to cell demise, prompting future studies on the relationship between cell survival and developmental cell death that involves autophagy in insects.

## 5. Conclusions and Perspectives

The work performed up to now has led to some general conclusions on the autophagic process in Lepidoptera that can be summarized as follows: (i) *ATG* genes identified so far share evolutionary conservation and have been proven to be essential to correctly initiate and complete autophagy; (ii) the remodeling of most larval organs during metamorphosis requires the intervention of autophagy, which is activated by 20E; (iii) despite the prominent role of developmental autophagy in the remodeling of the larval organs, autophagy can also be induced by starvation, thus supporting the notion that in larval tissues of Lepidoptera this "self-eating" mechanism can act also as a cytoprotective process as seen in other organisms; and (iv) autophagic and apoptotic features coexist within the same organs, suggesting a complex intertwining of these two processes during metamorphosis.

The growing number of publications dealing with autophagy in moths and butterflies has established this field as a promising research area that is progressively attracting the interest of an increasing range of researchers. We can envisage that a detailed molecular and functional characterization of autophagy in these insects may have a direct impact on different areas.

*(1) Knowledge of the Basic Mechanisms of Autophagy*. One of the most important advantages of studying autophagy in lepidopteran larval organs is that this process can be dissected in an articulated biological setting and thus its relation to other forms of cell death [33, 49], to metabolic requirements of the cell [85, 89], and to regeneration events (Franzetti et al., in preparation) can be delineated. In particular, the concomitant presence of autophagy and apoptosis within the same larval organ could help to assess the true role of autophagy in a complex developmental context and to determine in these tissues whether true autophagic cell death exists or if autophagy simply accompanies cell death processes.

*(2) Food and Sustainable Agriculture*. Reduction of pesticide distribution is one of the major objectives in sustainable agriculture and is largely being addressed by adopting the use of environmentally safe products, including biological control agents. To this end it must be considered that: (i) a number of antagonistic associations in insects represent underexploited sources of natural compounds which disrupt larval development, reproduction, and immune response of insect pests, frequently by inducing cell death processes [92]; (ii) the bacterium *Bacillus thuringiensis* produces a variety of entomocidal proteins, safe for vertebrates and almost exclusively active against larval stages of lepidopteran, dipteran, and coleopteran insects, which lead to

cytotoxicity and cell death events in the insect tissues [93, 94]; and (iii) there is increasing interest in the use of specific nanomaterials that show pronounced toxic effects on insects (the so-called "nanocides") [95]. All these nonconventional agents, potentially able to misregulate or induce cell death processes in lepidopteran tissues, could represent safe tools able to disrupt larval development. Thus, a complete view of the different cell death processes, and of autophagic events in particular, that occur in lepidopteran larvae could generate basic information of key importance for opening new frontiers in the field of biological control of pest insects in the postgenomic era.

*(3) Health*. Several reports have implicated autophagy, a major route for the bulk degradation of aberrant cytosolic macromolecules and organelles, in aging control. Accordingly, genetic studies showed that *ATG* genes are involved in lifespan control in nematodes [96] and maintenance of basal expression of *ATG8* in the nervous system of *Drosophila* extended the lifespan by 50%, thus demonstrating that autophagy regulates the rate at which the tissues age [97]. Moreover, an evolutionarily conserved role for autophagy in preventing neurodegeneration has been demonstrated in mice and flies. In these models, in fact, loss of autophagy causes an age-dependent accumulation of ubiquitin-containing inclusion bodies in neurons, which disrupt neural function [98–100]. The interaction of Atg proteins with p62, an ubiquitin-binding scaffold protein that accumulates in ubiquitinated protein inclusions [101, 102], may facilitate the selective degradation of these aggregates by autophagy [103]. In this context, silkworm can surely be established as an interesting model organism through which new insights can be gained on the regulation of autophagy via signals linked to oxidative stress, caloric restriction, and energy availability, thus contributing to a significant pool of information that is useful to understand the relationship between autophagy and aging.

*(4) Sericulture*. The silk gland is the largest tissue in the last instar of the silkworm *B. mori* and begins to degenerate shortly before pupation, once the silk cocoon has been completely spun. This degeneration process is driven both by apoptosis and by autophagy [53]. Recently, Ma and colleagues [104] demonstrated that transgenic silkworms with increased Ras activity show an enlargement of the posterior region of the silk gland; this leads to an increased production of fibroin, one of the two constituents of the silk thread. Since modulation of Ras signaling leads to striking consequences on the cell growth in the silk gland, we can foresee similar biotechnological approaches to antagonize autophagy and apoptosis in this organ that aim to reduce its deterioration, thus increasing its longevity and hopefully improving quantitatively the silk yield.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgment

This work was partially supported by FAR 2013 (University of Insubria) to GT.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 578698, 5 pages
http://dx.doi.org/10.1155/2014/578698

*Research Article*

# A Simple Method to Estimate the Number of Autophagic Elements by Electron Microscopic Morphometry in Real Cellular Dimensions

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Received 17 March 2014; Accepted 4 June 2014; Published 3 July 2014

Academic Editor: Ioannis P. Nezis



Autophagic elements typically appear as spherical bodies. During their life they undergo a series of changes (e.g., fusion, degradation of content, and swelling) which influence their size in a way that may be characteristic for cell type, stage of maturation, or various experimentally manipulated parameters. A simple and time efficient method is suggested here to use exactly calculated specific surface values and estimate average diameter and number of autophagic elements in real cellular dimensions. The method is based on the easiest morphometric determination of relative surface (surface density) and volume (volume density) data by electron microscopy. A series of data from real experimental samples of liver and exocrine pancreatic cells are offered to illustrate the potential of these measurements and calculations.

## 1. Introduction

The most frequently applied and easiest method for electron microscopic morphometry is the so-called volumetric measurement, when we determine the volume of some components within a unit of test volume [1–3]. With regard to autophagy it means the determination of the relative volume of an autophagic compartment ($V_v$) in a unit volume of cytoplasm ($V_c$), reasonably expressed as $V_v\ \mu m^3/\mu m^3$. It is also possible and easy to determine the relative surface of an autophagic compartment ($S_v$) within the same system of measurements expressed as $S_v\ \mu m^2/\mu m^3$ [1, 2]. In addition to traditional point counting, computer aided techniques are also available for this purpose (e.g., NIH ImageJ and Adobe Photoshop).

The values that we can get by these morphometric measurements include the total size of both the surface ($S_v$) and the volume ($V_v$) of the autophagic elements in $1\,\mu m^3$ of cytoplasm, also named, respectively, as surface and volume density. $V_v$ data in articles are usually given as multiplied with 100 to show the % volume of the autophagic compartment within the cytoplasm.

The theoretical background and the practical description of $S_v$ and $V_v$ measurements are beyond the scope of the present paper; however, they are available in a well-illustrated manner in several reviews and books like [1–3]. In short, for the determination of $V_v$ we measure areas and for $S_v$ length of limiting membranes of organelles on images from electron microscopic sections, as basic data for subsequent calculations (Figure 1).

While $V_v$ is good to express the total volume of the autophagic compartment in a given volume of cytoplasm, it does not provide any information about the number of autophagic elements behind it. In many cases it would be very useful to estimate at least the approximate number of various autophagic components. These types of data could help to develop, in real cellular dimensions, better understanding of qualities of autophagy which depend on the number of objects.

Methods to determine the exact number of components in a given volume (numerical density) have been worked out and are described in detail both in the older literature (based on the analysis of profile histograms [2]) and in the new era of morphometry using the disector technique [3]. However, autophagy is a special object for morphometry as autophagic elements usually comprise a rather small proportion of the cytoplasmic volume; therefore, a very large test area must be evaluated for the measurements by the histograms and

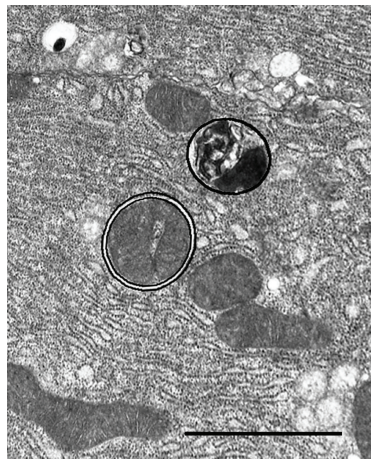

Figure 1: An electron microscopic picture showing a portion of a pancreatic acinar cell with an autophagosome encircled by double line and an autolysosome, encircled by single line, along their bordering membranes. Autophagosomes and autolysosomes are usually taken as two different categories of autophagic vacuoles. For $V_v$ data we measure the area within the membrane and for $S_v$ data the length of the bordering membrane separating the autophagocytosed material from the cytosol. These data are then related to the size of the surrounding cytoplasmic area during simple morphometric calculations. Scale bar 1 $\mu$m.

the dissector technique. The application of these accurate methods for autophagy studies would, therefore, be too time consuming and tedious for routine use. That is the most likely reason why, to my knowledge, such publications have not appeared so far. Here I suggest a simple and efficient approach which utilizes specific surface values ($S_{sp} = S_v/V_v$ $\mu m^2/\mu m^3$) for the determination of the number of autophagic elements. To illustrate its potentials I apply it to a large set of data from my previous measurements on liver and exocrine pancreatic cells with variable autophagic activity. The presented method offers approximations with a reasonable bias and can be utilized for a quick and low effort characterization of autophagy by the above parameters. The rough estimations obtained by this approach may also help to select specific cases to be evaluated by the accurate, high investment histogram and disector methods.

## 2. Results and Discussion

$S_{sp}$ gives us the surface of the (autophagic) compartment that belongs to a unit volume of the same (autophagic) compartment. The fact that the autophagic elements have the geometry of a sphere as a rule gives us the possibility to estimate their number with the help of their $S_{sp}$. The method of estimation is based on the simple geometrical fact that the surface/volume ratio of a certain sphere is exactly determined by its size. Therefore, for a homogenous population of spheres it is possible to calculate the diameter from their $S_{sp}$ value on the base of the following formulas:

$$S = D^2\pi; \quad V = \frac{D^3\pi}{6}; \quad S_{sp} = \frac{S}{V}; \quad D = \frac{6}{S_{sp}}, \qquad (1)$$

where $S$, $V$, and $D$ are the surface, volume, and diameter of a sphere, respectively.

To illustrate the technique for the determination of numbers of spheres, let us suppose that we have a homogenous population of spherical bodies in the cytoplasm. We measure $S_v$ and $V_v$ by simple morphometry and compute $S_{sp}(S_v/V_v)$ with them. With the diameter ($D_c$) calculated from $S_{sp}(D_c = 6/S_{sp})$, we can also calculate the individual volume of a single sphere in this homogenous population ($V_c = D_c^3\pi/6$). As we have the total of individual volumes ($V_v$), with a single division we can get the precise number of spheres in the unit volume containing those spheres. For example, if in a homogenous population we measure an $S_v$ and $V_v$ of 0,0351 $\mu m^2/\mu m^3$ and 0,0041 $\mu m^3/\mu m^3$, respectively, the $S_{sp}$ from them will be 8,55 $\mu m^{-1}$. This in turn gives a diameter of 0,7 $\mu$m with the help of the formula $D_c = 6/S_{sp}$. The volume of a sphere with a diameter of 0,7 $\mu$m is 0,180 $\mu m^3$. The total volume of the population of spheres in a cell with 5000 $\mu m^3$ size (the approximate average size of a rat hepatocyte [4]) will be $5000 \times 0{,}0041 = 20{,}5$ $\mu m^3$. A single division of the total by the individual volumes (20,5/0,180) will give us the number, which is 114 in this case. This calculation, as mentioned above, gives the precise number for a uniform population of spheres with equal diameter.

However, the autophagic elements have variable size which causes a bias in estimating numbers with the presented method. To estimate this bias we can make model calculations with sets of data in the range of real life samples. Although it is only an approximation, for our purpose it is possible to consider the distribution of the diameter values as closely Gaussian.

The typical diameter range for autophagosomes in mammalian cells, according to my own unpublished measurements and data derived from various articles [5–9], usually falls within 0,7–1,1 $\mu$m. Taking data in this range we can calculate the real average volume and the volume estimated with the help of the diameter derived from the $S_{sp}$ value based on the $D_c = 6/S_{sp}$ formula (calculations are presented in the Supplementary Material available online at http://dx.doi.org/10.1155/2014/578698). The result of this probe shows an underestimation of the number by a factor of 1,06. Further analysis reveals that the error of estimation depends on the changes in size distribution. Stimulation of autophagy, transition of autophagosomes to autolysosomes, and the following fusion events result in the widening of the distribution together with an appearance of categories with bigger size. The analysis of a probe with a range of 0,7–1,4 $\mu$m diameter, and a tail at the right end of the distribution, results in a 15,54% underestimation of the number (see the details of the calculations in the Supplementary Material).

Volumetric analysis of autophagy by point counting electron microscopic morphometry works with rather high standard errors [10–18], and sometimes only relatively big changes can be found statistically significant. Results presented here show that although the calculation of numbers of autophagic elements from $S_{sp}$ leads to underestimation, the error remains within a rather narrow range. In addition, if considered necessary, they might even be corrected with

Table 1: Approximate diameter ($D_c$), average volume ($V_c$), and number of autophagic elements ($N_c$) in a cell with 5000 $\mu m^3$ cytoplasm, calculated from the specific surface values ($S_v$ /$V_v$ = $S_{sp}$) from real experimental samples of liver and exocrine pancreatic cells. The details of calculation are described in the text.

| Experimental system and treatment | Category | $S_v$ $\mu m^2/\mu m^3$ | $V_v$ $\mu m^3/\mu m^3$ | $S_{sp}$ $\mu m^2/\mu m^3$ | $D_c$ $\mu m$ | $V_c$ $\mu m^3$ | $N_c$ in 5000 $\mu m^3$ |
|---|---|---|---|---|---|---|---|
| Isolated hepatocytes | | | | | | | |
| Plus amino acid mixture 30 min | Afs | 0,0023 | 0,0003 | 7,6 | 0,79 | 0,26 | 6 |
| | Al | 0,0147 | 0,0022 | 6,8 | 0,88 | 0,36 | 30 |
| Plus amino acid mixture 3 h | Afs | 0,0047 | 0,0006 | 7,4 | 0,81 | 0,28 | 11 |
| | Al | 0,0367 | 0,0072 | 5,1 | 1,18 | 0,85 | 42 |
| Minus amino acid mixture 3 h 30 min | Afs | 0,0472 | 0,0067 | 7,0 | 0,86 | 0,33 | 102 |
| | Al | 0,1333 | 0,0215 | 5,9 | 0,97 | 0,47 | 195 |
| Propylamine 10 mM 3 h | Afs | 0,0856 | 0,0124 | 6,9 | 0,87 | 0,34 | 180 |
| | Al | 0,0563 | 0,0084 | 6,7 | 0,90 | 0,38 | 112 |
| | Alam | 0,2925 | 0,1170 | 2,5 | 2,40 | 7,24 | 81 |
| Hepatocytes in vivo | | | | | | | |
| 3 h feeding | Afs | 0,0024 | 0,0003 | 7,9 | 0,76 | 0,23 | 7 |
| | Al | 0,0040 | 0,0005 | 7,9 | 0,76 | 0,23 | 11 |
| | Db | 0,0088 | 0,0008 | 11,0 | 0,55 | 0,08 | 47 |
| 24 h fasting | Afs | 0,0101 | 0,0014 | 7,2 | 0,84 | 0,31 | 23 |
| | Al | 0,0171 | 0,0022 | 7,8 | 0,77 | 0,24 | 46 |
| | Db | 0,0079 | 0,0008 | 9,9 | 0,61 | 0,12 | 34 |
| Ad libitum feeding | Afs | 0,0025 | 0,0003 | 8,5 | 0,71 | 0,18 | 8 |
| | Al | 0,0048 | 0,0006 | 8,0 | 0,75 | 0,22 | 14 |
| Vinblastine treatment 0,1 mg/g 2 h | Afs | 0,0778 | 0,0105 | 7,4 | 0,81 | 0,28 | 189 |
| | Al | 0,0648 | 0,0086 | 7,5 | 0,80 | 0,26 | 163 |
| Leupeptin treatment 0,12 mg/g 2 h | Afs | 0,0450 | 0,0054 | 8,3 | 0,72 | 0,20 | 138 |
| | Al | 0,1095 | 0,0278 | 3,9 | 1,52 | 1,85 | 75 |
| Exocrine pancreas cells in vivo | | | | | | | |
| 24 h fasting | Afs | 0,0050 | 0,0007 | 7,2 | 0,83 | 0,30 | 12 |
| | Al | 0,0061 | 0,0009 | 6,8 | 0,89 | 0,36 | 12 |
| Ad libitum feeding | Afs | 0,0016 | 0,0002 | 7,9 | 0,76 | 0,23 | 4 |
| | Al | 0,0024 | 0,0003 | 7,8 | 0,77 | 0,23 | 6 |
| Vinblastine treatment 0,1 mg/g 1 h | Afs | 0,0447 | 0,0063 | 7,1 | 0,85 | 0,32 | 99 |
| | Al | 0,0233 | 0,0034 | 6,8 | 0,88 | 0,35 | 48 |
| Vinblastine treatment 0,1 mg/g 1,5 h | Afs | 0,1073 | 0,0159 | 6,8 | 0,89 | 0,37 | 216 |
| | Al | 0,0401 | 0,0060 | 6,7 | 0,90 | 0,38 | 79 |
| Vinblastine treatment 0,1 mg/g 6 h | Afs | 0,3362 | 0,0518 | 6,5 | 0,92 | 0,41 | 626 |
| | Al | 0,3081 | 0,0517 | 6,0 | 1,01 | 0,53 | 484 |

Afs: autophagosome (early autophagic vacuole), Al: autolysosome (late autophagic vacuole), Db: dense body, Alam: swollen electron-lucent amine type of autolysosome.

The experimental animals were from male mice for in vivo treatments and from rats for isolated cells. The evaluated cytoplasmic area was in the range of 9–16000 $\mu m^2$. For further details of electron microscopy, morphometry, and experimental systems see, for example, in [5–10].

the help of size distribution data (see Supplementary Material). Being a sensitive indicator of changes related to the size of autophagic elements, $S_{sp}$ is a valuable parameter in itself. The derived $D_c$ and $N_c$ values also express quantitative changes with reasonably good approximation. In addition they help to depict the events during autophagy in real cellular dimensions.

In the following section I illustrate the utilization of this approach in selected autophagic processes. Some of them were previously described by volumetric ($V_v$) evaluation. For the present purpose a review and additional measurements were made to expand our database and support the calculation of $S_{sp}$.

Table 1 shows that $S_{sp}$ data are characteristically different for various categories of the autophagic-lysosomal compartment. The effect of various experimental treatments is also reflected in their values. The $S_{sp}$ is highest (9,9; 11,0) in the case of typical dense bodies resulting in small $D_c$ (0,61; 0,55 $\mu m$).

A rather wide distribution of $S_{sp}$ data for autolysosomes is revealed in different experimental systems and treatments. Low $S_{sp}$ values correlate well with swelling in 3 h amino acid (5,1) or propylamine treatment (2,5) and extensive fusion in leupeptin treatment (3,9). These features are only qualitatively indicated by the simple morphological evaluation of the pictures. It is the population of autophagosomes which appears to be the least heterogeneous. $S_{sp}$ values for autophagosomes are 6,5–8,5 which correspond to a $D_c$ range of 0,71–0,92 $\mu$m.

The last column of Table 1 shows the calculated number of autophagic structures in a real cellular volume of 5000 $\mu m^3$, an average rat liver hepatocyte [4]. For better comparison and simplicity, I have chosen the same volume for the exocrine pancreas cells.

The calculated numbers ($N_c$) are especially valuable to give a graphic quantitative picture of the autophagic lysosomal compartment in real cellular dimensions. The total number of autophagic elements may span a range of four orders of magnitude (1–1000) in a cell depending on experimental conditions. Immediately after feeding or amino acid treatment, when autophagy and lysosomal protein degradation are minimal, the number of autophagosomes may remain under or close to 10. Autolysosomes, however, are present in the lower range and dense bodies in the middle range of the $10^1$ order of magnitude. Fasting for 24 h in vivo increases the number of autophagosomes several times in liver cells. Nevertheless, the sum of autophagosomes and autolysosomes remains under 100. The number of dense bodies remains similar, although their size becomes bigger after fasting. Total amino acid withdrawal in vitro elevates the autophagosome number over 100 and that of autolysosomes close to 200.

The analysis of vinblastine treatments further illustrates the potential of the $S_{sp}$ values in approximate calculations of component numbers. This alkaloid disrupts microtubules and inhibits fusion of autophagosomes with endosomes and lysosomes [15, 19, 20]; in addition it also stimulates autophagosome formation [18, 19, 21]. In exocrine pancreatic cells after vinblastine treatment in vivo, we observed both the highest rate of accumulation and the total volume of the autophagic compartment [21]. After a review and additional measurements I calculated with the $S_{sp}$ method the component numbers of the autophagic compartment for certain time points.

The highest accumulation rate is seen between 1 and 1,5 h of vinblastine treatment while the highest volume at 6 h. The increase of the number of autophagic elements between 1 and 1,5 h is 148. This is the minimum number of autophagosomes generated during this 30 min. Supposing that each autophagosome is created from a single initiation event, we can calculate that the approximate frequency of initiations is 12 seconds in this case.

A calculation from the $S_{sp}$ values at the maximal volume of the autophagic compartment, 6 h after vinblastine treatment, shows that the number of autophagic elements can exceed 1000 in exocrine pancreatic cells.

The increasing interest in autophagy research goes along with the need to apply complex methodological approaches. In spite of many new possibilities [20, 22], electron microscopy remains an option and in some cases may prove to be indispensable. The $S_{sp}$ method might be a good and simple choice for solving problems where approximation of changes in size distribution and number is necessary.

## Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 832704, 11 pages
http://dx.doi.org/10.1155/2014/832704

*Review Article*

# The Role of the Selective Adaptor p62 and Ubiquitin-Like Proteins in Autophagy

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Received 4 April 2014; Revised 15 May 2014; Accepted 19 May 2014; Published 12 June 2014

Academic Editor: Ioannis P. Nezis



The ubiquitin-proteasome system and autophagy were long viewed as independent, parallel degradation systems with no point of intersection. By now we know that these degradation pathways share certain substrates and regulatory molecules and show coordinated and compensatory function. Two ubiquitin-like protein conjugation pathways were discovered that are required for autophagosome biogenesis: the Atg12-Atg5-Atg16 and Atg8 systems. Autophagy has been considered to be essentially a nonselective process, but it turned out to be at least partially selective. Selective substrates of autophagy include damaged mitochondria, intracellular pathogens, and even a subset of cytosolic proteins with the help of ubiquitin-binding autophagic adaptors, such as p62/SQSTM1, NBR1, NDP52, and Optineurin. These proteins selectively recognize autophagic cargo and mediate its engulfment into autophagosomes by binding to the small ubiquitin-like modifiers that belong to the Atg8/LC3 family.

## 1. Introduction

Two major pathways accomplish regulated protein catabolism in eukaryotic cells: the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal system. The UPS serves as the primary route of degradation for thousands of short-lived proteins and many regulatory proteins and contributes to the degradation of defective proteins [1]. Autophagy, by contrast, is primarily responsible for degrading long-lived proteins and maintaining amino acid pools during stress conditions, such as in chronic starvation [2]. The critical factors that direct a specific substrate to one degradation route or the other are incompletely understood. Protein degradations performed by the UPS and autophagy were regarded for a long time as complementary but separate mechanisms [3]. However, on the basis of recent studies, there are overlaps between them. The way of degradation of a misfolded, redundant, or unneeded protein may be often governed by the momentary activity or capacity of these systems or, in some cases, determined by strict regulation. Moreover, the two pathways use common adaptors capable of directing ubiquitinylated target proteins to both.

## 2. Ubiquitin-Proteasome System

The ubiquitin-proteasome pathway plays a crucial role in governing many basic cellular processes, such as normal protein turnover, protein quality control by degrading misfolded and damaged proteins, signal transduction, metabolism, cell death, immune responses, and cell cycle control [4]. Ubiquitin is a small, globular protein containing 76 amino acid residues (Figure 1). There are only three amino-acid changes from yeast to human, so ubiquitin is highly conserved within eukaryotes. Ubiquitinylation, the covalent conjugation of ubiquitin to other proteins, is a special posttranslational modification, which may either serve as an essential degradation signal for proteins or it may alter their localisation, function, or activity.

Before being covalently attached to other proteins, free ubiquitin is activated in an ATP-dependent manner with the formation of a thiolester linkage between a ubiquitin-activating enzyme (E1) and the carboxyl terminus of ubiquitin. Then, it is transferred to a ubiquitin-conjugating enzyme (E2). Finally, E2 associates with ubiquitin-ligases (E3s) which specifically bind the target substrate and attach ubiquitin

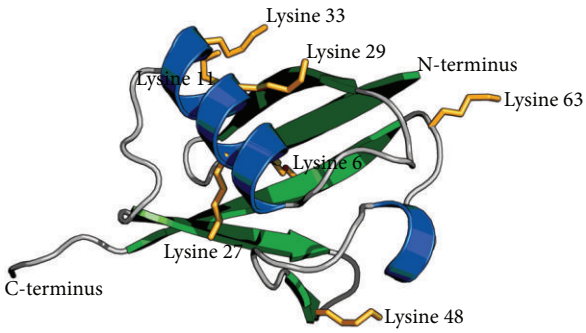


Figure 1: Ribbon model of ubiquitin exposing all the seven lysine side chains possibly involved in polyubiquitinylation reactions.

through its carboxyl terminal glycine to the $\varepsilon$-amino group of a lysine residue in the target protein (Figure 2). The exact details of ubiquitinylation biochemistry are determined by the type of E3 enzyme involved. E3s can be grouped into two major classes: HECT (homologous to E6-AP carboxyl-terminus) domain E3s and RING-finger (really interesting new gene) domain E3s [5]. The identification of E6-AP as the E3 responsible for the human papilloma virus E6-dependent ubiquitinylation of p53 led to the discovery of the HECT domain enzymes [6]. HECT domain is a conserved C-terminus of the molecule, which is about 350 amino acids long. HECT domain E3s form thiolester intermediates with ubiquitin through a conserved cysteine residue, like in case of E1 and E2 enzymes. By contrast, RING-finger E3s do not generate a thiolester intermediate but just simply act as a scaffold to hold a ubiquitin-E2 intermediate close to a substrate and catalyze ubiquitin transfer [7] (Figure 2).

The high specificity of the UPS system is tightly associated with the E3 enzymes, as they determine which substrate should be ubiquitinylated and hence usually degraded. Whether the attached ubiquitin is a modification signal or a sign for degradation depends on how it is linked to its substrates: conjugation of a single ubiquitin monomer (monoubiquitinylation) or sequential conjugation of several ubiquitin moieties (polyubiquitinylation) of variable length.

The ubiquitin chain could be lengthened by the E2 and E3, sometimes with the help of an accessory factor (E4). The carboxyl terminal glycine of the more distal ubiquitin molecule is bound to the previous ubiquitin molecule through an isopeptide bond with an $\varepsilon$-amino group of a lysine [8]. If the series of ubiquitin moieties is extended to at least four units, then it is sufficient to allow the ubiquitylated target protein to be recognized and degraded by the 26S proteasome [9].

The 26S proteasome is a 2.5 MDa multicatalytic multisubunit protease, which is made up of two subcomplexes: a barrel-shaped core particle (CP: also known as the 20S proteasome) and one or two 19S regulatory particle(s) (RP) on one or both ends of the core particle [10–12]. The 19S RP serves to recognize ubiquitinylated substrate proteins and plays a role in their unfolding and translocation into the interior of the 20S CP (Figure 2).

The 20S CP contains two outer $\alpha$-rings and two inner $\beta$-rings, each of which is made up of seven structurally similar $\alpha$ and $\beta$ subunits, respectively. The rings form an $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ structure creating three continuous chambers inside the particle. Only three of the $\beta$-type subunits ($\beta$1, $\beta$2, and $\beta$5) in each inner ring are catalytically active. They have threonine residues at their N-termini and show N-terminal nucleophile hydrolase activity. Such a "self-compartmentalized" structure keeps the proteolytic active sites separated in the central chamber and allows regulated substrate degradation only. The proteasome is a multicatalytic protease because the $\beta$1, $\beta$2, and $\beta$5 subunits are associated with caspase-like, trypsin-like, and chymotrypsin-like activities, respectively, which are able to cleave amide bonds at the C-terminal side of acidic, basic, and hydrophobic amino-acid residues, respectively.

The ubiquitin chains are called K6, K11, K27, K29, K33, K48, or K63 chains depending on which of the seven lysine (K) residues is involved in linkage of monomers in the polyubiquitin polymer (Figures 1 and 2). K48 ubiquitin chain was first identified as the signal to target proteins for proteasomal degradation. In contrast, K11 or K63 chains or single ubiquitin moieties (monoubiquitinylation) were thought to signal mainly for nonproteolytic functions [13]. These chain types are involved in controlling several processes such as gene transcription, DNA repair, cell cycle progression, apoptosis, and receptor endocytosis [14]. However, recent reports have demonstrated that all types of ubiquitin chains as well as monoubiquitinylation can target substrates for degradation via autophagy [15].

## 3. Ubiquitin-Like Proteins

There are more and more ubiquitin-like proteins (Ubls) identified and characterized. They resemble ubiquitin, as for all Ubls whose covalent attachment to other biomolecules has been experimentally demonstrated, the C-terminal residue is a glycine, and the carboxyl group of this glycine is the site of attachment to substrates [16]. On substrate proteins lysine side chains are the target sites so the Ubl and substrate are connected with an amide (or isopeptide) bond. Ubls also share a similar structural motif, the $\beta$-grasp fold, which contains a $\beta$-sheet with four antiparallel $\beta$-strands and a helical segment (Figure 3).

## 4. Autophagy

Autophagy is another degradative pathway that occurs in all eukaryotic cells. It is the main system for the degradation of bulk cytoplasmic components in the cell, and it is induced by nutrient starvation for example. Autophagy is crucial for homeostasis in the cell, as it recycles proteins and organelles. In addition, autophagy plays a critical role in cytoprotection by preventing the accumulation of toxic proteins and acting in various aspects of immunity, including the elimination of invading microbes and its participation in antigen presentation. Macroautophagy is the best characterized type of autophagy. In this case the cell forms a double-membrane sequestering compartment called the phagophore, which

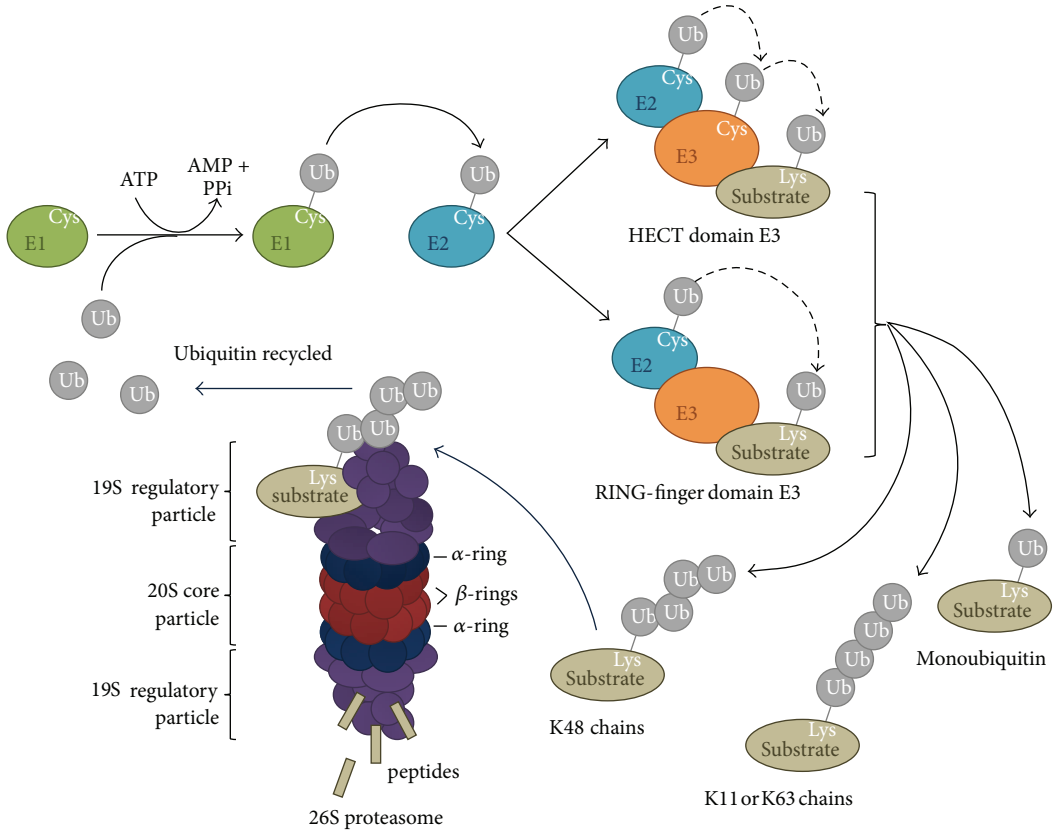


FIGURE 2: The ubiquitin-proteasome system. An enzyme cascade organizes the attachment of mono- or polyubiquitin to the substrates. Ubiquitin (Ub) is first activated in an ATP-consuming reaction by E1 (Ub-activating enzyme), to which it becomes attached by a high-energy thiolester bond. Then, the activated Ub is shifted to the active Cys residue of E2 (ubiquitin-conjugating enzyme). E2 catalyzes the transfer of ubiquitin to the substrate protein with the help of E3 (ubiquitin ligase). There are two major classes of E3 enzymes, characterized by the HECT domain or the RING-finger domain. In case of the HECT E3 enzymes, the activated Ub is transferred first to an active Cys residue in the HECT domain before it is finally moved to the substrate. RING-finger domain E3 enzymes bind to both the E2 enzyme and the substrate and catalyze the transfer of Ub directly from the E2 enzyme to the substrate. A polyubiquitin chain linked through Lys 48 is the signal for the proteasome to degrade the substrate. The 26S proteasome consists of the catalytic 20S core particle; a barrel of four stacked rings: two outer $\alpha$-rings (blue) and two inner $\beta$-rings (red); and the 19S regulatory particle. The polyubiquitin chain is recognized by the regulatory particle, which then binds, unfolds, and translocates the polypeptide into the catalytic core. The substrate is hydrolyzed by the enzymatically active $\beta$-subunits inside the core particle producing short peptides. Ubiquitin is recycled in the process [102, 103].

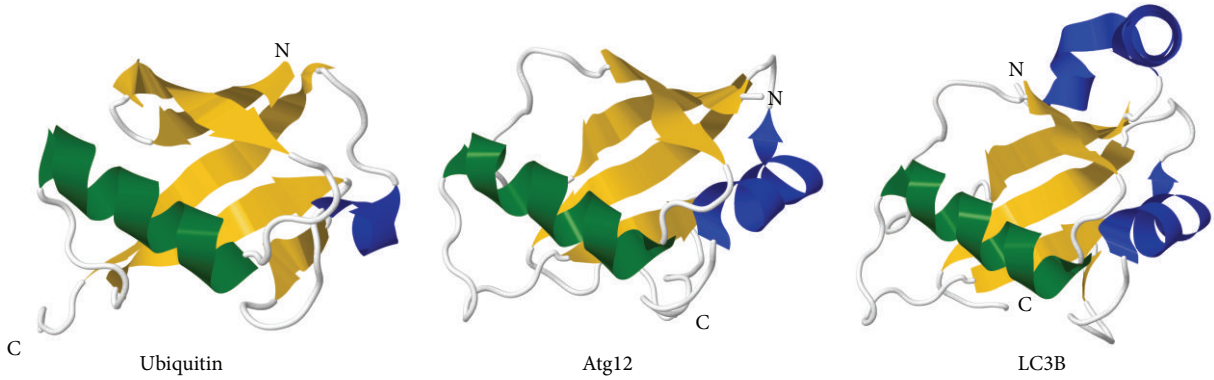


FIGURE 3: Structures of ubiquitin and the ubiquitin-like proteins (Ubls) Atg12 and LC3B, shown as ribbon diagrams generated by Jmol 13.0 [104] upon the structural data deposited in PDB. The characteristic Ubl $\beta$-grasp fold: a $\beta$-sheet with four antiparallel $\beta$-strands (yellow) and a helical segment (green) is well observable. Other helical structures are blue (Protein Data Bank (PDB) accession codes: 1UBQ [105], 4GDK [106], and 1UGM [107], resp.).

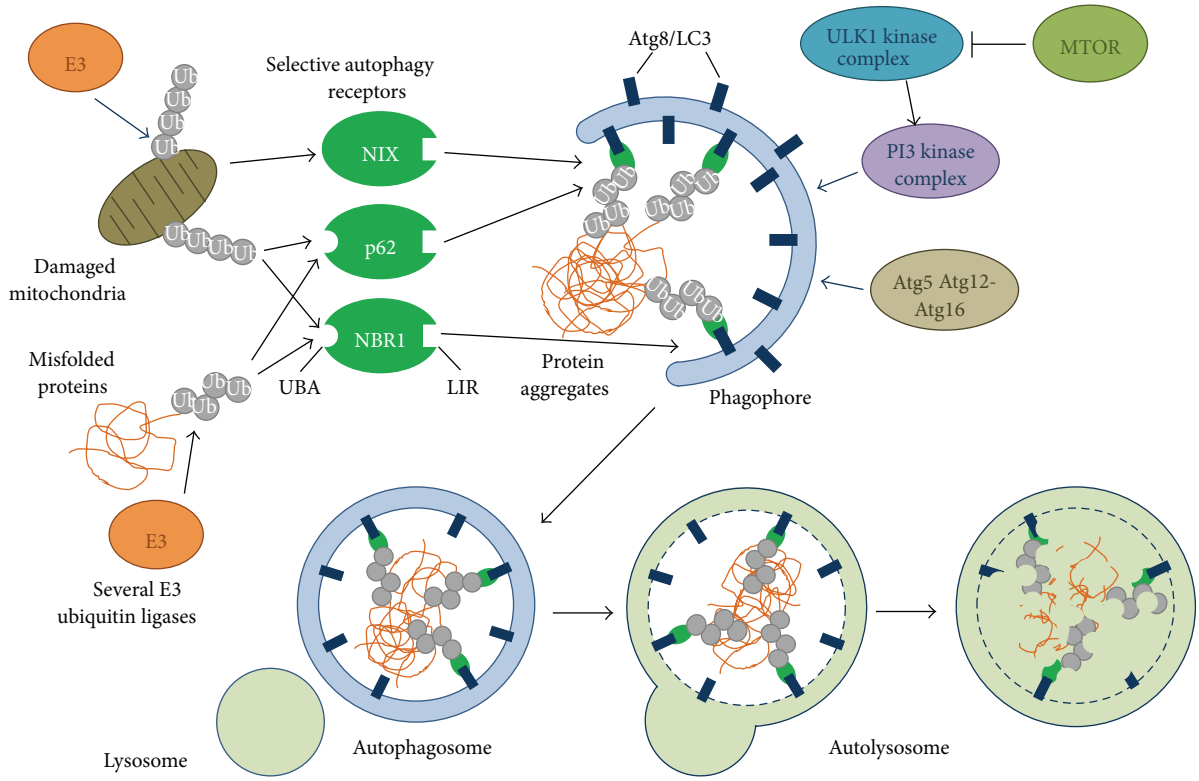


Figure 4: The process of autophagy. Initiation of autophagy is controlled by the ULK1 complex, followed by activation of the PI3-kinase complex leading to nucleation of the phagophore. Vesicle expansion is governed by two ubiquitin-like conjugation systems: the Atg5-Atg12-Atg16 and Atg8/LC3 pathways. Finally, autophagosomes fuse with lysosomes forming autolysosomes, where breakdown of the autophagic cargo takes place. Selective autophagy can distinguish and direct specific cargos to the lysosome. Autophagy receptors contain a short LIR (LC3-interacting region) sequence responsible for Atg8/LC3 binding. Recognition of ubiquitinylated proteins is mediated by interacting with ubiquitin noncovalently, via an ubiquitin-binding domain (UBA). NIX acts as a mitophagy receptor; it has a LIR motif but lacks an UBA domain and is localized within the mitochondrial outer membrane; this is why ubiquitinylation is not required for NIX-dependent delivery of damaged mitochondria to autophagosomes.

develops into an autophagosome. After fusion with lysosomes, the content of the resulting autolysosome is degraded and the newly generated monomers are released back into the cytosol for reuse [2, 17] (Figure 4).

There are 38 known autophagy-related (Atg) genes regulating the steps of autophagosome formation and breakdown. These were identified in yeast genetic screens but they are evolutionarily well conserved also in plants and animals, including *Drosophila* and mammalian cells [18, 19]. Initiation of autophagy is controlled by the Atg1/ULK complex, consisting of Atg1, Atg13, Atg17, Atg29, and Atg31 in yeast and ULK1/2, mAtg13, FIP200, and Atg101 in mammals. The ULK1/2, mAtg13, and FIP200 proteins form a complex independently of nutrient supply. MTORC1 (mechanistic target of rapamycin complex 1) phosphorylates and inhibits ULK1/2 and mAtg13 in nutrient-rich conditions, disrupting the contact between ULK1 and AMPK, an energy sensor kinase with activating effect on ULK1. On the contrary, MTOR is released from its complex under starvation, resulting in activation of ULK1/2 (Figure 4), which, in turn, phosphorylates and activates mAtg13 and FIP200 [20].

The transmembrane protein Atg9 and regulators of its trafficking (Atg2 and Atg18) play a role in membrane delivery to the expanding phagophore after the assembly of the Atg1 complex at the single phagophore assembly site (PAS), which is marked by the selective cargo proaminopeptidase I aggregate in yeast. Nucleation of the phagophore at the PAS is controlled by the phosphatidylinositol-3-kinase (PI3 K) complex (Vps34/hVPS34, Vps15/hVPS15, Vps30/Atg6/Beclin 1, and Atg14/ATG14L). Finally, there are two Ubl conjugation systems: the Atg12 (Atg5, Atg7, Atg10, Atg12, and Atg16) and Atg8 (Atg3, Atg4, Atg7, and Atg8) pathways which are responsible for vesicle expansion [18, 21] (Figure 4).

Autophagosomes undergo a maturation process in animal cells, which involves the recruitment of the SNARE protein syntaxin 17 [22–24]. Interaction of syntaxin 17 with the HOPS (homotypic fusion and vacuole protein sorting) tethering complex promotes the fusion of autophagosomes

with lysosomes, where breakdown of autophagic cargo takes place [25, 26] (Figure 4).

Macroautophagy has long been considered as a nonselective process responsible for bulk degradation of cytoplasmic components. The autophagy pathway appeared during evolution as an adaptation mechanism of the eukaryotic cell to starvation, allowing mobilization of nutrients in the cell by forfeit materials of the cytosol. Additionally, it became indispensable for specific degradation of unnecessary or toxic structures: proteins, organelles, and intracellular pathogens [27]. In contrast to the bulk autophagy, which ensures the more or less random sequestration of cytosol, selective autophagy operates under nutrient-rich conditions as well and is characterized by the presence of specialized autophagosomes. These autophagosomes lock up substrates in an exclusive way, which means that other parts of the cytoplasm are largely absent from them [18, 28, 29] (Figure 4).

*4.1. Atg12 and Atg8.* Autophagy requires the Ubls Atg12 and Atg8/LC3 (Figures 3 and 4). Atg12, which is 2.5 times larger than ubiquitin, was the first Ubl identified as a core autophagy protein [30]. It is synthesized in an active form that does not require proteolytic maturation. The C-terminal glycine of Atg12 is first activated by the E1 enzyme Atg7, and is then transferred to an E2 enzyme, Atg10, before finally forming a conjugate with Atg5 [30]. This Atg12-Atg5 conjugate is essential for autophagy. This system is well conserved in mammals; there is only one orthologue for each of the components of the Atg12 system in mice and humans [21].

Atg8, the other Ubl regulator of autophagy, is expressed with a C-terminal arginine residue in yeast, which is removed by the cysteine protease Atg4 leaving a glycine residue at the C-terminus [31]. Biochemical studies revealed the existence of another ubiquitinylation-like conjugation system [32]. The C-terminal glycine residue of Atg8 is activated by the same E1-like enzyme, Atg7, as in case of Atg12. Then Atg3, an E2-like enzyme, together with an Atg12-5-16 complex catalyzes the transfer of the activated Atg8 to phosphatidylethanolamine, the target lipid substrate. This way Atg8 becomes tightly membrane associated. Atg8 therefore can be utilized as a marker of the autophagosomal membrane and a key molecule during autophagosome formation (Figures 3 and 4). The conjugation of Atg8 to and its removal from phosphatidylethanolamine are essential for autophagy. There are three families of Atg8 homologues in mice and humans called LC3s, GABARAPs, and GABARAP-like proteins.

*4.2. Selective Autophagy and Its Specific Adaptors.* In the last decade, emerging evidence revealed that autophagy can distinguish and direct specific cargos to the lysosome. Different terms were coined to distinguish between different targets. The most investigated processes are mitophagy: the selective removal of defective or excess mitochondria [33]; aggrephagy: the disposal of aberrant, misfolded protein aggregates [34]; xenophagy: the selective autophagy of pathogenic intracellular bacteria, protozoa, or viruses [35, 36], and pexophagy: peroxisome autophagy first described in detail during peroxisome degradation in methylotrophic yeast species but also responsible for the destruction of 70–80% of the peroxisomal mass in mammalian cells [37]. The selective nature of autophagy is ensured mainly by specific adaptors, but direct interactions between the target molecule and the core autophagy machinery are also observed.

A molecule convenient to link a process with its substrate needs to carry at least two distinct functional domains: one that recognizes the target and another that transports it to the site of operation. How does it work in the case of selective autophagy? The best known mechanism to solve the problem of distinction between the different cytoplasmic components deemed for engulfment is to bring properly marked cargos to the inner surface of the growing phagophore. Accordingly, the precise delivery is generally ensured by interaction of the adaptor both with the membrane-anchored form of Atg8/LC3 and the main targets that are usually polyubiquitinylated (Figure 4).

The first clues for the role of protein ubiquitinylation as a signal for selective autophagy came from Atg knockout mice and some *Drosophila* experiments. They showed that the loss of basal autophagy in the brain resulted in large-scale accumulation of ubiquitinylated proteins [38–40].

Recognition of ubiquitinylated proteins during autophagy is mediated by ubiquitin receptors interacting with ubiquitin noncovalently, via their ubiquitin-binding domains. p62/SQSMT1 (hereafter p62), the first protein reported to have such an adaptor function [41], was originally discovered as a scaffold in signaling pathways regulating cell growth and proliferation; however, it was also detected in ubiquitinylated protein aggregates [42] (Figure 4). p62 possesses a C-terminal ubiquitin-binding domain (UBA) [43] and a short LIR (LC3-interacting region) sequence responsible for LC3 interaction [41]. In addition, it has a PB1 domain promoting self-aggregation and association with other adaptors such as NBR1, neighbour of BRCA1 gene 1 [15] (Figure 5). Knockout studies in mice and *Drosophila* revealed that p62 is required for the aggregation of ubiquitinylated proteins and thus plays essential roles for their autophagic clearance [44, 45]. The levels of p62 usually inversely correlate with autophagic degradation, as the loss of Atg genes or factors required for the fusion of autophagosomes with lysosomes all result in a marked increase of p62-positive aggregates [46, 47]. p62 can also deliver ubiquitinylated cargos to the proteasome, although they are mainly degraded by autophagy [48, 49].

Another adaptor used in selective autophagy is the above-mentioned *NBR1*, which, via its own PB1 domain, is able to interact with p62, and through its own UBA domain and LIR it can participate in the recruitment and autophagosomal degradation of ubiquitinylated proteins [50]. In plants, a functional hybrid homologue of p62 and NBR1 (NBR1 in Arabidopsis, Joka2 in tobacco) plays an important role in the disposal of polyubiquitinylated proteins accumulated under abiotic stress conditions [51, 52].

*Optineurin* and *NDP52* have been recently described as xenophagy receptors, utilizing the autophagic machinery for restriction of ubiquitinylated intracellular pathogens [53]. Both of them also participate in the clearance of protein

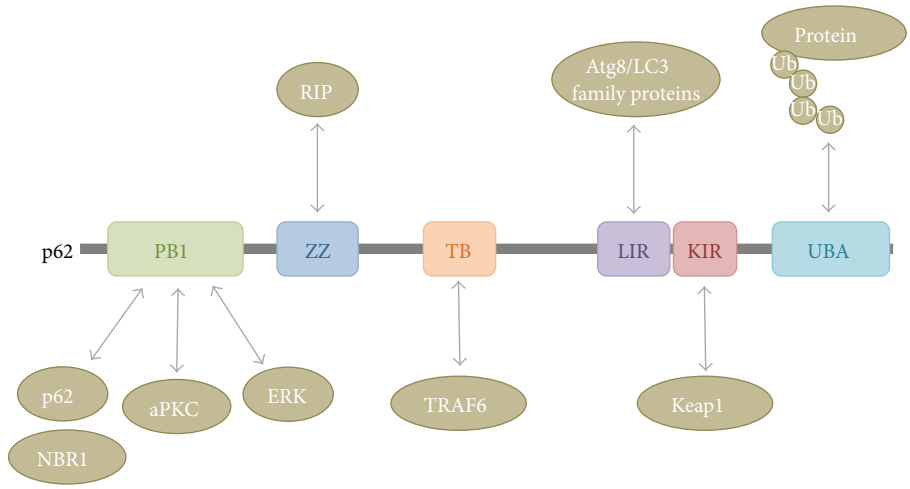


Figure 5: Domain structure of p62 and its interacting partners. There are six main domains/motifs in the p62 protein, necessary for its interaction with the autophagic machinery and with signaling pathways. The N-terminal Phox and Bem1 (PB1, 21-103 aa) domain is involved in the self-oligomerization of p62 or in heterodimerization with NBR1, a protein similar to p62. The PB1 domain is also responsible for the binding to atypical PKC (aPKC) or to ERK1. The central zinc finger ZZ domain (128-163 aa) and the TRAF6-binding domain (TB, 225-250 aa) interact with the RIP and TRAF6 proteins, respectively, to regulate the NF-$\kappa$B pathway. Through the LC3-interacting region (LIR, 321-345 aa) and the C-terminal ubiquitin-associated domain (UBA, 386-440 aa), p62 links the autophagic machinery to ubiquitinylated protein substrates to promote the selective degradation of these molecules. Finally, the Keap-interacting region (KIR, 346-359 aa) binds Keap1 leading to stabilization and nuclear translocation of the transcription factor Nrf2, engaged in the control of ROS level.

aggregates [54, 55] and are required for the regulation of NF-$\kappa$B signaling [56, 57].

While these receptors all mediate degradation of ubiquitinylated cargos, there are other more specific adaptors acting on removal of damaged or surplus mitochondria (e.g., Atg32 in yeast and NIX in mammals) or peroxisomes (such as Atg30 and Atg36). They recognize particular binding partners on the surface of their target organelle and, through their LIR sequence, ensure their delivery to the maturing autophagosome [58, 59]. It is worth noting that additional autophagic adaptors may be identified by software prediction of LIR sequences in suspected protein candidates [60] (see a recent review for more details on the structural basis of how the Atg8/LC3 and Atg12 Ubls interact with specific autophagy adaptors [21]).

*4.2.1. Role of p62 in Autophagosome Formation.* As individual p62-ubiquitin interactions are rather weak, the starting point of the polyubiquitinylated aggregate formation is presumably the p62 self-oligomerization via its PB1 domain [61]. However, the original "simple" concept of delivery through bridging the polyubiquitin side chain on the cargo and the Atg8/LC3 decoration on the phagophore surface by p62 is now changing. In fact, these aggregates containing p62 and ubiquitinylated proteins may even serve as a nucleating scaffold for autophagosome biogenesis, potentially by binding multiple Atg proteins [61–63].

Moreover, it was recently reported that phagophores may preferentially form at p62 aggregates near lysosomes in *Drosophila* cells, which is very similar to the location of PAS near the vacuole/lysosome in yeast [64, 65]. It is worth noting that p62 also associates with MTORC1 [66]. MTORC1 is active when bound to lysosomes and promotes cell growth and inhibits autophagy by phosphorylating Atg1 (ULK1/2) [67–69]. These data suggest the direct assembly of early autophagic structures on the surface of protein aggregates, which may be mediated by interactions between p62 and upstream Atg proteins. Later on, Atg8/LC3 will be recruited to the forming phagophore, and the growing double membrane will enclose the p62-containing aggregate due to interactions between p62, Atg8/LC3, and other Atg proteins [70, 71].

*4.2.2. p62 in Autophagy Regulation.* The role of p62 in the regulation of autophagy is controversial. It was suggested to promote MTORC1 activation by contributing to its translocation to the lysosomal surface. Therefore, p62 reduction, similarly to MTORC1 inactivation, may activate autophagy [72]. However, in HEK293 and HeLa cells p62 was suggested to liberate Beclin1 (an Atg6 homologue) by disrupting the association of Bcl-2 and Beclin1, and thus p62 may positively regulate the induction of bulk autophagy [73]. In addition, p62 interacts with and regulates the deacetylase activity of HDAC6, a known modifier of F-actin network involved in selective autophagy [74]. In carcinoma cells, while p62 silencing suppressed cell proliferation and induced autophagy, abnormal autophagosomes were found and p62 inhibition finally resulted in autophagic cell death [75]. We have recently found that p62 is not required for proteasome inhibition-induced autophagy in *Drosophila* fat body cells [76]. Thus, the role of p62 in autophagy induction appears to be complex and probably context-dependent.

As p62 can shuttle between the nucleus and the cytoplasm (in the nucleus it is thought to recruit proteasomes to nuclear

polyubiquitinylated protein aggregates), it can even export ubiquitinylated substrates from the nucleus into the cytosol, where autophagy offers a more robust degradative capacity [77].

*4.2.3. Cytoplasmic p62 Level as an Autophagy Indicator.* Since p62 itself is removed from the cytoplasm mainly by autophagy, its amount is generally considered to inversely correlate with autophagic activity [46, 47]. Accumulation of p62-positive inclusions during immunocytochemistry or elevated p62 levels on Western blots are frequently used as signs of autophagy impairment. In some cases, transgenic p62 reporter systems are also used to monitor the rate of autophagic degradation, although their use requires caution as overexpressed p62 tends to self-aggregate and may no longer indicate autophagy activity [78]. In addition, long term starvation may positively influence the amount of p62 in certain mammalian cell types, via both its transcriptional upregulation and promoting *de novo* p62 protein synthesis by providing autophagy-derived amino acids [49].

## 5. Interplay between p62 and Signaling Pathways

p62 was originally described as a scaffold protein ensuring the formation of signaling hubs, since, through different binding domains, it can establish interactions with many types of enzymes. As a consequence, it is able to integrate signaling routes involving particular kinases and ubiquitin-mediated pathways (Figure 5). This way, p62 regulates inflammatory processes in TNF$\alpha$-activated cells. The complex including the RIP kinase, atypical PKCs and TRAF6, and a K63 ubiquitin ligase (interactions formed through the ZZ, PB1, and TB domain of p62, resp.) plays a critical role in the phosphorylation of IKK$\beta$ leading to activation of the NF-$\kappa$B transcription factor [79]. Enhanced p62 level (under inflammatory conditions induced by impaired proteasomal degradation) was demonstrated to contribute to elevated IL-1$\beta$ production: p62 was found to bind the JNK and ERK kinases, hence further increasing NF-$\kappa$B activation and, as a consequence, pro-IL-1$\beta$ expression. In addition, p62 accumulation was found to promote caspase-1 activation in inflammasomes, which is required for IL-1$\beta$ proteolytic processing [80]. Interestingly, an opposite effect of p62 is suggested in Legionella-infected p62-deficient mice that showed more severe pulmonary inflammation than control animals, because the production and secretion of IL-1$\beta$ was significantly enhanced due to elevated caspase-1 activity in their macrophages [81].

p62, likewise in association with TRAF6 and aPKCs, is needed for the NF-$\kappa$B-mediated neuronal survival and differentiation in response to NGF [82] and also for osteoclastogenesis [83]. p62 mutations are among the genetic alterations that play a role in Paget disease of bone, where osteoclasts are overactive because of disturbed NF-$\kappa$B signalization [84]. The p62-NF-$\kappa$B connection has a role in tumorigenesis as well, since p62 is necessary to NF-$\kappa$B-dependent survival in Ras-transformed cells [85].

The autophagy adaptor function of p62 also has an impact on the NF-$\kappa$B signaling pathway. In human monocytes, high level of inflammation due to autophagy impairment is associated with p62 accumulation and the consequent overactivation of the NF-$\kappa$B pathway [86]. In accordance with the positive role of p62 in caspase-1 activation [80], a previous study demonstrated that stimulated autophagy, by enhanced degradation of p62, also eliminates activated inflammasomes and reduces inflammation, while blocking autophagy has an opposite effect [87]. In addition, NF-$\kappa$B signalization may be regulated directly by the rate of NF-$\kappa$B removal. Targeted degradation of the p62-NF-$\kappa$B p65 subunit complex by p62-mediated selective autophagy may play a key role in bone marrow derived macrophage differentiation [88].

The important role of p62 in innate immunity does not only rely on regulation of immune signaling responses. As an autophagy adaptor, p62 takes part in the elimination of ubiquitinylated intracellular pathogens; some infecting agents even target this step to escape from the defensive system of the cell. The coxsackievirus B3, through the activity of one of its proteases, cleaves p62 which results in impairment of selective autophagy and host defense [89]. Moreover, selective autophagy induced by pathogen-specific TLR4 activation requires transcriptional upregulation of p62 [90]. Interestingly, p62 also participates in the synthesis of neoantimicrobial peptides, by bringing inactive precursors such as Fau to autophagic degradation, where they are processed to active fragments [91].

p62 is also involved in the regulation of apoptosis. p62-mediated aggregation is needed for the activation of polyubiquitinated caspase-8 [92]. It was shown recently that caspase-8 colocalizes not only with p62, but also with Atg8/LC3 and Atg5, and its full self-processing requires the autophagosomal membrane as a platform for the assembly of the death-inducing signaling complex [93]. On the other hand, failure of autophagy may contribute to enhanced apoptosis because of impaired degradation of p62-complexed apoptosis proteins, as found in T-cells [94], while in autophagy-inhibited cancer cells, caspase-8 dependent cell death was mainly associated with the concomitantly elevated p62 level [95].

Another well-known signaling pathway influenced by p62 is the oxidative stress response, which is regulated by the Keap1-Nrf2 system. Through its KIR motif (Figure 5), p62 is able to bind to Keap1, a Cullin3-ubiquitin E3 ligase complex adaptor protein. In turn, Keap1-promoted polyubiquitinylation and subsequent proteasomal degradation of the transcription factor Nrf2 are inhibited. As a consequence, the expression of cytoprotective, antioxidant Nrf2 target genes is increased [96, 97]. Moreover, the p62 gene itself is a target for Nrf2; thus, the appropriate oxidative stress response is supported by a positive feedback regulation between p62 and Nrf2 [98]. Autophagy has a strong impact on Nrf2 activation, since p62 not only disrupts Keap1-Nrf2 interaction but also removes Keap1 from the cytosol via selective autophagy [99]. The well-known antioxidant effect of sestrins is, at least partly, due to their influence on the p62-dependent autophagic degradation of Keap1 [100]. In case of autophagy impairment, accumulation of p62 and the subsequent overactivation of

Nrf2 may contribute to development of liver carcinomas [96]. Interestingly, in these cancer cells, phosphorylation of p62 by the MTORC1 complex increases its affinity for Keap1, so MTORC1 activity further enhances stabilization of Nrf2 and the transcription of its target genes [101].

## 6. Conclusions

(i) Ubiquitin and ubiquitin-like proteins (Ubl) share functional similarity. The different Ubls are activated and conjugated to substrates by similar biochemical mechanisms.

(ii) Ubiquitinylation is frequently needed for substrate recognition and renders selectivity to autophagy in eukaryotes.

(iii) The connection between ubiquitinylation and autophagy is provided by autophagic adaptor proteins (or autophagy receptors), which bind both ubiquitin and autophagy specific Ubl modifiers like Atg8/LC3 family proteins.

(iv) Atg8/LC3 is required for the biogenesis of autophagosomal membrane and also mediates selective autophagy via the recruitment of LIR-containing autophagy receptors that recognize and select cargo.

(v) Autophagy receptors such as p62 regulate the selective autophagosomal degradation of large protein aggregates, mitochondria, and bacterial pathogens.

(vi) p62 may play an important role also as a regulator of autophagy; moreover, it may even be involved in the formation of the autophagosome.

(vii) As a scaffold protein, p62 operates in signaling pathways which, through the link provided by p62, can also be regulated by selective autophagy.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

The authors thank Vilmos Tóth for his excellent assistance in completing Figure 3. They apologize to the investigators whose works are not cited here.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 851349, 19 pages
http://dx.doi.org/10.1155/2014/851349

*Research Article*

# Atg6/UVRAG/Vps34-Containing Lipid Kinase Complex Is Required for Receptor Downregulation through Endolysosomal Degradation and Epithelial Polarity during *Drosophila* Wing Development

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Received 24 January 2014; Accepted 1 April 2014; Published 21 May 2014

Academic Editor: Gábor Juhász



Atg6 (Beclin 1 in mammals) is a core component of the Vps34 PI3K (III) complex, which promotes multiple vesicle trafficking pathways. Atg6 and Vps34 form two distinct PI3K (III) complexes in yeast and mammalian cells, either with Atg14 or with UVRAG. The functions of these two complexes are not entirely clear, as both Atg14 and UVRAG have been suggested to regulate both endocytosis and autophagy. In this study, we performed a microscopic analysis of UVRAG, Atg14, or Atg6 loss-of-function cells in the developing *Drosophila* wing. Both autophagy and endocytosis are seriously impaired and defective endolysosomes accumulate upon loss of Atg6. We show that Atg6 is required for the downregulation of Notch and Wingless signaling pathways; thus it is essential for normal wing development. Moreover, the loss of Atg6 impairs cell polarity. Atg14 depletion results in autophagy defects with no effect on endocytosis or cell polarity, while the silencing of UVRAG phenocopies all but the autophagy defect of Atg6 depleted cells. Thus, our results indicate that the UVRAG-containing PI3K (III) complex is required for receptor downregulation through endolysosomal degradation and for the establishment of proper cell polarity in the developing wing, while the Atg14-containing complex is involved in autophagosome formation.

## 1. Introduction

Autophagy mediates the degradation of cytoplasm and organelles in eukaryotic cells. A set of evolutionarily conserved Atg proteins is required for autophagosome formation in yeast, *Drosophila*, and mammals [1, 2]. Autophagosomes then fuse with lysosomes to deliver their cargo for degradation, which requires the autophagosomal SNARE syntaxin 17 [3–5]. Activation of the Atg1 kinase complex leads to the initiation of autophagy, which is followed by the action of a class III phosphoinositide 3-kinase (PI3K) complex [6]. This lipid kinase complex is involved in multiple vesicle trafficking processes in yeast in addition to autophagy, namely, endosome maturation and biosynthetic transport to the vacuole, the yeast equivalent of lysosomes [7].

Autophagy related gene 6 (Atg6/Vps30 in yeast, Beclin 1 in mammals) is a core component of the Vps34 complex and is best known for its crucial role in the induction of autophagy [8, 9]. In addition to Atg6, the PI3K (III) core complex is composed of a regulatory subunit (vacuolar protein sorting 15—Vps15) and a catalytic subunit (Vps34) responsible for the production of phosphatidylinositol-3-phosphate (PI3P) from phosphatidylinositol (PI) [7, 10–12]. This membrane lipid localizes to early endosomes and the internal vesicles of multivesicular bodies (MVBs) in mammalian cells [13]. Vps34 and thus PI3P are required for the sorting of hydrolytic enzymes to the lysosome/vacuole [14], autophagosome formation, endocytic trafficking, and the regulation of cell polarity [15–18].

The core PI3K (III) complex is able to bind multiple regulator proteins and forms distinct complexes in yeast and mammalian cell lines. An Atg14-containing complex I is proposed to function in autophagy, whereas the UVRAG- (ultraviolet radiation resistance associated-) containing complex

II is considered to be involved in endocytosis and vacuolar protein sorting [7, 10–12]. In yeast and mammalian cells, Atg14 is considered to be autophagy specific and is required for autophagosome formation [19–22], whilst UVRAG (also known as Vps38) has been shown to regulate late stages of autophagy and endocytic trafficking, and it may also interact with the class C Vps complex to promote vesicle tethering [12, 23–25]. However, Atg14 has recently been suggested to promote endocytic traffic based on shRNA experiments in cultured human cells [26].

*Beclin 1*, the mammalian homolog of yeast *ATG6*, is a potential haploinsufficient tumor suppressor gene, but such a role is not entirely evident based on data from human cancer patient samples [27–29].

Although the role of Atg6/Beclin 1 in autophagy is undoubted, its role in other processes, such as endocytosis, is less clear. It was shown that the expression of human Beclin 1 is able to rescue the autophagy, but not the vacuolar protein sorting defects in ATG6/VPS30 null mutant yeast [27]. This is further supported by the observation that in mammalian cells the maturation of cathepsin D in the lysosome is normal in cells that express little Beclin 1 [30]. This finding was supported by another group showing that the silencing of Beclin 1 suppresses autophagy, but not other PI3K (III) dependent processes [31]. This raises the question whether Beclin 1 is essential only for autophagy and dispensable for the role played by PI3K (III) in endocytic trafficking and lysosomal sorting [8]. In contrast, another work carried out on cultured human cells suggested that Atg6 is essential for endocytic degradation of epidermal growth factor receptor (EGFR) as a component of an UVRAG-containing PI3K (III) complex [32]. Although numerous key binding partners for Beclin 1 are involved in a variety of cell biological processes, such as endocytosis, endosomal sorting, and maturation, the direct evidence for the participation of Beclin 1 in these processes remains to be demonstrated *in vivo* [7]. *Drosophila* Atg6 was shown to be required for autophagy [33, 34]. Furthermore, fluid-phase endocytosis is interrupted in Atg6 mutant larval fat body cells, and the number of Rab5 positive early endosomes is markedly decreased [34]. This latter finding is in contrast with observations in pupal wing cells where the RNAi of Vps15 resulted in the accumulation of late endosomes [17]. These findings raise the possibility that the roles of Atg6 may differ in distinct cell types even within one organism and that multiple PI3K (III) complexes may exist in *Drosophila*.

In this paper we show that, in *Drosophila*, Atg6 is an essential regulator of endolysosomal maturation in the developing wing. We show that endocytosis is seriously impaired in the developing Atg6 depleted pupal wing cells: dense multivesicular bodies and multilamellar bodies accumulate, which represent aberrant late endosomes and endolysosomes. We show that Atg6 as an endocytosis regulator is essential for the downregulation of multiple signaling pathways regulating wing development such as Notch and Wingless. The knockdown of Atg6 results in several serious defects in the development of the wing tissue which is a consequence of disorganization of cell adhesion proteins and disturbed cell polarity. In addition, we show that RNAi knockdown of UVRAG, but not of Atg14, phenocopies the endolysosomal trafficking and cell polarity defects seen in Atg6 loss-of-function cells. In the case of autophagy, we find that both Atg14 and Atg6 are required, whereas UVRAG appears to be largely dispensable.

## 2. Materials and Methods

*2.1. Drosophila Strains and Genetics.* Fly stocks used in this study are listed in Table S1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2014/851349. Flies were raised on standard yeast/cornmeal/agar media, at 25°C, 50% humidity, and a 12-hour light/12-hour dark daily cycle, under uncrowded condition. To analyze the function of Atg6, UVRAG, and Atg14, transgenic RNAi flies were crossed to $Bx^{MS1094}$Gal4; UAS-Dicer2 lines. Progeny was used for the examination of the morphology of the adult wings and for electron microscopy. For the generation of Atg6 mutant wings, in which the large portion of the wing tissue is derived from homozygous Atg6 null-mutant clone cells, we used a modified method of Newsome and colleagues [35]. Briefly, we used $Bx^{MS1094}$Gal4 to drive the ubiquitous expression of FLP exclusively in wing disc cells, as we assumed that this system would provide continuous high levels of FLP activity throughout the proliferative phase of wing development, resulting in a high frequency of mosaicism. To increase the size of the clones, we used an FRT chromosome, where a Minute mutation was recombined onto the GFP marked FRT chromosome arm. As Minute mutations prevent the proliferation or survival of homozygous cells and retard the proliferation of heterozygous cells [36], we anticipated that wing tissue would mainly consist of Atg6 null-mutant cells. Therefore these wing discs or wings are referred to in the text as mutant discs or wings. In most cases, for fluorescent microscopy we used engrailed driven Gal4 (enGal4) to restrict the expression of the dsRNA-s to the posterior compartment of the developing wing, and since the area of the RNAi was marked by the expression of a fluorescent protein (GFP or RFP), the anterior part of the wing served as control. For null mutant clone generation P{neoFRT}82B$Atg6^1$/TM6Tb flies were crossed to hs-FLP; P{neoFRT}82B, P{w+ Ubi-GFP(S65T)nls}3R/TM6Tb flies. Progeny was heat shocked for 2 hours at 37°C at the second larval stage. Flies were then kept at 25°C.

*2.2. Antibodies.* Antibodies used in this study are listed in Table S2 with the corresponding dilutions, applications, and references.

*2.3. Histology and Microscopy.* 32 hour (after pupal formation (APF)) staged pupae were dissected and fixed with 4.0 (w/v)% paraformaldehyde (PFA) in PBS for 90 minutes at room temperature (RT), then were redissected in PBS to remove the wing cuticles. Third instar larval wing discs were dissected in ice cold PBS, then fixed with 4.0% PFA in PBS (60 min, RT). Pupal wings and wing discs were processed for immunofluorescence microscopy under the same conditions as follows: samples were incubated in 0.1%

(v/v) Triton X-100 in PBS (PBTX, 30 min, RT), then in blocking solution (5.0% (v/v) FCS in PBTX). Samples were then incubated in the blocking solution completed with primary antibodies (overnight (ON), 4°C). Samples were then rinsed (3×), washed in PBTX (3 × 10 min, RT), and incubated in blocking solution (30 min, RT). Samples were then incubated with the corresponding secondary antibodies diluted in blocking solution. Washing steps were repeated, nuclei were stained with Hoechst 33342 (10 $\mu$g/mL in PBS), and the samples were mounted in Vectashield (Vector). For rhodamine-phalloidin staining wings were fixed as described above, then were incubated in PBTX (10 min, RT). Wings were then incubated in blocking solution (1% FCS in PBTX), followed by rhodamine-phalloidin (0.5 $\mu$g/mL) in blocking solution completed with Hoechst (40 min, RT). Wings were then washed extensively with PBS, then examined. For *ex vivo* endocytic trafficking assay, wing imaginal discs were dissected in ice cold M3 medium, then incubated with anti-Notch extracellular domain antibody (in M3, 4°C, 10 min), chased for 3 hours (in M3, RT), then washed extensively in PBS, and fixed as described above. Incorporated antibody was detected with the corresponding secondary antibody as described above. All reagents used for light microscopy were obtained from Sigma-Aldrich, otherwise indicated. TUNEL-assays were performed as in [37]. To capture images, we used a Zeiss Axioimager Z1 microscope equipped with an ApoTome unit using AxioCam MRm camera with AxioVision 4.82 software. GFP intensity profiles were created using Image J software. Primary images were edited using Adobe Photoshop CS5 software: area of interest was cropped, and if it was necessary, brightness and contrast were adjusted.

### 2.4. Ultrastructural Analysis

*2.4.1. Transmission Electron Microscopy.* Thirty-hour (APF) staged pupae were dissected and fixed with 2% formaldehyde, 0.5% glutaraldehyde, 3 mM $CaCl_2$, and 1% sucrose in 0.1 M Na-cacodylate, pH 7.4 (overnight, 4°C). Samples were then redissected in Na-cacodylate to remove the wing cuticles, and then the dissected wings were postfixed in 0.5% osmium tetroxide (60 min, RT) and in half-saturated aqueous uranyl acetate (30 min, RT), dehydrated in graded series of ethanol, embedded in LR white according to the manufacturer's instructions, and cured for 24 hours at 60°C. Ultrathin sections were stained with 4% uranyl acetate in 50% methanol (for 8 min) and lead citrate (for 3 min). Grids were analyzed in JEOL JEM 1011 transmission electron microscope operating at 60 kV. Images were taken using Olympus Morada 11 megapixel camera and iTEM software (Olympus). All reagents and materials used for electron microscopy were obtained from Sigma-Aldrich.

*2.4.2. Acid Phosphatase Cytochemistry.* Pupal wings were fixed and dissected as described above, were washed in 0.05 M Na-acetate buffer pH 5.0 (3 × 5 min, RT), and then were incubated in Gömöri's medium (5 mM $\beta$-glycerophosphate and 4 mM lead nitrate dissolved in 0.05 M acetate buffer) for 30 min at RT. Samples were then washed in acetate buffer (3 × 5 min) and processed for electron microscopy as described above. Ultrathin sections were analyzed unstained. Substrate free medium was used for control experiments.

*2.4.3. DAB Staining.* Pupal wings were fixed and dissected as described above and were washed in quenching buffer (30 min, 50 mM glycine and 50 mM $NH_4Cl$ in 0.05 M Tris buffer (TB), pH = 7.6), followed by a 10 min wash in TB. Wings then were preincubated in 1% $H_2O_2$ in TB in order to block endogenous peroxidases. Next, wings were washed extensively (3 × 10 min in TB) and then were preincubated with DAB (0.5 mg/mL DAB in TB, 10 min). Wings were then incubated in DAB reaction buffer (0.01% $H_2O_2$ and 0.5 mg/mL DAB in TB, 20 min), and DAB reaction was terminated by extensive washing (3 × 10 min in TB). Wings were then processed for electron microscopy as described above. Ultrathin sections were analyzed unstained. $H_2O_2$-free DAB reaction buffer was used for control experiments.

*2.4.4. Immuno-EM.* Pupae were dissected and fixed with 4% formaldehyde, 0.05% glutaraldehyde, and 0.2% picric-acid in phosphate buffer (0.1 M PB, pH 7.4 overnight, and 4°C). Samples were then redissected in PB to remove the wing cuticles and were washed extensively with PB and free aldehyde groups were quenched with 50 mM glycine and 50 mM $NH_4Cl$ in PB. Wings were then postfixed in 1% uranyl acetate in 0.05 M maleate buffer (3 h, RT). Wings were then dehydrated in graded series of ethanol as follows: 25% EtOH (10 min, 0°C), 50% EtOH (10 min, 0°C), 70% EtOH (10 min, −20°C), 96% EtOH (20 min, −20°C), and Abs EtOH (2 × 60 min, −20°C). Samples were then infiltrated with pure LR white completed with 2% benzoyl peroxide as catalyst (24 hours, −20°C). Curing was performed with a homemade UV chamber using two 2 × 6 W UV lamp for 48 h (−20°C). Ultrathin sections (80–90 nm) were collected on formvar coated 100 mesh nickel grids. All the immunoreactions were carried out on humidified Parafilm coated 96 well plates on RT, otherwise indicated. The following procedure was performed: (1) 5% $H_2O_2$ for 1 min; (2) biDW (bidistilled water) for 3 × 5 min; (3) 0.1% $NaBH_4$ in TBS (pH: 7.6) for 10 min; (4) 50 mM glycine in TBS for 30 min; (5) TBS for 3 × 5 min; (6) 10% FCS in TBS for 30 min; (7) anti-GFP in 5% FCS-TBS overnight at 4°C; (8) 2% FCS in TBS for 3 × 5 min; (9) 18 nm gold-conjugated secondary antibody in 2% FCS in TBS for 90 min; (10) 3 × 5 min TBS; (11) 1% glutaraldehyde in TBS for 10 min; (12) extensive wash with biDW. Ultrathin sections were stained with uranyl acetate (for 15 min) and lead citrate (for 1 min).

*2.5. Quantification and Statistical Analysis.* From images of pupal wings we randomly took sample quadrates of 100 × 100 pixels using Adobe Photoshop CS5 extended v.12.0 software from both control and Atg6-, UVRAG-, or Atg14-depleted domains of the wings. Percentage of the area covered by the fluorescent markers indicated above was measured using Image J software. In case of wing discs, we used sample areas of 75 × 75 pixels from the wing pouch exclusively.

Percent of area covered by the signal of anti-Delta or anti-Notch antibodies was measured with the method described formerly. Effect of Atg6 null mutations on Notch EC domain localization was quantified by selecting 30 × 30 pixels measurement areas containing clone or control cells exclusively. In sampling, we chose the nearest neighboring area (in less than a distance of 100 pixels) of the selected clone cell group. The percentage of signal-covered area was measured using Image J. We performed the statistical analysis applying the indicated tests and constituted the box plot figures using IBM SPSS Statistics 21 software. On box plots, bars show the data ranging between the upper and lower quartiles; median is indicated as a horizontal black line within the box. Whiskers plot the smallest and largest observations, while dots and asterisks indicate outliers. $P < 0.05$ was considered to be significant. NS means $P > 0.05$, $*$ means $P < 0.05$, $**$ means $P < 0.01$, and $**$ means $P < 0.001$. For details and results of statistical analyses, see Table S3.

*2.6. RT-PCR.* RT-PCR experiments were performed following standard protocols. Total RNA and cDNA were prepared using Direct-zol RNA MiniPrep (R2051-Zymo Research) and RevertAid First Strand cDNA Synthesis Kit (K1621, Thermo Scientific) from RNAi and control L3 larvae, and these were used as template for PCR reactions with the following primers: Atg6: 5′-CGGAGTTATCTTTGCCCATCTA-3′ and 5′-GGCGTTGATCTCTGACCAGT-3′, UVRAG: 5′-CCACACTGGTGTTGGAGCTA-3′ and 5′-CCGAACGGC-AAATGCGTTGA-3′, and Atg14: 5′-CTGGGTCTTCTG-GACAGCAT-3′ and 5′-GAGTTTTCGTCCTCTGACTC-3′. Actin was used as loading control (5′-GTCGCTTAGCTC-AGCCTCG-3′ and 5′-TAACCCTCGTAGATGGGCAC-3′).

## 3. Results

*3.1. Drosophila Atg6 Is Required for PI3P Production, Endosomal Trafficking, and Lysosome Maturation, Similar to UVRAG.* To analyze the functional role of Atg6, UVRAG, and Atg14 in *Drosophila*, we first established that we can selectively inhibit the expression of these genes by transgenic RNAi. RT-PCR experiments revealed that systemic expression of Atg6, UVRAG, or Atg14 dsRNA strongly reduced the mRNA level of the corresponding genes. Interestingly, we found that the depletion of Atg6 also reduced the mRNA level of Atg14 (Figure 1), although not to the extent seen in the case of Atg14 RNAi. While the reason for this observation is not known, it is in line with a previous report showing that Beclin 1 siRNA treatment reduces Atg14 expression levels in cultured cells [32].

*3.1.1. Atg6 and UVRAG Are Required for PI3P Production.* As PI3K (III) core components Vps15 and Vps34 have been shown to promote endocytosis in *Drosophila* [15, 17] and Vps34 has been shown to physically interact with Atg6 [15], first we examined the *in vivo* activity of the PI3K (III) complex in Atg6 knockdown cells. For this purpose we used animals in which two FYVE domains fused to GFP (GFP-2xFYVE) was

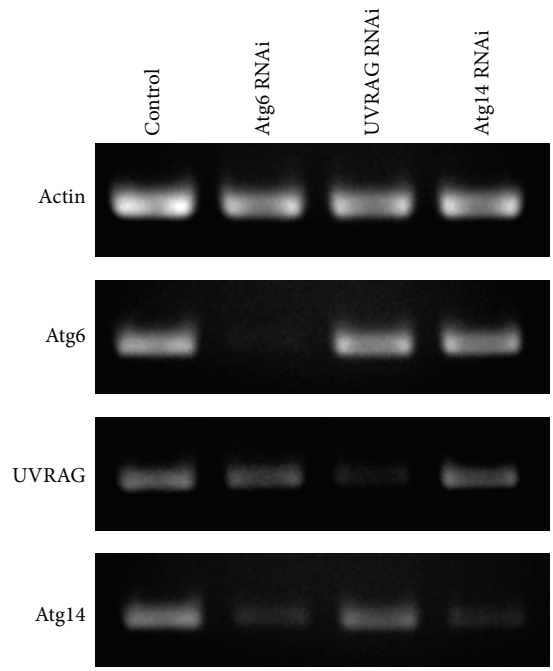


Figure 1: RT-PCR analysis of Atg6, UVRAG, or Atg14 transcripts from animals expressing transgenic RNAi constructs. Expression of Atg6, UVRAG, or Atg14 RNAi by tubGal4 strongly reduced the mRNA level of the corresponding genes. Interestingly, we found that the RNAi of Atg6 also decreased the mRNA level of Atg14.

expressed. This reporter protein selectively binds to PI3P containing membranes in wild type cells (Figure 2(a)). We found that in Atg6 RNAi pupal wing cells the GFP-2xFYVE loses its localization to endosomes but rather becomes dispersed within the cytoplasm. This suggests that PI3K (III) activity is dramatically reduced in pupal wing cells when cells lack Atg6, since in the absence of PI3P this marker is no longer able to associate with endosomal membranes (Figures 2(b) and 2(e)). This is in line with the results obtained by Shravage and colleagues, as Atg6 depleted larval fat body cells showed a very similar phenotype [34]. We found that in contrast to Atg6, Atg14 may not be required for PI3P production in pupal wing cells as Atg14 RNAi had no significant effect on GFP-2xFYVE localization, whilst UVRAG RNAi had very similar effect to Atg6 RNAi (Figures 2(c), 2(d), and 2(e)). Thus, PI3P is likely associated with endosomes rather than autophagosomes in this tissue.

*3.1.2. Depletion of Atg6 and UVRAG Results in the Accumulation of Defective Endolysosomes.* While there are some controversial data that Atg6 is required for endocytosis, detailed examination of the effect of Atg6 loss-of-function on different endosome populations is lacking. Therefore we next investigated the effects of Atg6 RNAi on several endosomal and lysosomal markers in pupal wings. First we used the endocytosis marker clathrin light chain-GFP (Clc-GFP) which labels clathrin-coated vesicles, and we could not detect any difference between Atg6 RNAi cells and control cells (Figures 2(f)–2(h)). Interestingly, that was not the case when later stages of endocytosis were examined.

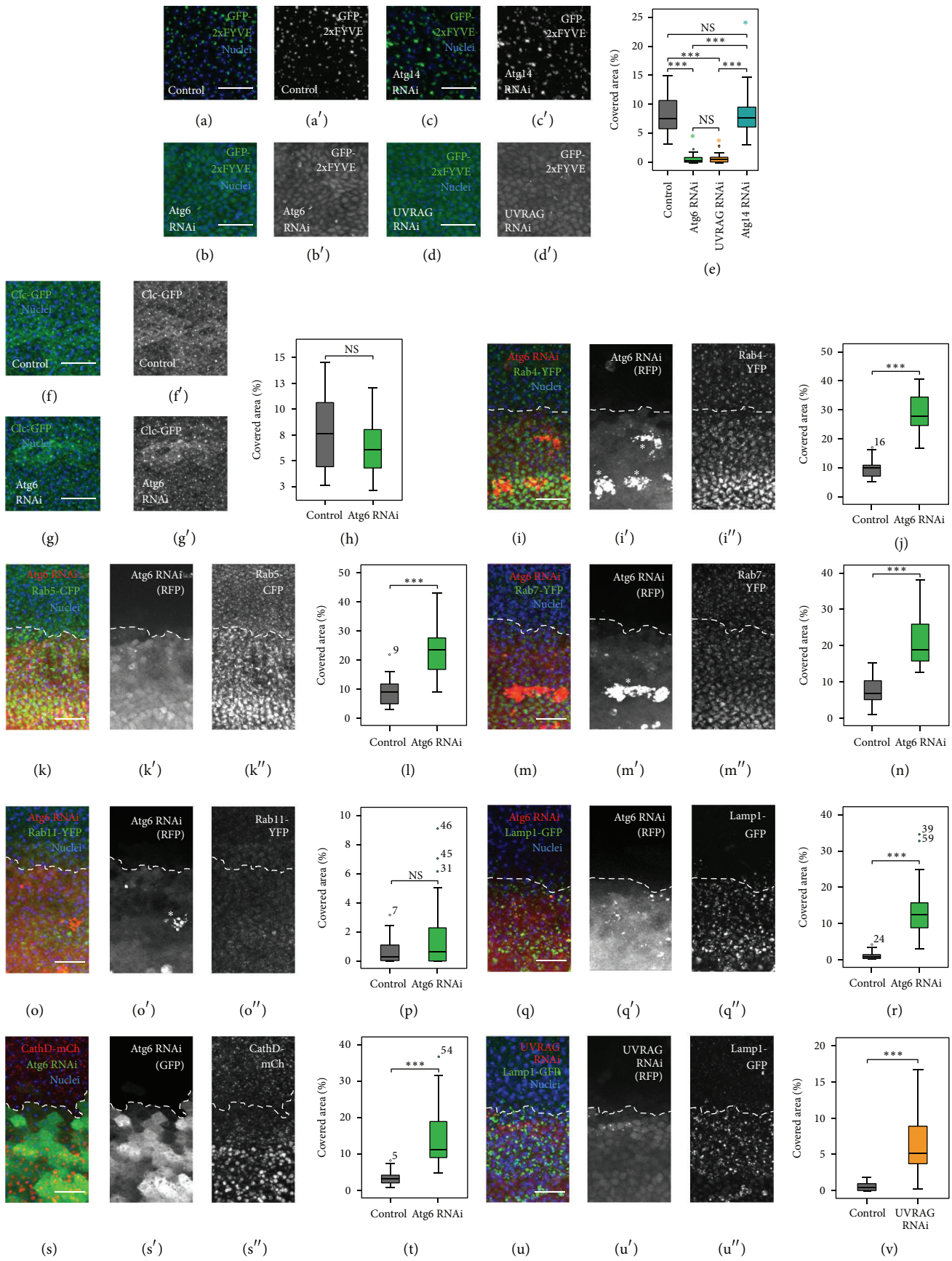


Figure 2: Continued.

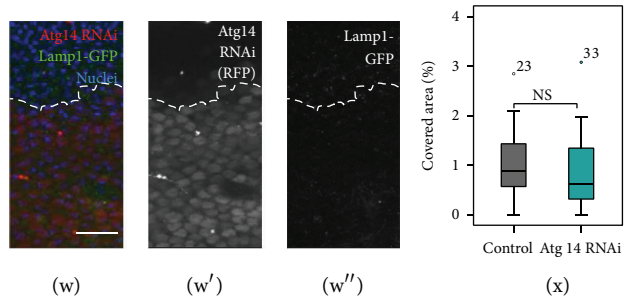


Figure 2: Knockdown of Atg6 and UVRAG. (a) The PI3P marker GFP-2xFYVE localized to dots and vesicle-like structures in wild type pupal wings. (b), (d) In Atg6 or UVRAG depleted wings the GFP-2xFYVE became dispersed within the cytoplasm, indicating a failure in PI3P production. (c) In contrast to Atg6 or UVRAG RNAi wings the localization of GFP-2xFYVE did not differ from controls in Atg14 RNAi wings. (e) Quantification of (a)–(d). (f), (g) In control wings clathrin coated vesicle marker Clc-GFP showed punctate pattern which was unaffected by Atg6 RNAi. (h) Quantification of (f), (g). (i)–(w) Images from pupal wings expressing endosomal and lysosomal fluorescent markers controlled by a constitutive promoter (tubulin promoter for Rab-FP-s and Lamp1-GFP, cathD promoter for CathD-mCherry) and expressing RNAi constructs by enGal4. The region of RNAi is marked by the coexpression of RFP or GFP and the borderline of the region of RNAi is indicated with a dashed white line. (i)–(p) The number of structures positive for endosomal markers ((i): Rab4-YFP; (k): Rab5-CFP; (m): Rab7-YFP, except (o): Rab11-YFP) was significantly increased in Atg6 RNAi cells. (j), (l), (n), and (p) Quantification of (i), (k), (m), and (o), respectively. (q)–(t) The number of lysosomes marked by Lamp1-GFP or CathD-mCherry was significantly increased in Atg6 depleted cells. (r), (t) Quantification of (q), (s), respectively. (u) UVRAG RNAi resulted in the accumulation of Lamp1-GFP structures very similar to Atg6 RNAi. (v) Quantification of (u). (w) RNAi of Atg14 did not alter the number of Lamp1-GFP positive dots. (x) Quantification of (w). On images from (i) to (w), the intensity of the markers is enhanced by immunostaining the wings with an anti-GFP or anti-mCherry. Note the presence of RFP positive hemocytes under the epithelia on (i), (m), and (o) (marked by asterisks). On box plots, bars show the data ranging between the upper and lower quartiles; median of the signal covered areas is indicated as a horizontal black line within the box. Whiskers plot the smallest and largest observations, while dots and asterisks indicate outliers. NS means $P > 0.05$ and $* * *$ means $P < 0.001$. For details and exact $P$ values of statistical analyses, see Table S3. For genotypes see Table S4. Scale bars represent 15 $\mu$m.

For this purpose we used animals expressing endosomal and lysosomal reporters controlled by a constitutive promoter and expressing RNAi constructs by enGal4. As the expression of the RNAi was restricted to the posterior compartment of the wing, the anterior part could serve as control. We found a significantly increased area of Rab4-YFP, Rab5-CFP, and Rab7-YFP positive dot-like structures, and mostly similar number of Rab11-YFP positive dots in the regions where the dsRNA of Atg6 was expressed. This implies that inactivation of PI3K (III) by Atg6 RNAi results in the accumulation of early and late endosomes (Figures 2(i)–2(p)). These results together suggest that Atg6 as a component of *Drosophila* PI3K (III) core complex is involved in endosomal maturation.

As late endosomes mature into lysosomes, their luminal pH continues to decrease and their membranes acquire lysosome specific proteins such as Lamp (Lysosome-associated membrane protein), and after fusion with lysosomes this process terminates as lysosomal hydrolases (such as cathepsins or acid phosphatases) degrade the luminal contents of these secondary lysosomes. Interestingly, it was found that depletion of Atg6 in pupal wing cells results in the massive accumulation of Lamp1-GFP positive and cathepsin D positive granules (Figures 2(q)–2(t)). A similar phenomenon was observed when the effect of the UVRAG RNAi was examined as cells lacking UVRAG accumulated numerous Lamp1-GFP positive granules (Figures 2(u) and 2(v)). In contrast, Atg14 RNAi cells had the same phenotype as control cells (Figures 2(w) and 2(x)). These results indicate that the UVRAG-containing lipid kinase complex is involved in endolysosomal maturation.

*3.1.3. Electron Microscopy of Cells Lacking Atg6 or UVRAG Reveals the Accumulation of Abnormal Endolysosomes.* Electron microscopy revealed that many aberrant late endosome-like structures, such as enlarged lucent or dense multivesicular body- (MVB-) like structures and multilamellar bodies (MLB) accumulated the apical cytoplasm of Atg6 RNAi pupal wing cells, whereas these structures were completely absent in control cells (Figures 3(a) and 3(b)). The ultrastructure of Atg6 null mutant cells was similar to Atg6 RNAi cells, further confirming this observation (Figure 3(c)). In order to decide whether this phenotype originates from the lack of autophagy, the RNAi of Atg8a was also examined. We found that the ultrastructure of the Atg8a RNAi cells was very similar to wild type cells (Figure 3(d)), suggesting that the abnormal structures in Atg6 depleted cells were not derived from the lack of autophagy. As it was expected from the Lamp1-GFP phenotype, the UVRAG RNAi pupal wing cells also accumulated numerous aberrant endolysosome-like structures, similar to Atg6 loss-of-function cells (Figure 3(e)). In contrast the ultrastructure of Atg14 RNAi cells was completely indistinguishable from wild type or Atg8a RNAi cells (Figure 3(f)).

Postembedding immunocytochemistry showed that the aberrant late endosomes and endolysosomes in Atg6 depleted cells were positive for LAMP1-GFP, indicating that they could be immature or malfunctioning lysosomes (Figures 4(a) and 4(b)). Interestingly, many of the MVBs were often found in the close vicinity of small dense vesicles. These small structures always showed acid phosphatase activity and were also found in wild type cells; therefore they likely represent

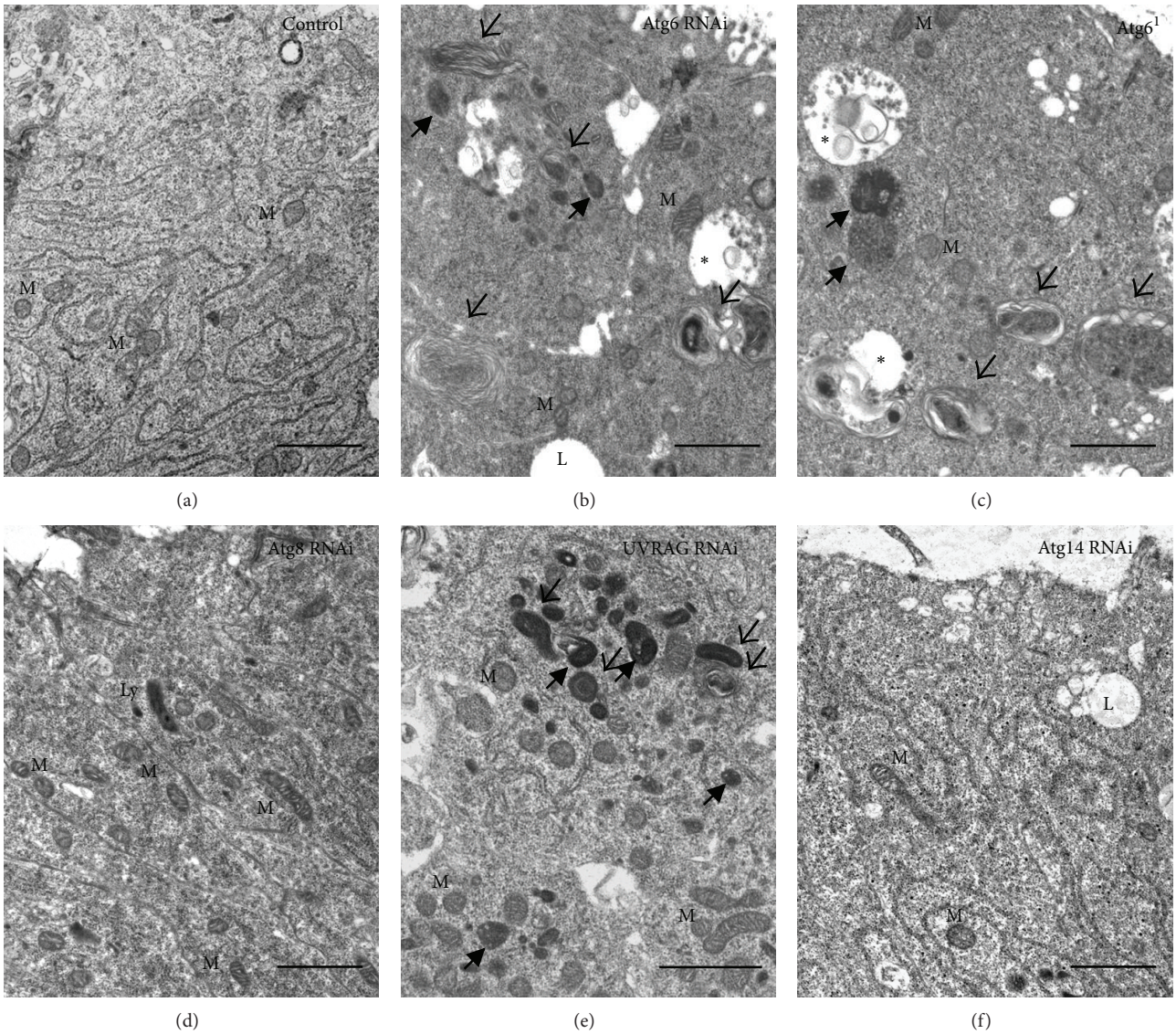


Figure 3: Electron microscopy of pupal wings. (a) Ultrastructure of control cells. (b) Depletion of Atg6 by RNAi resulted in the massive accumulation of enlarged multivesicular bodies (asterisks), dense multivesicular-body like structures (arrows), and multilamellar bodies (open arrows). (c) All of these structures could be found in Atg6 mutant pupal wings. (d) The depletion of Atg8a did not alter the ultrastructure of pupal wing cells. (e) Similar to Atg6, UVRAG RNAi also resulted in the accumulation of aberrant endolysosome-like structures (MVBs: arrows, MLBs: open arrows). (f) The depletion of Atg14 did not alter the ultrastructure of pupal wing cells. M: mitochondria, L: lipid droplet, and Ly: lysosome. For genotypes, see Table S4. Scale bars represent 1 $\mu$m.

primary lysosomes (Figures 4(c) and 4(d)). Furthermore, enzyme cytochemistry also revealed that a notable portion of the dense MVBs (58%, $N = 85$) and all of the MLBs showed acid phosphatase activity as well, whilst the electron-lucent MVBs never did (Figures 4(d)–4(f)). This raised the possibility that the sorting of lysosomal enzymes into MVBs and/or the maturation dynamics of MVBs into secondary lysosomes were seriously compromised in Atg6 depleted cells. As disrupted primary lysosomes or Golgi vesicles may also show acid phosphatase activity, a plasma membrane localized horseradish peroxidase enzyme (HRP-CD2) was expressed in the developing wing, to confirm the endosomal origin of the aberrant lysosome-like compartments in Atg6 depleted cells. This reporter protein can be visualized with routine diaminobenzidine (DAB) staining and due to the stability of the protein, this method allows one to label all membranes with plasma membrane origin, including all kinds of endosomes and secondary lysosomes as well [38]. We found that the aberrant structures accumulated in the Atg6 RNAi cells were all positive to DAB; therefore their endosomal origin is clearly confirmed (Figures 4(g) and 4(h)). Taken together our results strongly suggest that Atg6 has essential functions in endosomal and lysosomal maturation and sorting.

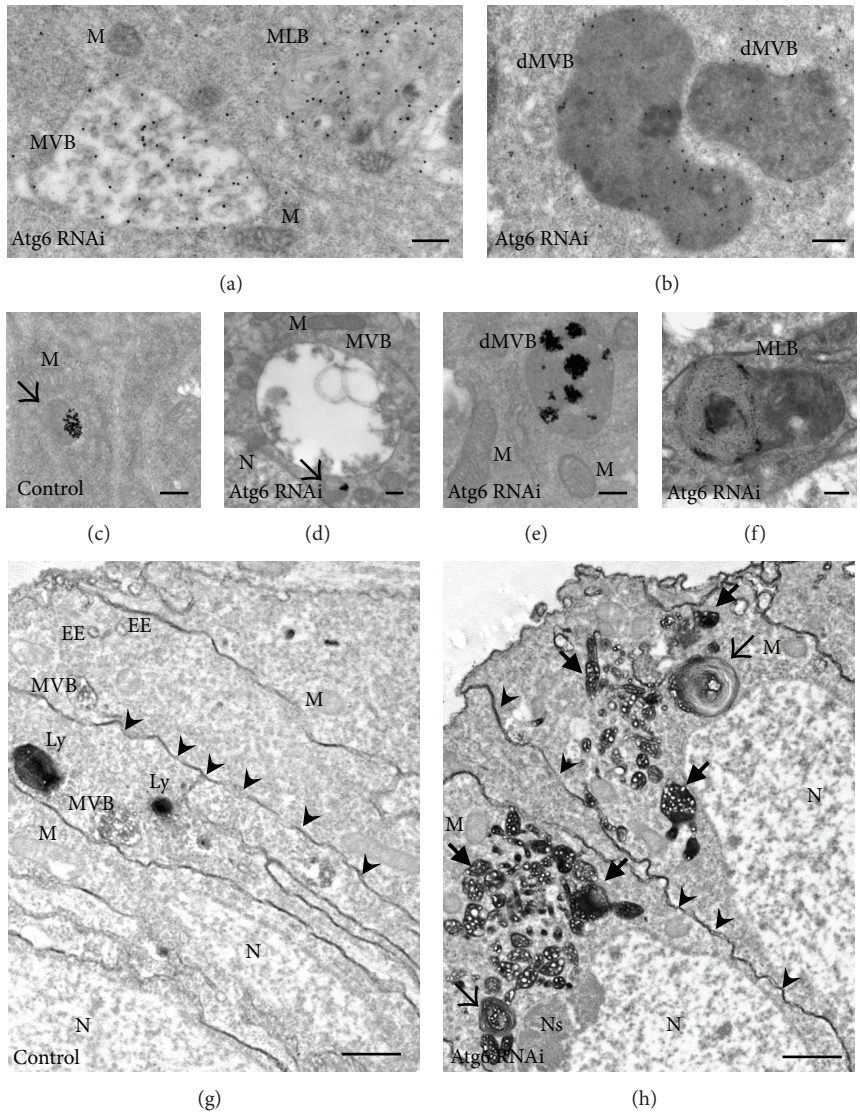


Figure 4: Identification of the abnormal organelles of Atg6 RNAi cells. (a), (b) Immunogold labeling of Atg6 RNAi cells with anti-GFP antibody to detect Lamp1-GFP. MVBs, dMVBs, and MLBs were positive to Lamp1-GFP. (c) Enzymecitochemistry revealed small, dense acid phosphatase positive structures (open arrow) in wild type pupal wing cells. Based on the size and morphology, these structures were considered as primary lysosomes. All other organelles were devoid of reaction product. (d) Electron-lucent MVBs in Atg6 depleted cells never contained acid phosphatase reaction product but were often found in the vicinity of acid phosphatase positive primary lysosomes (open arrow). (e) A dense multivesicular body-like structure (dMVB) showing acid phosphatase reaction product in an Atg6 RNAi pupal wing cell. (f) A multilamellar body (MLB) in Atg6 depleted cell positive to acid phosphatase. (g), (h) DAB-staining of pupal wings expressing plasma membrane localized horseradish peroxidase enzyme (HRP-CD2). (g) In control cells plasma membrane showed a prominent DAB staining (arrowheads), just as organelles with plasma membrane origin, such as early endosomes (EE), multivesicular bodies (MVB), and secondary lysosomes (Ly) as well. (h) The accumulated organelles in Atg6 depleted cells: the multivesicular bodies (arrows) and multilamellar bodies (open arrows) were all positive to DAB. M: mitochondria, MVB: multivesicular body, dMVB: dense multivesicular body-like structure, MLB: multilamellar body, N: nucleus, Ns: nucleolus, and Ly: lysosome. For genotypes, see Table S4. Scale bars represent 200 nm in (a)–(f) and 500 nm in (g), (h).

*3.2. Atg6 and Atg14 Are Required for Autophagy in the Wing, Unlike UVRAG.* As Atg6 and Atg14 have been shown to be required for autophagy in the larval fat body [34, 39], next we examined the effect of Atg6, Atg14, and UVRAG depletion on autophagy in the wing imaginal disc and pupal wing. *Drosophila* Myc has been shown to efficiently induce autophagy in various *Drosophila* tissues [40]. We overexpressed Myc in the patched (ptc) domain of the wing disc along with the autophagy marker mCherry-Atg8a, using ptcGal4. We found that compared to controls (Figure 5(a)), RNAi of Atg6 or Atg14 inhibited Myc-induced autophagy (Figures 5(b) and 5(c)), whilst UVRAG RNAi wing discs showed a phenotype similar to controls (Figure 5(d)). The role of Atg6 and Atg14 but not UVRAG in autophagy was

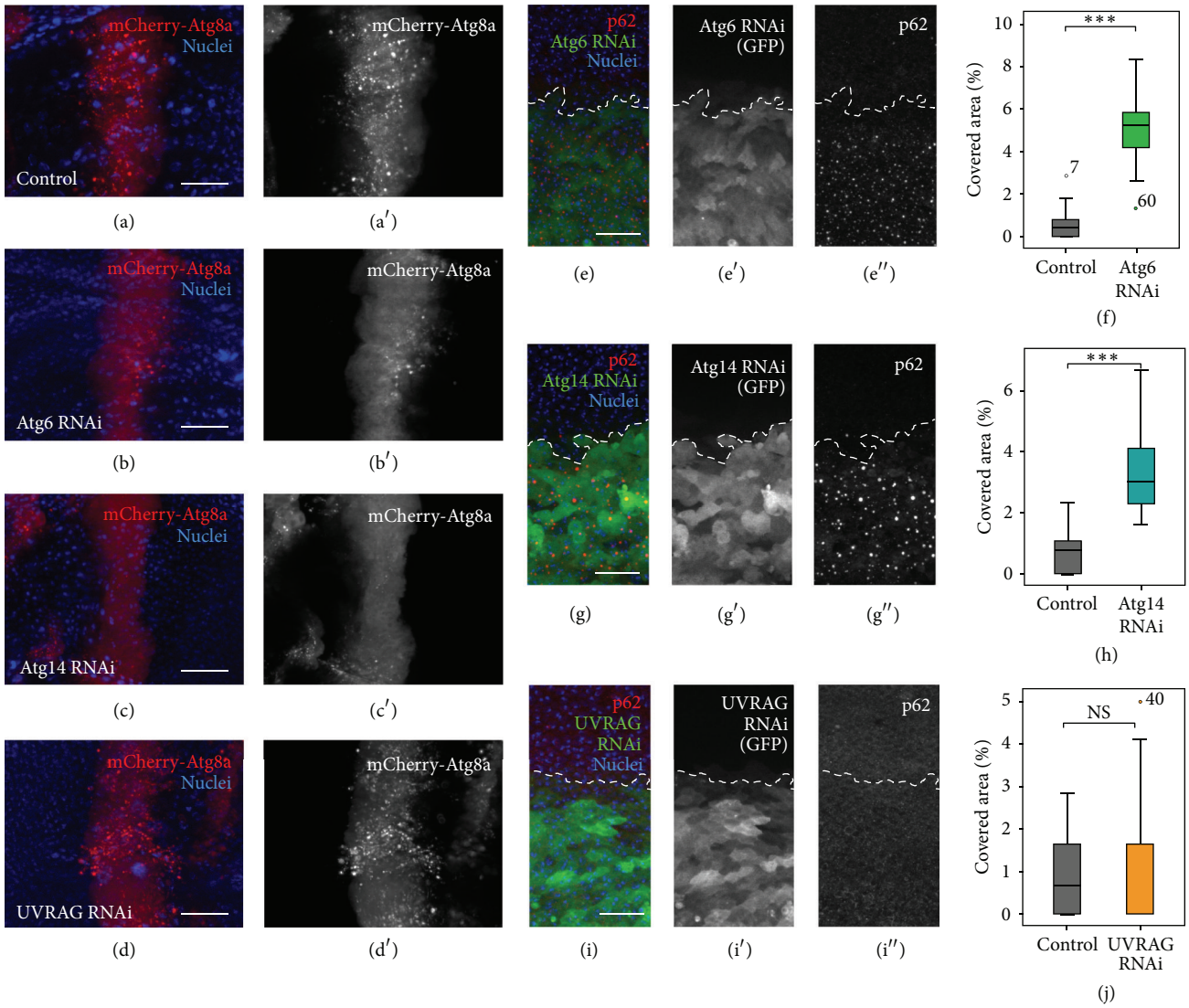


FIGURE 5: Atg6 and Atg14 are required for autophagy in contrast to UVRAG. (a)–(d) Larval wing discs expressing RNAi with transgenic Myc along with the autophagy marker mCherry-Atg8 in the patched (ptc) domain using ptcGal4. (a) In controls the expression of Myc induces massive autophagy in the ptc domain. (b) Atg6 or (c) Atg14 RNAi inhibited Myc-induced autophagy as the pattern of mCherry-Atg8 became dispersed in the citoplasm, whereas (d) UVRAG RNAi did not alter the pattern of the autophagy marker. (e), (g), and (i) Pupal wings expressing RNAi constructs by enGal4, immunostained against the selective autophagic cargo protein p62. (e) Atg6 or (g) Atg14 RNAi resulted in the massive accumulation of p62 aggregates. (i) UVRAG RNAi cells did not accumulate p62 aggregates. (f), (h), and (j) Quantification of (e), (g), and (i), respectively. On box plots, bars show the data ranging between the upper and lower quartiles; median of the signal covered areas is indicated as a horizontal black line within the box. Whiskers plot the smallest and largest observations, while dots and asterisks indicate outliers. NS means $P > 0.05$ and $***$ means $P < 0.001$. For details and exact $P$ values of statistical analyses, see Table S3. For genotypes, see Table S4. Scale bars represent 25 $\mu$m in (a)–(d) and 15 $\mu$m in (e), (g), and (i).

confirmed by immunostaining pupal wings against the selective autophagy cargo p62/Ref(2)P. This protein can be used to detect autophagy defects, as in such cases cells accumulate p62 aggregates [41–43]. We found a significantly increased number of p62 positive aggregates in the regions where the dsRNA of Atg6 (Figures 5(e) and 5(f)) or Atg14 (Figures 5(g) and 5(h)) was expressed. In contrast, UVRAG RNAi cells did not accumulate p62 (Figures 5(i) and 5(j)). These results together suggest that Atg6 and Atg14 are required for autophagy in wing discs and wings, while UVRAG is dispensable for autophagy in these tissues.

### 3.3. Atg6 Depletion Results in Defective Endocytic Degradation of Signaling Molecules and Enhances Notch Signaling, Similar to UVRAG

*3.3.1. Depletion of Atg6 or UVRAG Increases the Endosomal Retention of Notch, Unlike Atg14.* As it has been shown that Notch signaling is enhanced in mutants that increase endosomal retention [44], we assumed that the accumulation of late endosomes/endolysosomes in Atg6 depleted cells could result in enhanced Notch signaling. To assess this possibility, first we examined the cellular localization of Notch and Delta

(the ligand of Notch). As expected, Notch and Delta both accumulated in small, numerous puncta in the absence of Atg6, indicating that these molecules could be restrained from lysosomal degradation (Figures 6(a)–6(d), Figures S1(a) and S1(b)). Whilst UVRAG RNAi resulted in a similar phenotype to Atg6 RNAi, the depletion of Atg14 had no detectable effects on the localization of Notch (Figures 6(e)–6(h)). To further examine the effects of Atg6 RNAi, time-course *ex vivo* experiments were carried out. We incubated an antibody against the extracellular domain of Notch with live wing imaginal disc cells, and the surface bound antibody was allowed to get internalized and degraded. We found that the cell surface localized Notch was internalized normally but became trapped in vesicular structures in Atg6 depleted cells even at 3 h of chasing (Figure 6(i)). On the other hand, control cells successfully took up and degraded Notch over this time. Many of the Notch containing granules colocalized with the lysosome marker Lamp1-GFP in Atg6 RNAi cells, further suggesting that the degradation of Notch is impaired when cells lack Atg6 (Figure 6(i)).

*3.3.2. Notch Signaling Is Enhanced in Atg6 or UVRAG Loss-of-Function Cells, Unlike Atg14.* To examine Notch signaling activity in Atg6, UVRAG, or Atg14 loss-of-function cells, a Notch response element, EGFP (NRE-GFP), was used. The expression of this reporter depends on the transcriptional activity of Notch; therefore it can be used to show Notch signaling activity [45]. We found that compared to controls, the RNAi of Atg6 results in the enhancement of the reporter expression, which observation was very similar to the effect of UVRAG RNAi or wild type Notch protein overexpression (Figures 7(a), 7(b), 7(d), and 7(f)). The effect of Atg6 RNAi on the reporter expression was remarkably enhanced when wild type Notch protein was coexpressed in the wing imaginal disc cells (Figure 7(c)), further indicating that Notch signaling is highly activated in the lack of Atg6. In contrast to Atg6 or UVRAG, the RNAi of Atg14 had no significant effect on the reporter expression (Figure 7(e)).

*3.3.3. Depletion of Atg6 or UVRAG Increases the Endosomal Retention of Wingless, Unlike Atg14.* As endocytosis is required for proper Wingless (Wnt) signaling as well [46–48], the localization of this protein was also examined. We found that similar to Notch, Wnt also accumulated in small puncta in Atg6 or UVRAG RNAi cells, while Atg14 RNAi had no detectable effect on the pattern of Wnt (Figure 8). This suggests that Atg6 and UVRAG may be required for regulating several other signaling pathways besides Notch, unlike the Atg14-containing PI3K (III) complex.

*3.4. Depletion of Atg6 and UVRAG Causes Similar Malformations in the Developing Wing.* As mutations in the endocytotic machinery can lead to the disturbance of cell polarity as well [17, 49] and these kinds of mutations commonly affect Notch signaling as well [44, 50], next we examined the overall wing morphology of RNAi or Atg6 null mutant animals. We found that compared to controls, the wing specific depletion of Atg6 and UVRAG by RNAi causes severe malformations of the tissue (Figures 9(a)–9(d), 9(f)–9(h), Figure S2), as the wing became blistered or heavily creased. This effect was very similar to Vps15 and Vps34 RNAi (Figures 9(e) and 9(i)), which were previously shown to be required for wing development [17]. As the Atg6 null mutant animals die during the late third larval or early pupal stages [34], mitotic recombination technique was used to generate completely null mutant adult wings. We found that Atg6 null mutant wings also exhibited a heavily creased morphology, which effect could be rescued by the expression of an Atg6 transgene (Figures 9(k)–9(m)). In contrast, Atg14 RNAi caused a vestigial-like effect rather than blistering or creasing (Figure 9(j), Figure S2). As the malformations of the RNAi wings could be the consequence of increased cell death in the developing wing tissue, wing discs were stained against cleaved Caspase-3, and TUNEL assays were performed to detect apoptosis. We found that, in Atg14 RNAi discs, numerous cells underwent apoptosis (Figures S3(a), S3(b), S3(e), and S3(f)), which could explain the wing phenotype of Atg14 RNAi adults. In contrast, in Atg6 or UVRAG RNAi discs no cleaved Caspase-3 or TUNEL positive cells could be detected (Figures S3(c), S3(d), S3(g), and S3(h)).

Vps15, an adaptor subunit of PI3K (III), was shown to be required for the transport and sorting of several membrane proteins to the appropriate cell adhesion structures [17]. This raised the possibility that Atg6 and UVRAG could function together with Vps15 in this setting as well. As the blistering and creasing observed in the adult wing experiments could be a result of disrupted epithelial polarity of the wing cells, immunofluorescence microscopy was performed on the developing pupal RNAi wings against several cell adhesion molecules. For this purpose we used engrailed (en) promoter driven Gal4 to restrict the expression of the RNAi constructs to the posterior compartment of the developing wing; therefore the anterior compartment served as control (Figure 10(a)).

*3.4.1. Atg6 and UVRAG Regulate the Localization of Zonula Adherens Proteins.* First, the zonula adherens (ZA) components were examined and the developing wings were immunostained against Flamingo (Fmi), DE-cadherin (DE-cad), and Armadillo (Arm) [51, 52]. We found that due to the knockdown of Atg6, the major components of the ZA were seriously mislocalized and accumulated in small intracellular compartments in the apical region (Figures 10(c)–10(e)). As Fmi was shown to regulate planar cell polarity [51], rhodamine-phalloidin staining was used to examine the condition of the wing hairs in Atg6 RNAi cells. 32 hours after pupal formation the control wing hairs were well-developed and very regularly oriented towards the distal end of the wing (Figure 10(a)). In contrast, the wing hairs were poorly developed or completely absent in the Atg6 RNAi region of the wing (Figure 10(b)). Similar to Atg6, UVRAG RNAi also altered the localization of Arm and disoriented the pattern of the wing hairs, whilst Atg14 RNAi had no noticeable effect on these parameters (Figures 11(a)–11(d)).

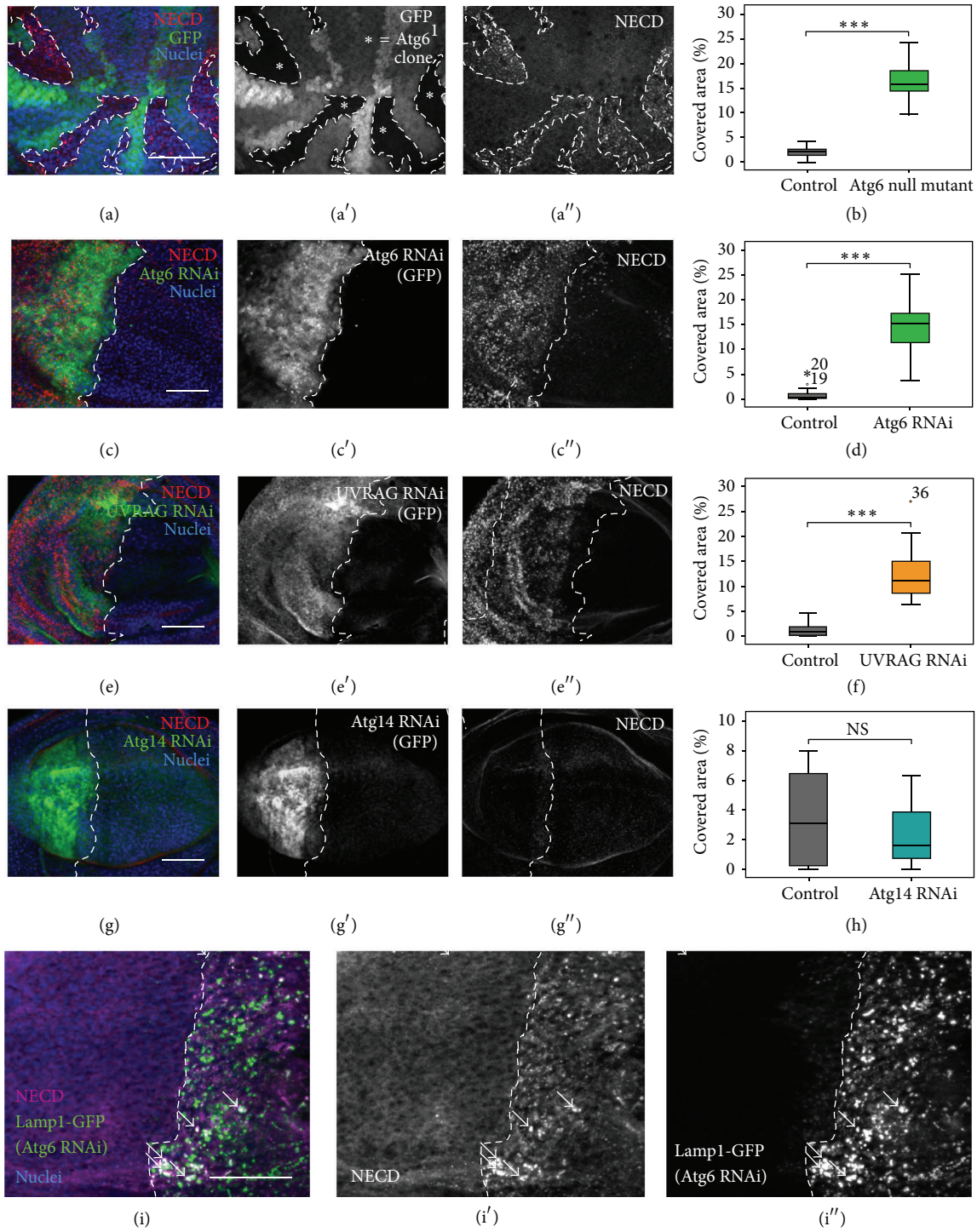


Figure 6: Impaired endocytic degradation of Notch in Atg6 depleted cells. (a) In 3rd instar larval wing discs Atg6 null mutant clones (marked by the absence of GFP, asterisks) accumulate the extracellular domain of Notch (NECD). (b) Quantification of (a). (c), (e), and (g) Imaginal wing discs expressing dsRNA (Atg6, Atg14) or siRNA (UVRAG) by enGal4 were immunostained against NECD. The region of RNAi is marked by the coexpression of GFP and the borderline of the region is indicated with a dashed white line. Immunostain revealed that NECD accumulated in small, numerous puncta in the case of (c) Atg6 RNAi and (e) UVRAG RNAi, (g) but not in Atg14 RNAi. (d), (f), and (h) Quantification of (c), (e), and (g), respectively. (i) Live trafficking assay for Notch in cultured wing imaginal discs. Atg6 RNAi region is marked by the coexpression of the lysosome marker Lamp1-GFP. In controls, surface-bound Notch was internalized then degraded after 3 h. In contrast, in Atg6 depleted cells Notch was internalized normally but trapped in vesicular structures even at 3 h of chasing. Arrows indicate Notch signal colocalizing with Lamp1-GFP. On box plots, bars show the data ranging between the upper and lower quartiles; median of the signal covered areas is indicated as a horizontal black line within the box. Whiskers plot the smallest and largest observations, while dots and asterisks indicate outliers. NS means $P > 0.05$ and $***$ means $P < 0.001$. For details and exact $P$ values of statistical analyses, see Table S3. For genotypes, see Table S4. Scale bars represent 50 $\mu$m.

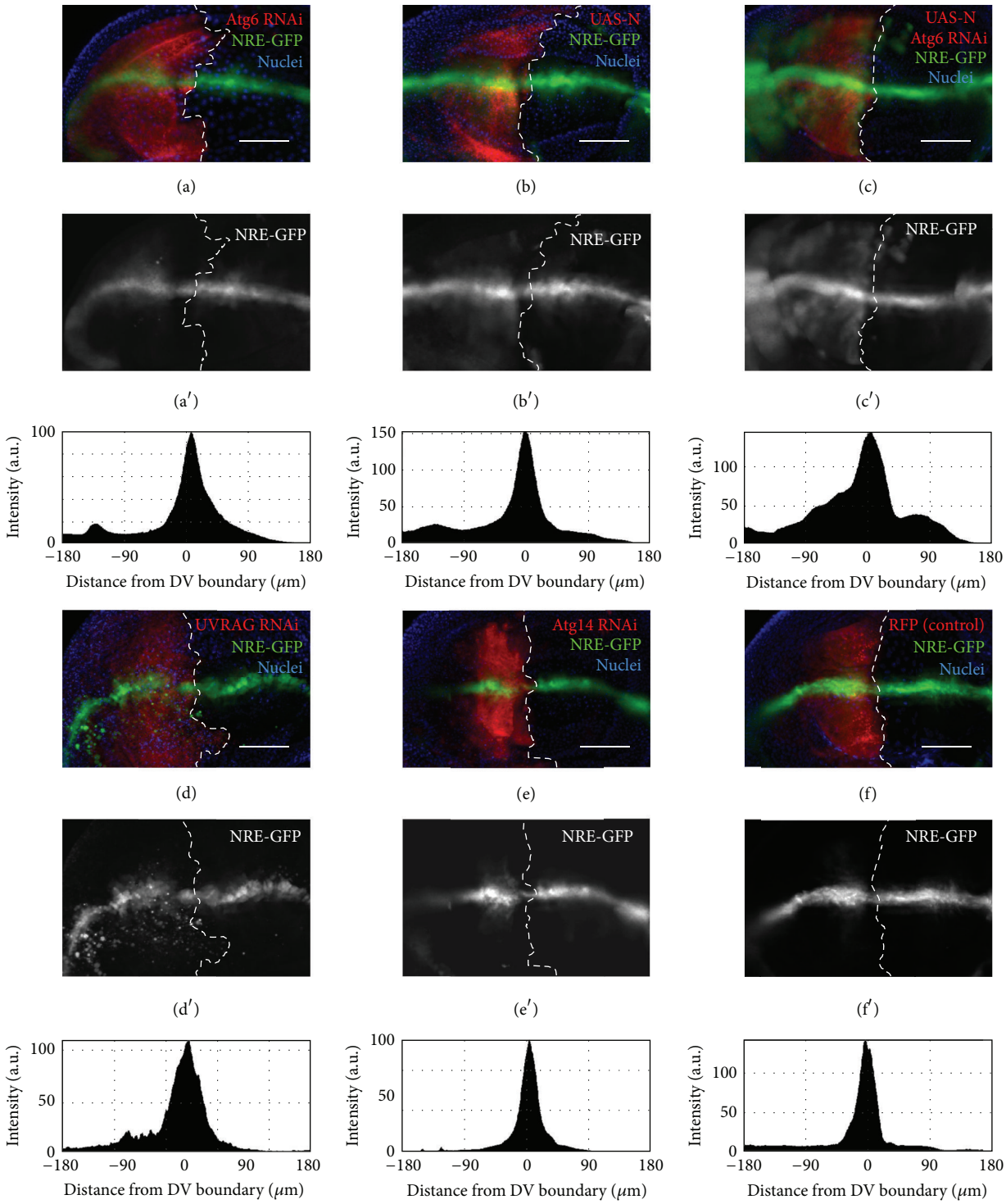


Figure 7: Enhanced Notch signaling in Atg6 RNAi and UVRAG RNAi cells. Larval wing discs expressing RNAi with or without transgenic Notch. UAS-myrRFP was used to mark enGal4-specific region. Notch response element- (NRE-) EGFP was used to show Notch transcriptional activity. (a) Atg6 RNAi and (b) Notch overexpression lead to enhanced Notch activity. (c) This effect was further increased as coexpression of Atg6 RNAi and Notch strongly enhanced the reporter expression. (d) UVRAG enhanced Notch transcriptional activity similarly to Atg6 RNAi (e) RNAi of Atg14 did not alter the reporter expression. (f) Control disc. Histograms show the intensity profiles of the NRE-GFP expression in the enGal4-specific regions. Note that the peaks are broader in panels (a)–(d), compared to panels (e), (f). For genotypes, see Table S4. Scale bars represent 50 $\mu$m.

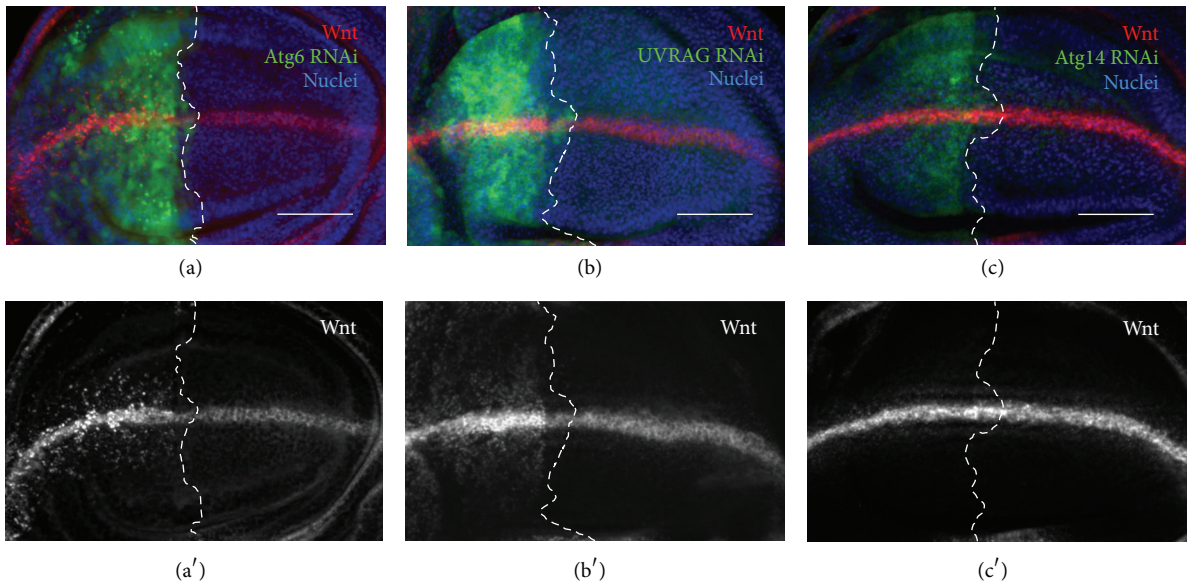


Figure 8: Impaired endocytic degradation of Wnt in Atg6 or UVRAG depleted cells. Imaginal wing discs expressing RNAi by enGal4 were immunostained against Wnt. The region of RNAi is marked by the coexpression of GFP and the borderline of the region is indicated with a dashed white line (a), (b) RNAi of Atg6 and UVRAG resulted in the accumulation of Wnt in small, numerous intracellular puncta. (c) RNAi of Atg14 did not affect Wnt localization. For genotypes, see Table S4. Scale bars represent 50 $\mu$m.

*3.4.2. Atg6 and UVRAG Regulate the Localization Of Basolateral Membrane Proteins.* Next, the localization of basolateral membrane proteins was examined in the pupal wings. At this developmental stage, discs large (Dlg) is localized at the apical regions of septate junctions (SJ), whilst the domain of Fasciclin III (Fas III) is expanded throughout the whole SJ [53]. At the same time the level of these proteins is relatively lower in other plasma membrane regions. We found that the RNAi of Atg6 results in the broadening of the Fas III and Dlg containing plasma membrane area, while the detectable amount of these proteins in the SJ is markedly reduced (Figures 10(f) and 10(g)). Likewise, the RNAi of UVRAG also resulted in the disturbance of Fas III localization (Figure 11(f)). Similarly to SJ proteins, the localization of the basal junction (BJ) protein $\beta$-integrin is also seriously affected by Atg6 or UVRAG knockdown. At this developmental stage the prospective wing intervein cells form large, $\beta$-integrin containing basal junctions [54], but this process was dramatically blocked when the level of Atg6 or UVRAG was reduced (Figures 10(h) and 11(h)). Since Atg6 or UVRAG RNAi wing intervein cells were unable to form basal junctions between the two epithelial layers of the wing, this suggests that the blistering of RNAi adult wings was due to the lack of proper basal cell adhesion structures. In contrast, Atg14 RNAi had no noticeable effect on the localization of basolateral membrane proteins (Figures 11(e) and 11(g)).

Collectively, our results demonstrate that the UVRAG and Atg6 containing PI3K (III) complex II is essential for the proper localization of membrane proteins and is required for the establishment of epithelial cell polarity, unlike the complex containing Atg14.

## 4. Discussion

Although the key role of Atg6 in mediating autophagy is obvious [8, 9, 33], its role in other processes such as endocytosis is less clear. For example, while several papers dispute the endocytic role of Atg6 [27, 30, 31], others suggest that Atg6 may regulate endocytosis and other processes as well [32, 34]. As many of these studies were carried out in cell culture, the direct participation of Atg6 in other processes in an *in vivo* system had to be demonstrated [7]. For this purpose, we used the well-acclaimed animal model *Drosophila melanogaster,* and we showed that the Atg6, UVRAG, and Vps34-containing PI3K (III) complex is required for multiple cell biological processes.

As Vps34 and Vps15 have been shown to mediate multiple vesicle trafficking events and cell polarity [14–18], we assumed that Atg6, as a component of the PI3K (III) core complex should also mediate such processes. Our data presented here suggest that *Drosophila* Atg6 is an essential endocytosis regulator, as cells lacking Atg6 are unable to produce PI3P and fail to progress endosomes into fully functioning endolysosomes. We showed that this failure ultimately results in the accumulation of abnormal endolysosomal compartments in the apical regions of the cells. These results are in line with the studies on *Drosophila* Vps34 and Vps15 in which animals lacking Vps34 or Vps15 showed a very similar phenotype [15, 17]. Furthermore, our results are very similar to the results obtained in mice lacking Vps15, as the mutant animals accumulate abnormal lysosomes and develop lysosomal storage diseases [55]. *Drosophila* Vps15 was shown to regulate the localization of plasma membrane proteins and thus is required for cell polarity [17]. In line

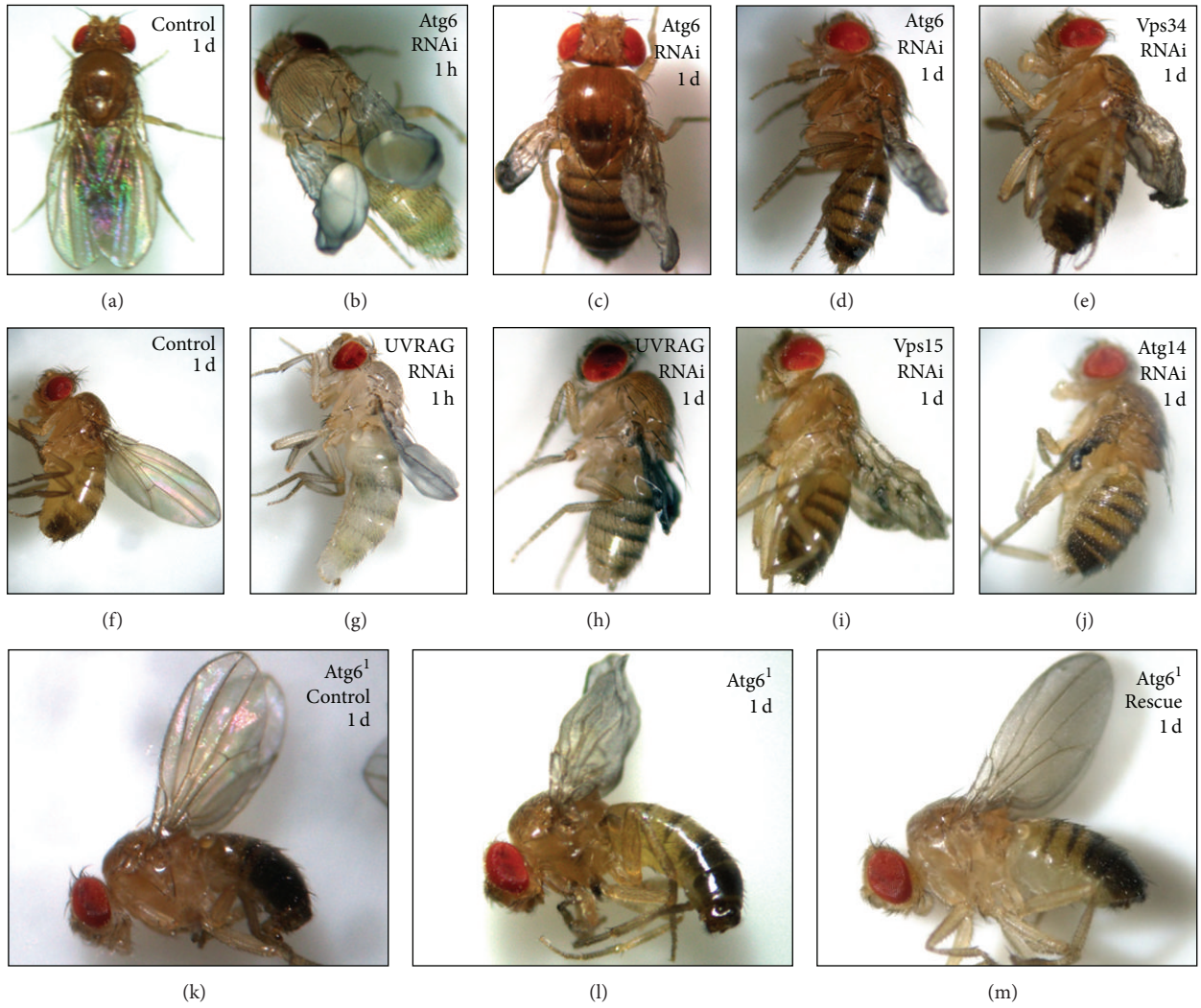


Figure 9: Atg6 and the other members of PI3K (III) are required for normal wing development: (a), (f) 1-day-old wild type flies. (b) Newly hatched Atg6 RNAi animals developed serious malformations, blisters in their wings. (c), (d) In 1-day-old Atg6 RNAi animals, the blisters had become collapsed; thus the wings were creased and heavily distorted. (g), (h) The RNAi of UVRAG completely mimiced the effect of Atg6 RNAi. (e), (f) Positive control animals: Vps34 RNAi and Vps15 RNAi flies, respectively. (j) Unlike the other members of PI3K (III) complex, the RNAi of Atg14 causes a vestigial-like morphology rather than blistering or creasing. (k) The Atg6 null mutant wings are heavily distorted and creased, (l) compared to the control wings lacking the mutation or (m) expressing an Atg6 transgene. 1 h: 1 hour; 1 d: 1 day after emerging from puparia, respectively. For genotypes, see Table S4.

with this, we demonstrated that *Drosophila* Atg6 also acts as a cell polarity regulator, since cells lacking Atg6 fail to form junctional complexes and show disturbed basolateral and planar cell polarity as well.

Notch signaling is a very extensively studied pathway [56] in which endocytosis is a key process for Notch activation and also for downregulation [44]. *Drosophila* Vps34 mutant cells were shown to accumulate Notch-positive punctae in the eye imaginal disc [15]; therefore it can be assumed that the result of the depletion of Atg6 should be similar. Indeed, we found that Atg6 is required for the endocytic degradation of Notch and Wnt as well; thus possibly Atg6 is required also for downregulation of several other signaling pathways. It has been shown that Notch activation is greatly reduced in mutants that block entry into the early endosome but is enhanced in mutants that increase endosomal retention [44]. This is in line with our observations, as depletion of Atg6 results in slightly enhanced Notch signaling. The data presented above provide further evidence that Atg6 is essential for downregulating this pathway. Mammalian cells in culture accumulate EGFR in small intracellular compartments when the members of PI3K (III) complex are silenced [32]. As in *Drosophila*, Atg6 depleted cells accumulate not only Notch but also Wingless; we assume that Atg6 as a component of PI3K (III) is essential for regulating several important signaling pathways by degrading the endocytosed receptors complexed with their ligands, and this function is greatly conserved among eukaryotes.

UVRAG is a tumor suppressor [21], and as a component of PI3K (III) complex II, it has been suggested to regulate

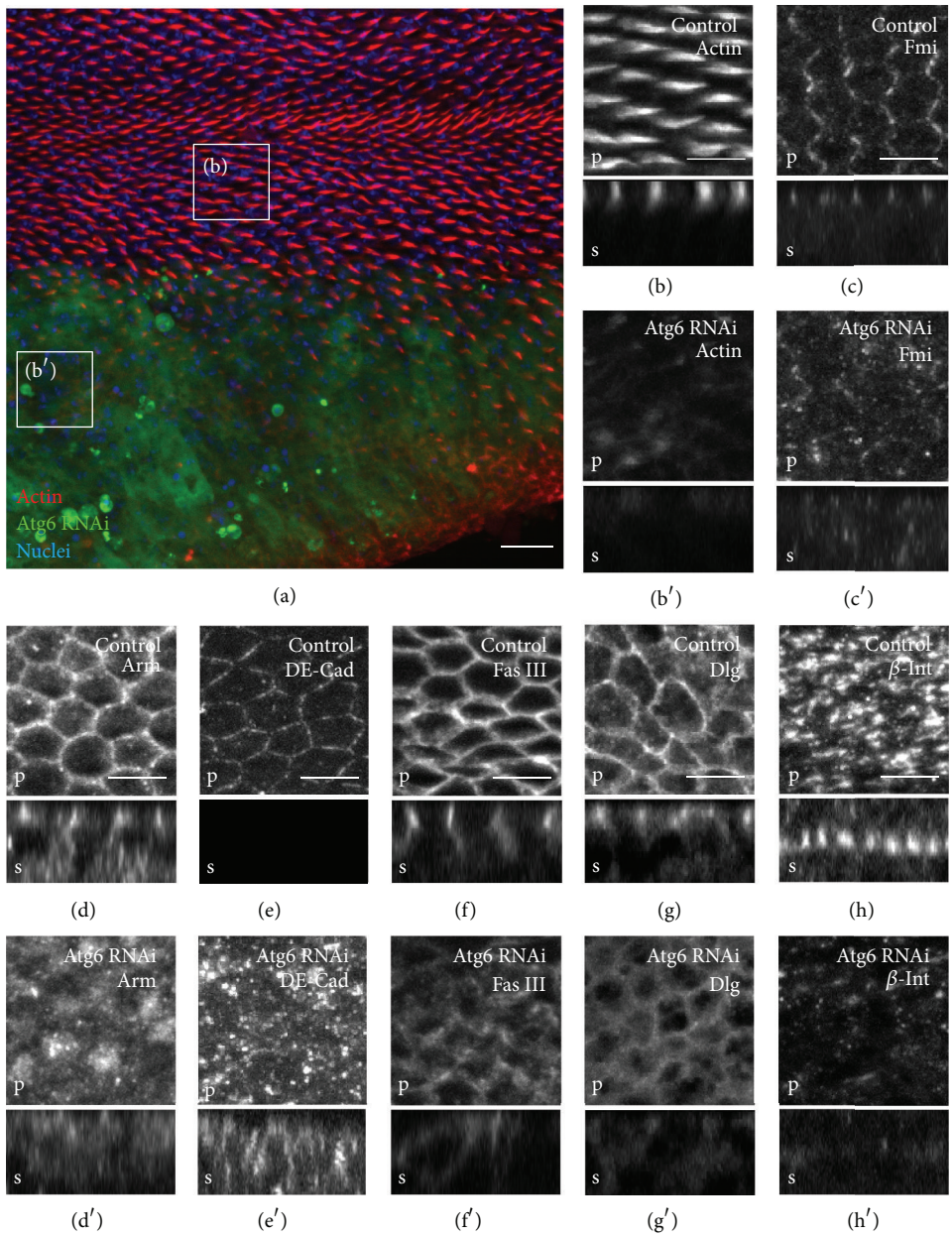


Figure 10: Polarity and membrane protein localization defects of Atg6-knockdown pupal wings. Semiconfocal immunodetection showing XY (marked by a letter p (plan view) at the bottom left corners) and XZ sections (marked by a letter s (side view) at the bottom left corners) in the plane of pupal wings at 32 h APF. Regions of controls are shown in boxes marked by a single character at the upper left side, whereas the regions where dsRNA for Atg6 was expressed by en-Gal4 driver are shown in boxes marked by a character at the upper left side with an apostrophe. (a) Low magnification image of a pupal wing with rhodamine-phalloidin stain showing the regions from where the high magnification images were taken. The expression of the dsRNA by enGal4 is restricted to the posterior compartment of the wing (marked by the coexpression of GFP), and anterior side serves as control. Small boxes surround areas of images (b) and (b′) in which rhodamine-phalloidin stain reveals that control wing hairs are well-developed and very regularly oriented towards the distal end of the wing. In contrast the wing hairs are poorly developed or completely absent in the regions of the RNAi. (c)–(e) In controls Fmi, Arm, and DE-Cad show a very pronounced zonula adherens (ZA) localization; furthermore Fmi shows a very regular planar cell polarity pattern as well, whereas all of them show intracellular punctuation and irregular localization in the Atg6 depleted wing regions. (f), (g) Septate junction proteins Fas III and Dlg show very typical lateral localizations as Dlg is mainly localized at the apical regions of septate junctions (SJ), whilst the domain of Fas III is expanded throughout the whole SJ. In contrast, in Atg6 RNAi cells the amounts of these proteins lowered and both show less pronounced SJ localization. (h) At this developmental stage the prospective wing intervein cells form large, $\beta$-integrin ($\beta$-int) containing basal junctions between the two epithelial layers of the wing, but this process is dramatically blocked when the level of Atg6 is reduced and wing cells seem to be unable to develop these structures. For genotype, see Table S4. Scale bars represent (a) 10 $\mu$m and (b)–(h) 5 $\mu$m.

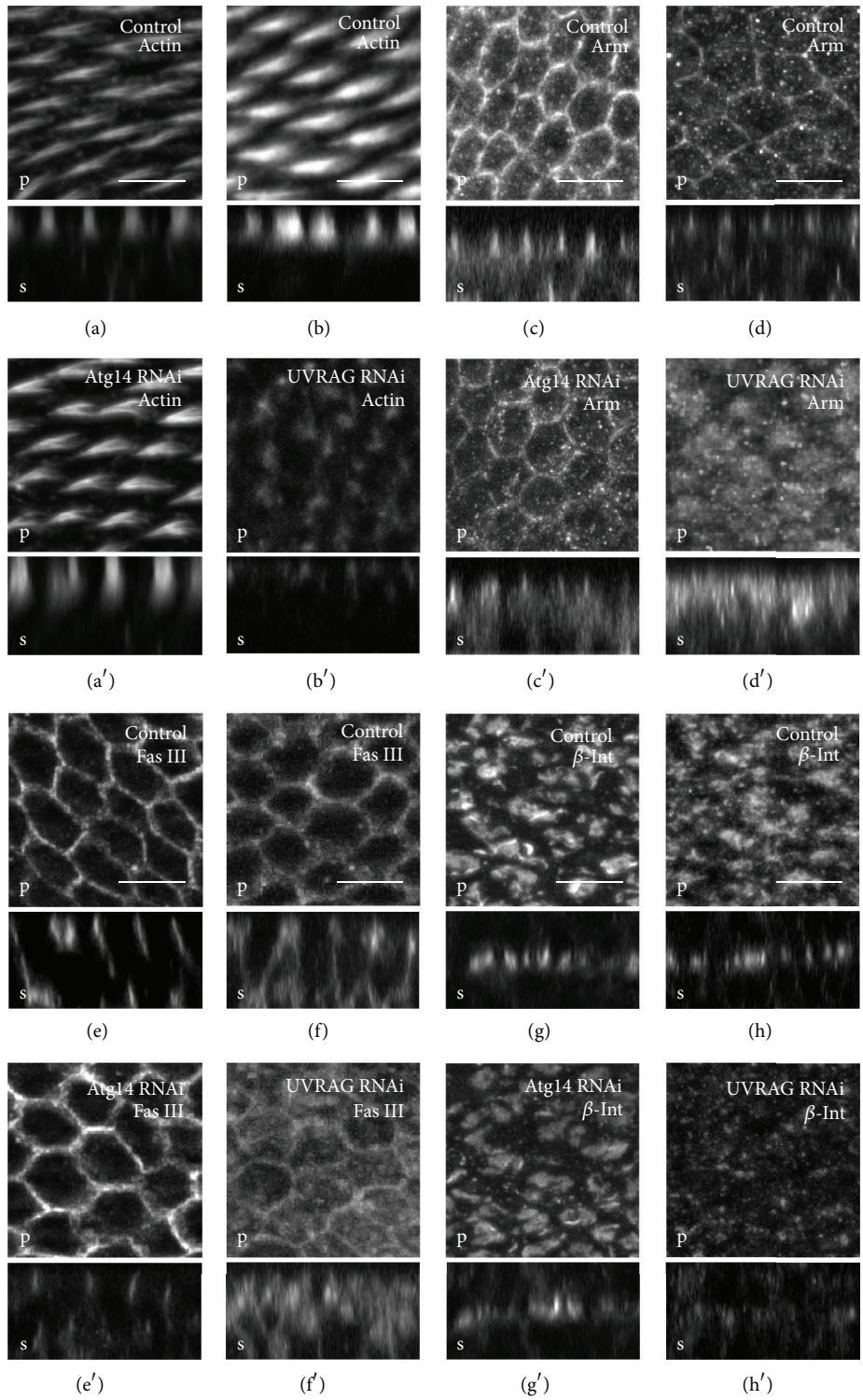


Figure 11: RNAi of UVRAG but Atg14 alters cell polarity and membrane protein localization. Semiconfocal immunodetection showing XY (marked by a letter p (plan view) at the bottom left corners) and XZ sections (marked by a letter s (side view) at the bottom left corners) in the plane of pupal wings at 32 h APF. Regions of controls are shown in boxes marked by a single character at the upper left side, whereas the regions where dsRNA for Atg6 was expressed by en-Gal4 driver are shown in boxes marked by a character at the upper left side with an apostrophe. (a), (c), (e), and (g) Immunodetection reveals no obvious differences of the pattern of wing hairs and the localization of Fas III, Arm, and $\beta$-int between control and Atg14 depleted regions of pupal wings. (b), (d), (f), and (h) In contrast to Atg14, the RNAi of UVRAG dramatically alters wing hair development and the localization of membrane proteins (Fas III, Arm, and $\beta$-int) and these phenotypes are very similar to those induced by Atg6 RNAi as was shown in Figure 2. For genotypes, see Table S4. Scale bars represent 5 $\mu$m.

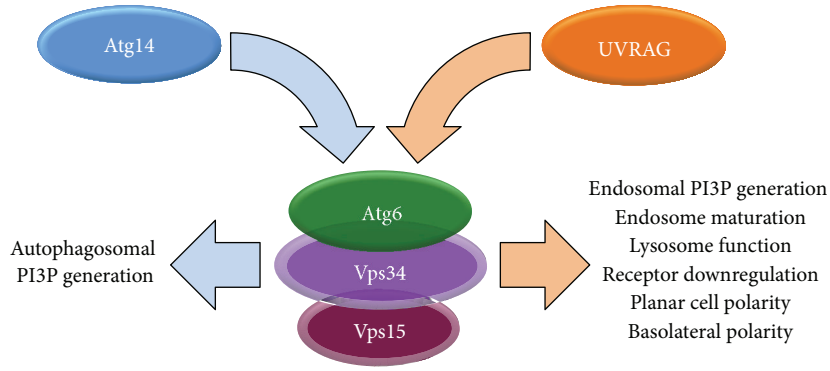


Figure 12: Schematic representation summarizing the multiple roles of the two PI3K (III) complexes in *Drosophila*. See text for details.

autophagy and vesicle trafficking in mammalian cells [12, 23]. An orthologue of UVRAG was identified in *Drosophila* and was shown to control organ rotation by regulating the degradative endocytic traffic of Notch [57]. Based on a large-scale proteomic study, UVRAG likely binds to Atg6 in *Drosophila* as well [58]. As UVRAG has been speculated to form other complexes in mammals besides PI3K (III) complex II such as with class C Vps [12], the question remained open whether UVRAG regulates Notch signaling as a member of PI3K (III) complex II or this function of UVRAG is independent from this complex. We found that the phenotype of UVRAG RNAi is similar to the phenotype of Atg6 RNAi, as the depletion of either results in enhanced Notch signaling due to the accumulation of Notch. This suggests that Atg6 is responsible for regulating endocytosis and thus Notch and other signaling pathways as well, as a central component of UVRAG-containing PI3K (III) complex II. This presumption is supported by our observation that cells lacking UVRAG accumulate abnormal endolysosomal compartments in a very similar manner to Atg6 RNAi cells. Similarly, in UVRAG depleted cells the selective PI3P marker GFP-2XFYVE is also unable to associate with endosomal membranes, indicating a failure of PI3P production. This raised the possibility that, in other autophagy-independent processes mediated by an Atg6, the PI3K complex II is the key player. Indeed, we found that UVRAG depleted wing cells develop similar polarity defects as Atg6 RNAi cells.

Atg14/Barkor was identified as the mammalian autophagy-specific factor for Beclin 1 and PI3K (III) [19] and is a component of PI3K (III) complex I [11]. In mammalian cells Atg14 is required to recruit PI3K (III) to the formation site of autophagosomes [22]. Although Atg14 is considered to be autophagy specific, there are results showing that Atg14 may also participate in the regulation of endocytosis [26]. The *Drosophila* orthologue of Atg14 was shown to be also essential for autophagy in the fat body [39, 42], but the participation of Atg14 in other PI3K (III) mediated processes remained unclear. We found that although depletion of Atg14 causes very serious malformations in the wing, this effect is neither the consequence of altered cell polarity nor the consequence of endocytosis defects. Atg14 RNAi wing cells develop normal cellular junctions, their endosomal compartments did not differ from control cells, and the ultrastructure of Atg14 depleted pupal wing cells appeared to be normal. Notch signaling and Notch and Wingless localization were completely identical to controls, and cells lacking Atg14 were able to produce PI3P as the distribution and localization of PI3P marker GFP-2XFYVE was similar to wild type cells. Our results suggest that Atg14 may not be required for other PI3K (III) mediated processes other than autophagy in *Drosophila* wing.

## 5. Conclusions

Our data presented here suggest that in *Drosophila*, an UVRAG-containing PI3K (III) complex II acts as an essential regulator of endocytosis, membrane trafficking, and is required for downregulating several signaling pathways (Figure 12). Due to these functions, Atg6 is indispensable for proper organ development and cannot be considered as an exclusive autophagy related protein. We propose that the UVRAG-containing PI3K (III) complex II may act as a tumor suppressor, and such a role of Beclin 1 may be masked by its essential function during autophagy, as established cancer cells often depend on autophagy [29, 59].

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

The authors would like to thank Gabor Juhasz, Eric H. Baechrecke, Marcos Gonzalez-Gaitan, Suzanne Eaton, Thomas Klein, and Helmut Krämer for stocks and reagents (listed in Tables S1 and S2) and Zsófia Kováts for technical help. This work was supported by a Grant from the Hungarian Scientific Research Funds (NK78012) provided to M. Sass. The European Union and the European Social Fund have provided financial support to this project under the Grant agreement no. TAMOP 4.2.1./B-09/KMR-2010-0003.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 273473, 24 pages
http://dx.doi.org/10.1155/2014/273473

*Review Article*

# Autophagy in *Drosophila*: From Historical Studies to Current Knowledge

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Received 23 January 2014; Accepted 17 April 2014; Published 18 May 2014

Academic Editor: Rodney J. Devenish



The discovery of evolutionarily conserved Atg genes required for autophagy in yeast truly revolutionized this research field and made it possible to carry out functional studies on model organisms. Insects including *Drosophila* are classical and still popular models to study autophagy, starting from the 1960s. This review aims to summarize past achievements and our current knowledge about the role and regulation of autophagy in *Drosophila*, with an outlook to yeast and mammals. The basic mechanisms of autophagy in fruit fly cells appear to be quite similar to other eukaryotes, and the role that this lysosomal self-degradation process plays in *Drosophila* models of various diseases already made it possible to recognize certain aspects of human pathologies. Future studies in this complete animal hold great promise for the better understanding of such processes and may also help finding new research avenues for the treatment of disorders with misregulated autophagy.

## 1. Introduction

Autophagy collectively refers to a group of intracellular degradation pathways that mediate the breakdown of intracellular material in lysosomes. This definition could as well include the endocytic downregulation of transmembrane proteins in the plasma membrane, but for historical and mechanistic reasons, that pathway is not considered to be part of autophagy. Different routes have evolved to solve the same topological issue; that is, cytoplasmic material including proteins, lipids, nucleic acids, and whole organelles including ER and mitochondria needs to be transported into the lumen of lysosomes. Three main subtypes are usually distinguished based on how cargo reaches the lysosome.

(A) During chaperone-mediated autophagy, a subset of individual proteins bearing a KFERQ amino acid sequence are unfolded and translocated across the lysosomal membrane through a channel consisting of LAMP2A proteins [1]. This pathway was described in cell-free systems and in cultured mammalian cells and its existence has not been shown in invertebrates yet.

(B) During microautophagy, invaginations of the lysosomal membrane pinch off portions of the cytoplasm. The resulting intraluminal vesicles are then broken down inside lysosomes. While the topology of this pathway resembles multivesicular endosome formation, genetic studies in yeast revealed that it requires a subset of the same genes that mediate the main, macroautophagic pathway. Although a morphological account of microautophagy is already found in a 1965 paper on the premetamorphotic insect fat body [2], this process is still difficult to study in metazoans, as no specific genes and reporters have been described yet. Thus, it is not discussed further here, and interested readers are suggested to consult a recent review on this topic [3].

(C) During macroautophagy, membrane cisterns called phagophores (also known as isolation membranes)

assemble and capture cargo to be degraded. The resulting double-membrane autophagosomes then fuse with endosomes or lysosomes to give rise to amphisomes or autolysosomes, respectively. Autophagosome formation is enhanced in response to certain stress conditions such as starvation or during physiological changes triggered by hormonal cues [4, 5]. Thus, the degradative capacity of macroautophagy is the highest of the three pathways. As it is also the best studied route, it is usually simply referred to as autophagy, including the rest of this review.

## 2. Historical Early Studies

During the first 35–40 years of autophagy research, only a very limited methodological repertoire was available to study this process. The most commonly used technique was transmission electron microscopy (TEM), sometimes used together with cytochemical detection or biochemical measurement of lysosomal enzyme activities, and classical histological staining methods for light microscopy.

The first report with properly interpreted ultrastructural images of autophagic structures dates back to 1959 by Novikoff [6]. In the epithelial cells of proximal convolutions of kidneys in experimental hydronephrosis (caused by ligation of the ureter), mitochondria could be found in dense bodies that were positive for acidic phosphatase, a typical lysosomal enzyme [6, 7]. In 1962, Ashford and Porter published ultrastructural images of vesicles observed in hepatic cells of rats treated with glucagon, which obviously contained cytoplasmic material in various stages of degradation [8]. Subsequently, work in the laboratory of Christian de Duve, the biochemist famous for identifying and naming lysosomes, revealed that glucagon induced the relocalization of lysosomes to mediate glucagon-induced autophagy in rat liver [9]. Pfeifer published complementary studies on suppression of liver autophagy by insulin [10, 11]. Furthermore, starvation was already reported to be a strong enhancer of autophagy in rat liver back in 1964 [12]. It was de Duve who recommended to refer to the process of progressive degeneration of mitochondria and other organelles in cytolysosomes as autophagy (literally meaning "self-eating" in Greek), at a scientific meeting held in 1963 [13], and later described it in a widely cited review article [14]. It is worth noting that he also coined the names for processes now known as endocytosis (or heterophagy, which means "different eating" in Greek) and exocytosis in his lecture. A variety of terms were used initially for vesicles involved in autophagy, including initial and degrading autophagic vacuoles; these structures are now usually referred to as autophagosomes and autolysosomes, respectively.

Many of the pioneering early studies were carried out on insects other than *Drosophila*, as the fruit fly was not as popular before the revolution of molecular genetics as it is today. It was already shown in 1899 that in certain insects, the larval fat body (an organ with metabolic and storage functions similar to our liver and fat tissues) contains storage granules of proteins [15], and it was later described that honey bee larvae accumulated such granules just prior to pupation [16, 17]. The first recognition of autophagy in *Drosophila* melanogaster was published in 1963, showing TEM images of large autolysosomes containing ER and mitochondria in fat body cells of larvae approaching the time of puparium formation [18]. This programmed wave of autophagy in the larval fat body of holometabolous insects (those undergoing complete metamorphosis) is now known as an example of developmental autophagy.

In 1965, Locke and Collins provided a very detailed ultrastructural description of this process in the larva of the butterfly *Calpodes ethlius* [2]. Similar to the above examples, a large number of granules (which are essentially vesicles with a high protein content) form prior to metamorphosis in these animals. Three types could be distinguished: granules composed almost entirely of densely packed proteins that often form crystals, granules containing isolated regions of ER and mitochondria, and granules of a mixed type. This pioneering study published ultrastructural images that beautifully demonstrate phagophores in the process of capturing cytoplasmic contents such as a mitochondrion, double-membrane autophagosomes containing ER and mitochondria, and autolysosomes within which organelles are seen in various stages of degradation. Moreover, the authors properly recognized that the outer membrane of autophagosomes is involved in fusion with lysosomes (or first with each other), and after loss of the internal membrane, ER and mitochondria coalesce due to degradation by lysosomal enzymes. It is important to emphasize that the densely packed protein granules generated during this period originate in large part from the endocytic uptake of blood proteins when such holometabolous insect larvae (including *Drosophila*) are preparing for metamorphosis and that the heterophagy and autophagy pathways converge at the level of lysosomes [19–21]. It became clear that increases in the steroid hormone ecdysone trigger larval molts in these insects at a high concentration of juvenile hormone, and the drop in juvenile hormone concentration allows for the larval-pupal molt [22]. Note that in flies including *Drosophila*, first the larval cuticle hardens during puparium formation, and the actual molt only happens 5-6 h later, when the adult appendages such as legs and wings are everted from their primordia found as imaginal disks within the larval body. As early as in 1969, ligation and decapitation experiments (separating the ecdysone-producing endocrine organ from the larval fat body) were shown to prevent storage granule formation in *Calpodes*, and this effect could be rescued by injection of ecdysone [23]. In this report, Janet Collins already correctly hypothesized that ecdysone triggers autophagy only when juvenile hormone concentration is low, which was later confirmed in other insects including *Drosophila* [21, 24, 25].

Autolysosomes were also observed in ultrastructural images of *Rhodnius* larval fat body cells during prolonged starvation, published in 1967 by Wigglesworth [26]. Two years earlier, Francis Butterworth and colleagues reported that a 3-day starvation of early third instar *Drosophila* larvae induced massive granule formation in the fat body based on light microscopy [27], although this effect may have been due to the fact that once larvae reach the so-called 72 h checkpoint

counted from the time of egg laying, they are able to initiate metamorphosis (and thus turn on developmental autophagy and heterophagy in the fat body) following acute starvation [28].

These early studies were not limited to the insect fat body. An ultrastructural analysis of eye development of wild-type and eye color mutants of *Drosophila* was published in 1966, demonstrating that the so-called type IV granules form in the pigment cells of various colorless mutants [29]. These granules are essentially autolysosomes as they were found to be positive for acid phosphatase and contained ribosomes, myelin-like membranes, glycogen, and ferritin [29]. In 1965, Lockshin and Williams showed that during the elimination of intersegmental muscles following adult ecdysis in silkmoths, increased activity of lysosomal cathepsins and acid phosphatases can be detected biochemically, and lysosome-like organelles abound which were later found to contain mitochondria [30–32]. These findings led to the morphological classification of this histolysis as a type II (or autophagic) cell death, to distinguish it from type I cell death events, which are characterized by the classical apoptotic morphology such as chromatin condensation, cell shrinkage, and blebbing [33].

## 3. Genetic Control of Autophagy in *Drosophila*

Multiple genetic screens carried out in the 1990's identified a core set of about 20 evolutionarily conserved genes required for autophagy in yeast [34–36]. Since different names were proposed often for the same genes in each screen, a consensus nomenclature for these *Atg* (autophagy-related) genes was adopted in late 2003 [37]. Note that the first study to demonstrate that an *Atg* gene homolog is also required for autophagy in a complete animal was published in *Drosophila* earlier that year, that is why it did not follow the agreed-upon naming conventions and referred to the fly homolog of *Atg3* as *Drosophila Aut1* [38]. It is commonly accepted that Atg gene products assemble into functional protein complexes, and several attempts have been made to establish their hierarchy during autophagosome formation in various models [39–41]. Such genetic epistasis analyses have proven difficult based on data from yeast and cultured mammalian cells, which is likely explained by the emerging connections between Atg proteins that were originally grouped into separate complexes, by temporal differences in the recruitment of various Atg proteins to phagophore assembly sites (PAS), and by differences in the localization of proteins thought to act as part of the same complex [4, 42, 43]. Nevertheless, we will discuss the role of these proteins according to the canonical classification in this review for clarity (please see also Figure 1).

The Atg1 complex is usually considered to act most upstream in the hierarchy of Atg gene products in all eukaryotic cells and contains the serine/threonine kinase Atg1 (the homolog of mammalian ULK1 and ULK2 proteins), Atg13, Atg101, and FIP200 (also known as RB1CC1 in mammals and Atg17 in flies) in metazoans. Of these, neither Atg101 nor FIP200 has clear homologs in yeast based on sequence

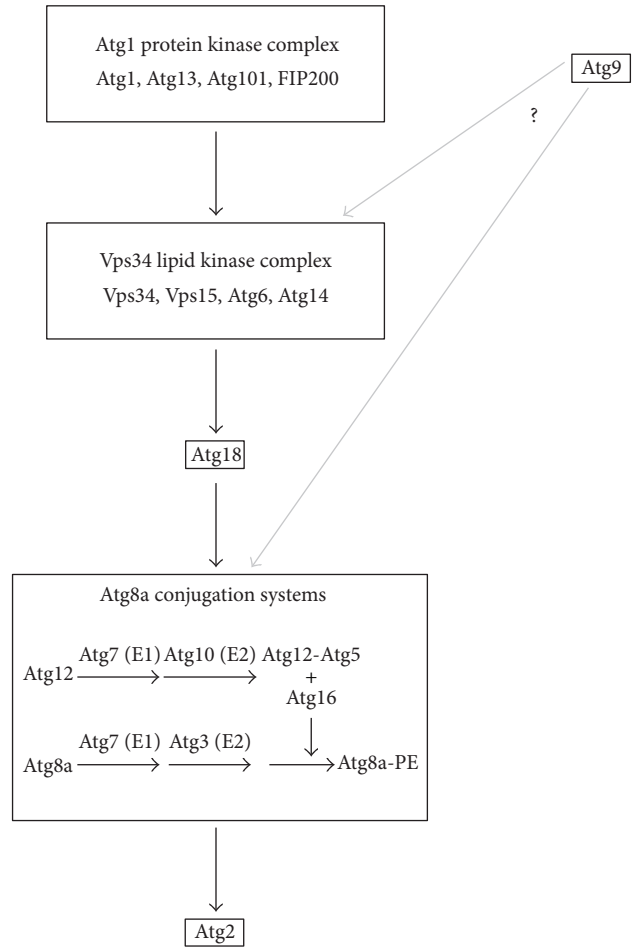


Figure 1: A model for the hierarchical relationships of Atg proteins in *Drosophila*. PE: phosphatidyl-ethanolamine. See text for details.

comparisons, although FIP200 is thought to act similar to the scaffold protein Atg17 [44]. Biochemical studies in flies and mammals show that Atg13 directly binds to the other three subunits, and that it undergoes Atg1-mediated hyperphosphorylation upon starvation in *Drosophila* [44–46]. The catalytic activity of Atg1 seems to be especially important for autophagy induction. First, expression of kinase dead Atg1 inhibits autophagy in a dominant-negative fashion [47]. Second, overexpression of Atg1 strongly induces autophagy, which eventually culminates in cell death due to activation of caspases [47]. Third, Atg1 undergoes limited autophosphorylation during starvation, which is thought to increase its activity [44]. Interestingly, expression of dominant-negative, kinase dead Atg1 still shows a low-level rescue of the lethality of Atg1 null mutants [47]. Moreover, Atg1 was found to localize to the whole phagophore in yeast while all other subunits of this complex remain restricted to the initially appearing PAS area, indicating that Atg1 may also function independent of its canonical binding partners [43].

Both autophagosome and endosome membranes are positive for phosphatidylinositol 3-phosphate (PI3P), a phospholipid generated by the action of similar lipid kinase complexes. The core complex contains Atg6 (known as

Beclin-1 in mammals), the catalytically active class III phosphatidylinositol 3-kinase (PI3K) Vps34, and its regulatory subunit Vps15, which has a serine/threonine kinase domain. A catalytically inactive point mutant of Vps15 was shown to lose Vps34 binding in yeast [48], but the significance of its putative protein kinase activity is poorly understood. The identity of the fourth subunit is critical: Atg14 is present in the autophagy-specific complex while the other complex involved in endocytosis contains UVRAG/Vps38, and the binding of these subunits to the core complex has been shown to be mutually exclusive in mammalian cells [49, 50]. Starvation-induced autophagy is severely impaired in Vps34 null mutant or dominant-negative Vps34 overexpressing cells, although some autophagosomes form at a reduced rate [51]. This may be explained by the activity of the class II PI3K, which was suggested to partially compensate for the loss of Vps34 during autophagy in mammalian cells [52, 53]. Similarly, deletion of *Drosophila* Vps15 or Atg6 results in a block of starvation-induced autophagy [54, 55]. In line with the distinct roles of different Vps34 complexes in mammals and yeast, it has been shown that *Drosophila* UVRAG is involved in endolysosome maturation and is dispensable for autophagosome formation or fusion with lysosomes, whereas studies using RNAi or hypomorphic mutants suggested that Atg14 is required for autophagy in larval fat body cells [56–59].

It is commonly accepted that PI3P found on phagophore and autophagosomal membranes recruits and activates phospholipid effectors. One class of such proteins includes the metazoan homologs of the yeast WD40 domain protein Atg18, which are called WIPI1-4 in mammals [60, 61]. In *Drosophila*, Atg18 has been shown to be required for autophagy, whereas the function of its closely related paralog CG8678 (also known as Atg18b) is not known [62]. DFCP1 (double FYVE containing protein 1) was characterized as another phospholipid effector, and it translocates to a putative subdomain of the ER during autophagy induction [63]. This structure is called the omegasome, and it is also positive for VMP1 (vacuole membrane protein 1), an ER-localized, six transmembrane domain containing protein of poorly characterized function [40, 64]. Interestingly, VMP1 has been found to interact with Beclin-1, suggesting that it may modulate phospholipid production [65]. The fly homolog of VMP1 is called Tango5 (Transport and Golgi organization 5), as it was recovered in a cell culture-based RNAi screen as required for ER to Golgi trafficking in the secretory pathway [66]. Interestingly, the gene encoding DFCP1 has been lost multiple times during evolution as it is missing from all *Caenorhabditis* and most *Drosophila* species including *Drosophila melanogaster*, but its homolog can be clearly identified in *Drosophila willistoni* and the *virilis* subgroup using bioinformatic searches, in addition to more ancient species such as *Trichoplax* and *Hydra*. The role of DFCP1 is also unknown in mammals, and it is mostly used as a marker along with VMP1 for the PAS [40, 42].

Atg9 is the only transmembrane protein among the Atg gene products identified in yeast, and it likely plays a critical role in the membrane transport events during phagophore assembly in all eukaryotes studied so far [42, 67–69]. The source of autophagic membranes has been debated since the discovery of this process, and practically all membrane compartments were suggested to contribute, including endosomes, ER, Golgi, mitochondria, and plasma membrane [70–72]. *Drosophila* Atg9 is still largely uncharacterized, with only a few RNAi studies showing that it is also required for autophagy in various settings [57, 73–75]. Yeast Atg9 physically binds to Atg18 and Atg2, and these proteins are required for the retrograde traffic of Atg9 from the PAS in yeast [76]. Atg9 also binds to fly Atg18, and it has recently been shown that Atg9 accumulates on protein aggregates containing the autophagy cargo Ref(2)P (also known as p62/SQSTM1) in starved Atg7, Atg8a, and Atg2 mutants, but not in Atg18 mutants [75].

Structural studies of Atg8 and Atg12 revealed that these proteins belong to the family of ubiquitin-like modifiers, and these are involved in two related ubiquitin-like conjugation systems [77]. First, the C-terminal amino acid(s) following a glycine residue of Atg8 and its homologs are cleaved by the Atg4 family of cystein proteases. Subsequently, the exposed glycine is conjugated to the E1-like enzyme Atg7, followed by its transfer to the E2-like Atg3 (also known as Aut1 in flies). In parallel, Atg12 is activated by Atg7 as well, and then the E2-like Atg10 catalyzes the formation of an Atg5-Atg12 conjugate [77]. Atg5 contains two ubiquitin-related domains flanking a helical region [78]. Then, a multimeric complex of Atg5-Atg12 and Atg16 forms, which enhances the covalent conjugation of Atg8 to the membrane lipid phosphatidylethanolamine (PE) [78]. Atg8 and its homologs (Atg8a and Atg8b in flies, and LC3 and GABARAP family proteins in mammals) are the most commonly used markers in autophagy studies [40, 79]. First, Atg8 is covalently bound to phagophore and autophagosome membranes, making it possible to visualize these structures using tagged reporters or by immunostaining using antibodies against endogenous proteins (Figure 2). Second, the processing of Atg8 can be followed by Western blots, as unconjugated Atg8 (usually referred to as Atg8-I or LC3-I) migrates slower than the lipid-bound form (Atg8-II or LC3-II). Autophagy induction usually increases the amount of the processed form relative to tubulin or actin, which becomes even more obvious if the fusion of autophagosomes with lysosomes is blocked by bafilomycin, or genetically by loss of the autophagosomal SNARE Syntaxin 17 [79–82].

It is clearly established that Atg2 and Atg18 function together in yeast, acting most likely in parallel to the Atg8 and Atg12 conjugation systems [39, 83]. In mammals, depletion of the Atg18 homolog WIPI2 suppressed LC3 puncta formation [61]. In contrast, its putative binding partner Atg2 appears to function most downstream of the core Atg genes in mammals and worms, similar to VMP1 homologs, as Atg8-positive structures with some characteristics of phagophores form in cells upon silencing of these genes [40, 41, 64, 84]. Atg18 also shows an interaction with Atg2 in *Drosophila*, although it is weaker than that observed between its paralog CG8678 and Atg2 [75]. Interestingly, *Drosophila* Atg2 acts downstream of, or parallel to, the Atg8 systems in *Drosophila* as well, as it is dispensable for Atg8a dot formation in the fat body [75, 80]. In contrast, no GFP-Atg8a puncta were seen in Atg2 mutant

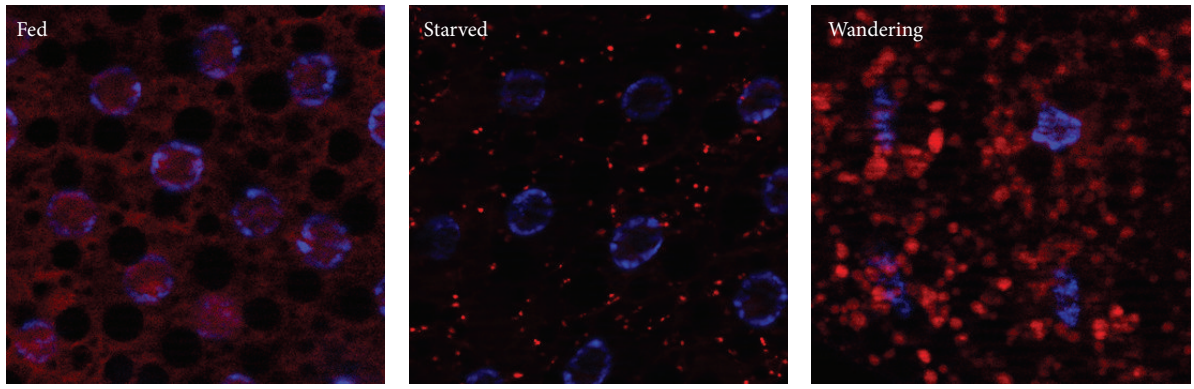


Figure 2: Autophagy induction in the larval *Drosophila* fat body. Dots positive for mCherry-Atg8a (red), representing autophagosomes and autolysosomes, are rarely seen in fat body cells of well-fed larvae (a). Punctate mCherry-Atg8a structures form in response to starvation (b) or during the wandering period (c). DNA is stained blue.

prepupal midguts [85], suggesting that either tissue-specific differences exist, or that a GFP-Atg8a reporter expressed at very low levels is not as potent as anti-Atg8a immunolabeling for the visualization of these aberrant structures that are apparently seen in most metazoan cells. This issue clearly warrants further studies.

*Drosophila* Atg18 appears to function upstream of Atg8 recruitment during phagophore formation similar to worms and mammals, as punctate Atg8a localization is lost in Atg18 mutant or RNAi cells [41, 61, 75, 84]. Interestingly, protein aggregates positive for ubiquitin and Ref(2)P show a near complete colocalization with FIP200 and Atg9 in *Drosophila* mutants lacking more downstream players, raising the possibility that such protein aggregates may serve as an organizing centre during autophagosome formation [46, 75]. This hypothesis will need further testing.

A complicated network of core Atg proteins coordinates the process of autophagosome formation, a process that is still not completely understood. Autophagosomes must fuse with lysosomes and endosomes to deliver their cargo for degradation. In yeast, direct fusion of the autophagosome with the vacuole is achieved by a tethering factor called HOPS (homotypic fusion and vacuole protein sorting) complex, which facilitates membrane fusion catalyzed by SNARE proteins Vam3, Vam7, and Vti1 [86]. Interestingly, autophagosome fusion in *Drosophila* appears to depend on the amphisome pathway, as a genetic block of multivesicular endosome formation results in large-scale accumulation of autophagosomes [51, 87]. Recent studies identified Syntaxin 17 as the autophagosomal SNARE protein, both in flies and mammals [80, 81]. Syntaxin 17 binds to ubisnap, an ortholog of mammalian SNAP-29, to mediate fusion by forming a ternary complex with late endosomal/lysosomal VAMP7 (VAMP8 in mammals) [80, 81]. Fusion is facilitated by the binding of HOPS to this SNARE complex, both in *Drosophila* and mammalian cells [58, 88]. In the final steps following fusion, cargo is degraded inside acidic autolysosomes by the action of hydrolases such as cathepsins, and the breakdown products are recycled back to the cytosol to fuel synthetic and energy producing pathways.

## 4. Regulation of Autophagy during *Drosophila* Development

The best known examples for stimulus-induced autophagy in *Drosophila* larvae are the starvation response during the feeding stages and developmental autophagy triggered by hormonal cues around the start of metamorphosis in polyploid tissues. The role and regulation of autophagy have also been studied in a developmental context in adult ovaries and in the extraembryonic tissue called amnioserosa during early embryogenesis. The following paragraphs summarize the major regulatory pathways regulating autophagy in these settings.

Autophagy is controlled by the main nutrient and energy sensor in all eukaryotic cells, a serine/threonine kinase called TOR (target of rapamycin) [89]. TOR activity is increased by the presence of nutrients and growth factors and promotes cell growth in part through the phosphorylation and activation of S6k (RPS6-p70-protein kinase) and phosphorylation and inactivation of Thor (also known as 4E-BP for Eukaryotic translation initiation factor 4E binding) [90]. TOR not only enhances general protein synthesis this way, but it may also increase net cell growth by actively repressing autophagy through the direct phosphorylation and inhibition of Atg1 in metazoans [45, 91–93]. Inactivation of TOR during starvation, growth factor withdrawal, or impaired lysosomal function rapidly results in the shutdown of cap-dependent translation and in the activation of autophagy, which is likely also facilitated by the poorly characterized action of phosphatases such as PP2A that may antagonize TOR [52, 56, 62, 91–94]. Interestingly, the serine/threonine kinase Atg1 and its mammalian homologs are able to directly phosphorylate TOR, which may act as a feedback mechanism to inhibit cell growth and further enhance autophagy induction [47, 95].

Growth signaling pathways are remarkably active in the larva, a specialized life stage of holometabolous insects. Larvae basically just eat and grow throughout the feeding stages to acquire and store as many nutrients as possible in a relatively short time, mostly in the form of polyploid cells and tissues besides the hemolymph. Notably, the size of the larval fat body (a metabolic organ similar to our liver and white fat tissues) increases more than 200-fold between the first and mid-third instar stages in *Drosophila*. This process generates polyploid cells of enormous size, reaching a ploidy level of 256–512 n for fat cells and 1,024 n for salivary glands. As expected, autophagic activity is very low during these stages (Figure 2). Initiation of wandering behavior, when larvae crawl out of the food in search of a dry place to pupariate around 108 h after egg laying (AEL), or starvation before this time results in a remarkable induction of autophagy in polyploid tissues (Figure 2), but not in diploid cells. This response is thought to serve as a nutrient reallocation mechanism, as breakdown products released from polyploid cells likely feed diploid tissues that will give rise to the adult fly by the end of metamorphosis. Mechanistically, growth signaling mediated by the insulin-like receptor is rapidly inactivated during starvation or at the beginning of metamorphosis in polyploid tissues [62, 96]. Diploid tissues such as the brain and wing disc appear to be able to grow and proliferate thanks to maintained activation of TOR signaling by sustained receptor Tyrosine kinase signaling, originating from Alk in neurons and Stit in future wing cells, respectively [97, 98]. In addition, the larval fat body secretes an insulin-like peptide (dilp6) during nonfeeding stages to maintain insulin signaling in diploid tissues [99].

As described briefly in the chapter on historical early studies, autophagy of the polyploid tissues including fat body and midgut cells is induced by a small peak of the molting hormone ecdysone towards the end of the last larval instar [20, 96]. Interestingly, there is a preprogrammed anterior-posterior gradient in the magnitude of autophagy in the fat body [100]. This is also observed for the separation of fat cells and kynurenine synthesis during metamorphosis, potentially due to the extremely low blood circulation in sessile prepupae and pupae, which necessitates the coordination of all these responses with respect to the location of nearby imaginal organs [100, 101]. Autophagy is induced in fat body cells as a cell-autonomous response, as overexpression of dominant-negative forms of the ecdysone receptor in mosaic animals maintains insulin signaling and blocks developmental autophagy in these cells [96]. Massive induction of autophagy is not seen during earlier ecdysone peaks that trigger larval molts, because high concentration of the juvenile hormone during the first and second larval stages inhibits autophagy. It is not known yet how juvenile hormone may inhibit autophagy. One candidate mechanism involves the peptidyl-prolyl cis-trans isomerase FKBP39. FKBP39 is a juvenile hormone target gene, and it has been shown to inhibit autophagy likely by preventing the translocation of the transcription factor FOXO into the nucleus [102, 103]. The presence of FOXO in the nucleus during starvation or at the beginning of metamorphosis likely promotes transcription of genes involved in autophagy, and its loss strongly impairs autophagic responses [103, 104]. It is worth mentioning that metamorphosis is not the only developmentally programmed starvation period in *Drosophila*, as larvae are also essentially immobile and do not feed during periods of molting that separate L1/L2 and L2/L3 stages, leading to increased autophagy in fat body (Gábor Juhász, unpublished data). This response is similar to the induction of autophagy observed during molting in worms [105].

Polyploid cells that account for the majority of larval masses undergo programmed cell death during metamorphosis. Initially, the larval fat body disintegrates into individual trophocytes following puparium formation, which is triggered by a prominent ecdysone peak at the end of the last larval instar [106]. Interestingly, approximately half of the larval fat cells survive until eclosion of adult flies and are only eliminated by caspase-dependent cell death during the first two days of adult life, promoting the survival of starved young adults [107, 108]. Salivary glands are also almost entirely composed of polyploid cells in the larva, with the exception of a ring of diploid imaginal cells surrounding the ducts of the paired glands. Larval gland cells are eliminated around 13–18 h after puparium formation, and both autophagy and activation of apoptotic caspases have been shown to facilitate histolysis, although the relative importance of each pathway is not fully understood [109–114]. A wave of autophagy is also seen in larval midgut cells of wandering larvae, but their elimination begins only after puparium formation, and it is not completed until after adult flies eclose [96, 115]. Groups of diploid imaginal cells (scattered throughout the larval gut) proliferate and replace polyploid cells during this process. Thus, polyploid cells are extruded into the lumen of the future adult gut, which is accompanied by caspase activation, DNA fragmentation, and autophagy-mediated shrinkage of these larval cells [85, 110, 112, 113, 115]. Remnants of the larval midgut form the meconium, the waste product that adult flies get rid of during the first defecation.

There is some discrepancy regarding the role of the apoptotic and autophagic pathways during larval *Drosophila* midgut degeneration. Two papers suggested that midgut shrinkage is blocked by expression of the caspase inhibitor p35, or by simultaneous loss of two proapoptotic genes Rpr and Hid [110, 112]. Importantly, RNAi depletion of the caspase inhibitor DIAP1 leads to premature caspase activation and death of larval midguts and salivary glands [110]. In contrast, midgut shrinkage was suggested to proceed largely independent of caspase activation based on experiments carried out on animals with a combination of mutations for certain caspases, whereas midgut cells fail to shrink properly if certain *Atg* genes are silenced or mutated [85, 115]. Interestingly, overexpression of Hid in *Drosophila* larvae triggers apoptosis in diploid cells of the developing eye and brain, but it leads to the induction of autophagy in polyploid cells of the fat body, salivary glands, and midguts [116], also indicating tissue-specific differences in the mechanism of action of certain proapoptotic genes.

In contrast to ecdysone-mediated shutdown of insulin signaling, which is responsible for the initial wave of autophagy in wandering animals, death of polyploid cells in salivary glands and midguts appears to be regulated by

a complex transcriptional cascade. As mentioned earlier, the elimination of about half of the fat body cells takes place in the pupa in a seemingly random manner, and surviving cells only die in young adults [108]. In prepupal midguts and pupal salivary glands, binding of ecdysone (or more likely its active form 20-hydroxyecdysone) activates the heterodimeric steroid receptor complex consisting of EcR and USP (the homolog of mammalian retinoid X receptor). Activation of this complex by ecdysone is necessary to trigger salivary gland cell death by inducing transcription of insect-specific target genes such as E93, E74A, and BR-C, but this process also requires a competence factor: the nuclear receptor $\beta$FTZ-F1 [117]. E93 is a transcription factor acting as a master regulator of the complex genetic programme involved in the death of both larval salivary glands and midgut in *Drosophila* [114, 118]. The role of autophagy in dying salivary gland and midgut cells may not be restricted to the recycling of building blocks to support diploid cells. Autophagy in dying mammalian cells is known to promote the release of so-called "eat me" and "come get me" signals to attract engulfing macrophages [119]. While larval midgut cells are situated inside the adult gut and are therefore protected from hemocytes, clearance of salivary gland cell fragments may be facilitated by macrophages in the pupa. This hypothetical scenario would explain why salivary glands undergo complete histolysis, whereas midgut cell remnants remain in the lumen of the adult gut until excreted.

Given the seemingly important role of autophagy during *Drosophila* development, it is surprising that null mutants for different genes show large differences regarding viability. Null mutants of *Atg1*, *Atg13*, and *FIP200* display a highly penetrant pharate adult lethality: adult flies form completely inside the pupal case, but almost all of them fail to eclose [45–47, 120]. The lipid kinase complex subunit null mutants (*Atg6, Vps34*, and *Vps15*) die much earlier (as L3 stage larvae), and only a few *Atg6* mutants are able to initiate pupariation [51, 54, 55]. This is not surprising considering that these gene products are involved in endosome maturation and biosynthetic transport to lysosomes acting in a complex with UVRAG. It is worth noting that UVRAG null mutants also die as late L3 stage larvae, even though UVRAG is dispensable for autophagosome formation or fusion with lysosomes [58, 121]. It will be interesting to see the phenotype of flies null mutant for *Atg14*, which encodes the autophagy-specific subunit of this complex, as these should behave similar to Atg1 kinase complex subunits in showing pharate adult lethality. Similarly, both *Atg2* and *Atg18* mutants are late pupal/pharate adult lethal. In contrast, all null mutants identified so far in genes encoding proteins involved in the ubiquitin-like conjugation systems are viable, including *Atg7* [113], *Atg8a* [57, 122], and *Atg16* (Gábor Juhász, unpublished data). Moreover, these null mutants can be maintained as viable stocks over multiple generations despite their shorter lifespan and increased stress sensitivity. The reason why null mutations affecting conjugation system components are viable in *Drosophila* is not known. A recent paper showed that prepupal midgut shrinkage requires Atg8a and Atg16, but not Atg3 or Atg7 [115], suggesting that Atg8a promotes cell shrinkage in a lipidation-independent manner. Still, these results do not explain the lethality data described above. Potential explanations can be that certain *Atg* genes are not required for autophagy in certain key developmental settings (such as *Atg3* and *Atg7* in midgut shrinkage), or that the ones that are lethal also have important roles independent of autophagic degradation (similar to *Vps34*, *Vps15,* and *Atg6*). It is important to note that *Atg3, Atg5, Atg7, Atg9,* and *Atg16L1* knockout mice complete embryonic development and are born at expected Mendelian ratios and only die due to suckling defects, whereas the loss of beclin *1/Atg6* leads to lethality during early embryogenesis [4].

Another role of autophagy has been described in the *Drosophila* ovary. During oogenesis, 15 nurse cells transfer a large part of their cytoplasm to the single oocyte through interconnecting cytoplasmic bridges called ring canals. Nurse cells die after the oocyte has matured, which is accompanied by caspase activation and DNA fragmentation. Caspase activation is reduced in nurse cells lacking Atg1, Atg13, or Vps34, and both DNA fragmentation and cell elimination are reduced [123]. Interestingly, the antiapoptotic protein Bruce accumulates in these mutant cells. Bruce colocalizes with GFP-Atg8a in wild-type ovaries, and loss of Bruce restores nurse cell death in autophagy mutants [123]. These observations suggest that autophagic elimination of Bruce may contribute to caspase activation and cell death in late stage *Drosophila* ovaries. However, mutation of either core autophagy genes or caspases, or the simultaneous loss of both autophagy and caspases still results in only a partial inhibition of developmental nurse cell death [124]. In contrast, hypomorphic mutation of dor/Vps18, a subunit of the HOPS complex, blocks nurse cell elimination much more efficiently, suggesting that lysosomes or endocytosis may play a more important role in developmental nurse cell death than autophagy or caspases [124, 125].

Autophagy can also be induced in the ovary during two earlier nutrient status checkpoints in germarium and mid-oogenesis stages, both in nurse cells and follicle cells, somatic epithelium surrounding germ cells [126–128]. This autophagic response requires core Atg genes and the caspase Dcp-1, and it can be suppressed by overexpression of Bruce [126, 127]. Interestingly, oogenesis is impaired in chimeric ovaries lacking autophagy in a subset of follicle cells but not in the germline, which may be caused at least in part by precocious activation of Notch signaling in mutant follicle cells [127, 129].

Another example for developmentally programmed autophagy is seen in the amnioserosa, a polyploid extraembryonic tissue of the developing embryo. Autophagy is induced prior to, and independent of, the activation of a caspase-dependent cell death programme in these cells [130]. Autophagy is also activated in a subset of amnioserosa cells that undergo extrusion during dorsal closure, but it is not required for the death of these cells [131].

In contrast with the paradigm of the inverse regulation of cell growth and autophagy by TOR signaling, autophagy has been shown to be required for cellular overgrowth driven by the evolutionarily conserved transcription factor Myc. Myc is required for autophagy, both in *Drosophila* and mammalian cells [73, 132]. Conversely, overexpression of this well-known

oncogene not only enhances cell growth, but it also leads to autophagy induction through activation of PERK, an ER-associated kinase involved in the unfolded protein response (UPR). Importantly, blocking PERK or autophagy prevents Myc-induced overgrowth in *Drosophila* and inhibits Myc-induced tumorigenesis in mouse models [73, 133]. These results suggest that inhibition of PERK or autophagy may be a potential therapeutic strategy in the context of Myc-dependent cancers.

## 5. Autophagy Implication in the Immune Response, Aging, and Neurodegeneration

Autophagy plays an important role in development, cellular differentiation, and homeostasis. Defects in autophagy are associated with many diseases including neurodegeneration, ageing, pathogenic infection, and cancer [5]. *Drosophila* melanogaster has been shown to be an excellent model system to study such cellular processes. The key advantages of using *Drosophila* as a disease model organism are short life cycle, small body size, ability to produce large number of progeny, availability of powerful genetic tools, and less redundant genome than that of mammals. Moreover, more than 70% of human disease genes have orthologues in *Drosophila* [134].

Autophagy has also been proposed to play a role in the removal of pathogens, given that it is the only degradative system in the cell which is able to handle cargo that is too large for proteasomal degradation. Evidence shows that autophagy is able to capture and degrade multiple categories of pathogens, including bacteria, viruses, and parasites [135]. This is not, however, a universally effective defence system, as some pathogens have developed resistance against it, or even learnt how to use autophagy in order to enhance their own replication [135, 136]. This interplay between host defences and infective agents suggests that autophagy, as an intracellular immune response, has exerted strong selective pressure on pathogens over the course of a long evolutionary time [137]. Flies lack an adaptive immune system, which facilitates the study of autophagy-derived innate immunity at the cellular level, without added complexity [138].

*Drosophila* has also been used successfully to study of the effects of pharmacological modulators of autophagy in neurodegenerative disease models. The available *Drosophila* disease models successfully recapitulate many of the symptoms associated with human diseases, and these can be used to identify new factors with a role in diseases [134].

*5.1. Autophagy-Derived Innate Immunity.* In mammals, pathogen recognition activates the antimicrobial response of the host, using transcription level regulators [137]. So far, two well-characterised nuclear factor-$\kappa$B (NF-$\kappa$B) pathways are known in flies: the Toll and immune deficiency (IMD) pathways, which are key to regulating the immune response against bacterial and fungal infections, by means such as the secretion of antimicrobial peptides (AMPs) [138, 139]. The Jak-Stat pathway, native to higher organisms, also plays a role in the immune defence response in flies, and all of the aforementioned pathways have been observed to mediate antiviral responses at the level of transcription [140, 141]. There are many aspects of the innate immune response in insects which are yet to be elucidated, and the role of autophagy in the antimicrobial response is only beginning to be deciphered. Striking parallels were observed between flies and mammals in terms of antimicrobial functions of autophagy [137]. A new aspect in mammalian antimicrobial autophagy, which is quickly gaining visibility, is the role of pattern recognition receptors (PRRs) in the activation of autophagy [135, 142]. These receptors work by recognising well-conserved molecular signature sequences, called pathogen-associated molecular patterns (PAMPs) [143]. The *Drosophila* protein Toll was first used to pinpoint the mammalian Toll-like receptors (TLRs) by virtue of homology, which make up the canonical pattern recognition system [137, 138]. These membrane receptors can induce autophagy upon binding to a cognate ligand [144]. Their cytoplasmic counterparts, the NOD-like receptors (NLRs), can activate autophagy as well [145, 146]. The importance of autophagy control by PRRs in mammalian host defence is certainly an interesting research avenue, despite the difficulty of assessing its *in vivo* potential during infection in mice. *Drosophila*, on the other hand, offers a much more genetically malleable system for such studies. The relationship between autophagy and PRRs has been found to be critical in preventing the host from succumbing to viral and bacterial infections [137]. Hence, it is likely that antimicrobial autophagy is an ancient cellular response to invading pathogens.

Autophagy genes have been shown to confer resistance to parasites (*Toxoplasma gondii*), bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella enterica*, *Typhimurium,* and *Mycobacterium tuberculosis*), and viruses (Sindbis virus, vesicular stomatitis virus (VSV), and herpes simplex type 1) [147–154]. Importantly, a landmark study recently showed that parkin, a gene implicated in the pathogenesis of Parkinson disease by promoting the selective autophagic elimination of mitochondria, is also important for the recognition and subsequent autophagic degradation of infecting intracellular bacteria in mice and *Drosophila* [155].

In terms of bacterial resistance, the *Drosophila* immunity comes equipped with two previously mentioned major response pathways: the Toll pathway, which is usually activated by Gram-positive bacteria, and the IMD pathway, which mainly handles Gram-negative bacteria [138]. Activation of either of these systems depends on the receptors' ability to detect PAMPs, such as the bacterial cell wall component peptidoglycan (PGN) [138]. This process and the subsequent release of AMPs are vital given that flies that are deficient in either the IMD or Toll pathway display hypersusceptibility to bacterial infection [156].

There are, however, species that show resistance to such a host response. Both the IMD and Toll signalling pathways are dispensable for controlling intracellular *L. monocytogenes* in flies. Instead, once bacteria have escaped to the cytoplasm, autophagy restricts their replication. *L. monocytogenes* replication takes place in the cytoplasm of *Drosophila* blood cells, termed "haemocytes" [157]. It has been observed that *L. monocytogenes* induces autophagy, which was visualised by the appearance of GFP-fused LC3 puncta that colocalised

with internalised bacteria [157]. This study showed that RNAi-mediated silencing of core autophagy genes causes increased bacterial replication and reduces fly life expectancy in infected adultsvspace2pt

In mammalian cells, autophagy can also degrade *L. monocytogenes*, but this process is normally blocked by the release of ActA, which inhibits the host's ability to ubiquitinate the pathogen and target it for autophagosomal degradation [153]. A similar autophagy evading behaviour has been independently observed in conjunction with protein InlK, although the mechanism is yet unexplained [158]. Failure to successfully resist the host's response, such as in the unnatural host *Drosophila*, reveals restrictive pathways that the *L. monocytogenes* cannot evade and highlights the constant adaptations that the bacterium must undergo in order to effectively counteract the immune responses of the host [137]. Upstream of the IMD pathway is the PGN recognition protein (PGRP) family receptors, which recognize bacterial PGN structures. PGRP-LC is a transmembrane sensor, which recognises monomeric and polymeric diaminopimelic acid-(DAP-) type PGN at the cell surface. PGRP-LE comes in two forms that have both cell-autonomous and non-cell-autonomous functions [159]. It is constitutively secreted into the open circulatory system, where it activates the IMD pathway [160]; it is also found within immune cells and acts as an intracellular receptor for the detection of the PAMP tracheal cytotoxin, a monomeric DAP-type PGN, initiating the release of the listericin AMP [161, 162]. Loss of either of the two receptors confers susceptibility to infection by *L. monocytogenes*, but only PGRP-LE initiates autophagy as an immune response. Unexpectedly, PGRP-LE can signal via the IMD pathway, components of which are not required either for autophagy induction or intracellular bacterial sequestration, suggesting that an unknown signalling pathway links PRR engagement to antimicrobial autophagy in *Drosophila*. Autophagy is observed to play an important regulatory role against a variety of bacterial invaders. Multiple hosts have been found to utilise autophagy to control the growth of *Wolbachia*, a common endosymbiotic bacterium, found in arthropods and filarial nematodes. Activation of autophagy by starvation or rapamycin treatment was found to reduce the rate of bacterial replication; conversely, siRNA-mediated depletion of Atg1 in flies was associated with enhanced bacterial replication [163].

In addition to controlling bacterial infection, autophagy was found to impact viral replication and pathogenesis in some mammalian infections [137]. Overexpression of beclin-1 (mammalian homologue of Atg6) in neonatal mice protects neurons against Sindbis virus infection-induced pathogenesis [164]. Loss of Atg5 expression accelerates the development of Sindbis-associated symptoms, due to failed viral capsid clearance, even though autophagy does not appear to affect viral replication proper [150]. A range of other viral agents are ostensibly managed by autophagy, such as HIV, encephalomyocarditis virus, and human papilloma virus in mammalian cells, although the *in vivo* significance has not been weighed [165, 166].

Recent data demonstrates that autophagy is a key element of the innate antiviral response against (−) ssRNA Rhabdovirus VSV in flies [151]. Negative sense viral RNAs must be first converted into mRNA-like positive-sense strands by an RNA polymerase, before they can be translated. Depletion of core autophagic machinery genes in *Drosophila* S2 cells leads to increased viral replication. Along the same lines, RNAi silencing of autophagy genes was associated with increased viral replication and mortality after infection of flies, directly linking autophagy with an important antiviral role *in vivo* [151]. VSV was observed to induce PI3 K-Akt regulated autophagy in primary haemocytes and in adult flies [151]. Similar to the immune response against *L. monocytogenes* infection, antiviral protection is also initiated by the recognition of PAMPs [151]. An active response against UV-inactivated VSV suggested that nucleic acids are not the targeted markers; rather, the viral glycoprotein VSV-G was sufficient to induce autophagy. Eventually, the *Drosophila* Toll-7 receptor was identified as the PRR, which identifies VSV as a trigger for an autophagic response [167]. Toll-7 is localised to the plasma membrane in order to interact with the virions, suggesting that the roles of Toll-7 and the mammalian TLRs are similar. Toll-7 restricts VSV replication in cells as well as in adult flies, as deficiency of Toll-7 leads to significantly increased mortality after infection [167]. Recent work has drawn in other Toll receptors as likely participants in the host's immune response. Tollo (Toll-8) has been shown to negatively regulate AMP expression in *Drosophila* respiratory epithelium [168]. Many antiviral factors are upregulated during infection; given that *Drosophila* Toll and Toll-7 receptors have been recently shown to be transcriptionally induced upon infection, it is possible that the other less characterised Toll receptors may also play a role in antiviral defences (Figure 3).

There is an overlap in the mode of action of Toll receptors and mammalian TLRs in triggering autophagy. A number of studies using model ligands and in vitro systems have shown autophagy induction via the TLR pathway (such as lipopolysaccharide, a ligand for TLR4, by looking at the colocalisation of autophagosome markers and intracellular bacteria) [169]. Autophagic activation can be observed using canonical ligands for TLR1, TLR3, TLR5, TLR6, and TLR7 [144, 170]. TLR8 was revealed in a recent study to activate vitamin D-dependent autophagy in human macrophages, in order to restrict HIV replication [137, 171].

*5.2. Autophagy in Ageing and Life Span Extension.* Ageing is a complex process that involves a progressive decline in physiological functions of an organism, eventually causing disease and death [172]. During this decline, cellular and molecular damage accumulates such as deleterious mutations, shortening of telomeres, accumulation of ROS, damaged organelles, and misfolded proteins. Aged individuals have increased sensitivity to environmental stress and a decreased capacity to maintain cell and tissue homeostasis. Prevalence of many diseases such as neurodegeneration, cardiovascular dysfunction, and cancer increases with age [173].

Autophagy maintains cellular homeostasis by targeting unwanted and deleterious intracellular materials to the lysosome for degradation. Autophagy has been implicated

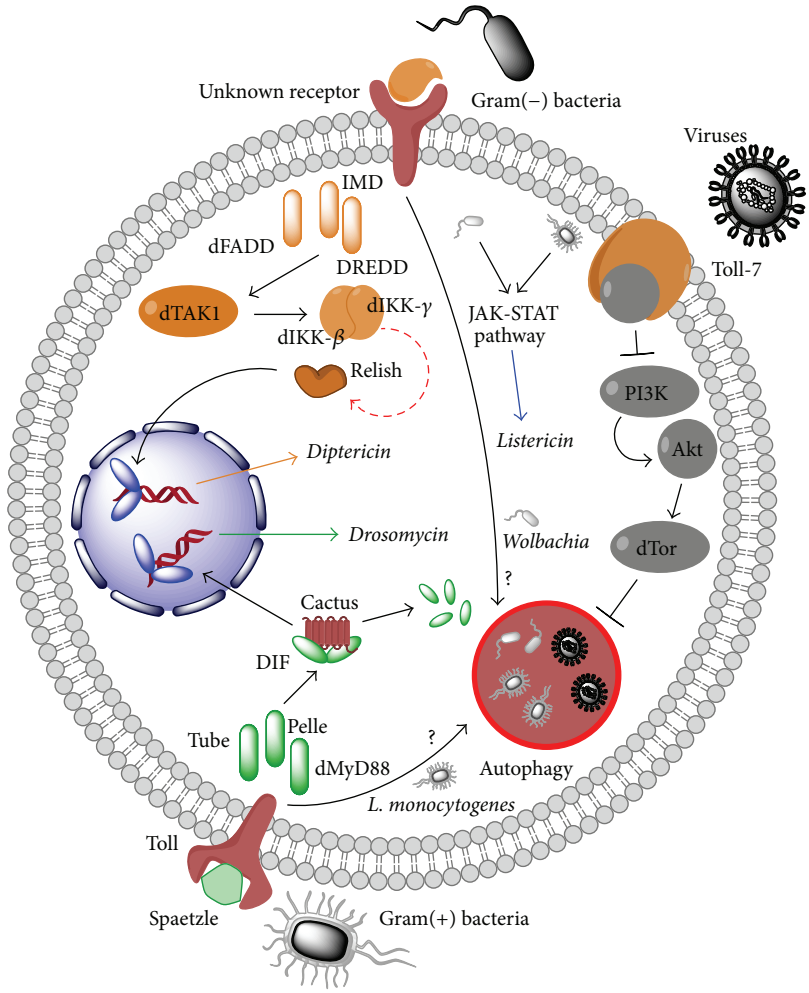


Figure 3: *Drosophila* immunity response pathways. A robust innate immunity system confers *Drosophila* protection against a variety of pathogens. Autophagy has been suggested to play a role in restricting infections, but the exact pathway of this response has yet to be deciphered. In addition there have been observations of a number of antimicrobial peptides (e.g., Diptericin) being expressed in response to immunological challenge.

in numerous diseases [5]. Accumulating evidence indicates that the efficiency of autophagy decreases with age, and the induction of autophagy delays aging-associated symptoms and extends life span [172]. In addition to the direct effect of autophagy on ageing, cellular pathways with a role in regulating ageing are shown to induce autophagy as their downstream targets [174–176]. These highly conserved pathways are insulin/insulin like growth factor (Igf) (ISS) pathway, the TOR pathway, c-Jun N-terminal kinase (JNK) signaling, and histone deacetylation [174, 177].

During ageing, the expression levels of several autophagy genes are downregulated in mammals. Autophagy mutants often exhibit phenotypes such as the accumulation of ubiquitinated protein aggregates, damaged organelles, increased sensitivity to oxidative stress, abnormal motor function, and short life span that are similar to those observed during ageing [172]. The expression level of Atg5, Atg7, and Beclin-1 is downregulated in human brains during ageing [178, 179]. Furthermore, a decrease in Beclin-1 expression has been reported in the brains of patients with Alzheimer's disease (AD) and Huntington's disease (HD) [179, 180]. Disruption of autophagy by reducing Beclin-1 expression enhances the severity of neurodegenerative phenotypes in transgenic APP (amyloid precursor protein) mice, and overexpression of Beclin-1 was sufficient to rescue the adverse effects in APP transgenic mice [180]. Suppression of basal autophagy in the central nervous system causes neurodegenerative phenotypes in mice even in the absence of a toxic protein: mice lacking Atg5 or Atg7 specifically in the central nervous system exhibit behavioural defects, motor dysfunction, accumulation of protein aggregates, and reduced life span [181, 182]. Chaperone-mediated autophagy (CMA) has been shown to be downregulated in rat livers during ageing as well. Restoring the level of chaperone-mediated autophagy by overexpressing LAMP2a, a CMA receptor, decreased the accumulation of damaged proteins and increased organ function [183]. A reduction in autophagy levels is also observed in mice during ageing. The heart-specific deletion of Atg5 causes abnormal heart morphology and the accumulation of

abnormal protein aggregates and damaged mitochondria in mice [184].

Similar to these observations in mammals, the expression of several autophagy genes (Atg2, Atg8a, Atg18, and bchs) is reduced in *Drosophila* during ageing. This correlates with an increase in accumulation of insoluble ubiquitinated protein aggregates (IUP) in the ageing brain [122]. *Drosophila* Atg8a mutants exhibit reduced autophagy, increased accumulation of IUP, increased sensitivity to oxidative stress, and reduced life span. Overexpression of Atg8a in adult brains decreased the incidence of IUP and increased oxidative stress tolerance and life span [122]. Similarly, *Drosophila* Atg7 null mutants are hypersensitive to nutrient and oxidative stress. Atg7 null mutants exhibit reduced life span and progressive neurodegeneration, which is characterized by the accumulation of ubiquitinated proteins [113]. Overexpression of Atg7 increases life span in wild-type flies and also rescues the age-related phenotypes caused by the knockdown of Hsp27 chaperone in *Drosophila*. Interestingly, overexpression of Hsp27 also extends life span in wild-type flies and rescues the neurodegenerative phenotypes caused by mild polyQ toxicity. The Hsp27-mediated rescue effect is abolished in flies lacking Atg7 [185]. Loss of the autophagosomal SNARE Syntaxin 17 has severe consequences: young mutant adults perform extremely poor in standard climbing tests that measure neuromuscular function and die within 3-4 days of eclosion. This is potentially due to large-scale accumulation of autophagosomes in neurons which causes neuronal dysfunction, rather than to cell death, as the lethality and behavior defects cannot be rescued by genetic inhibition of caspases in Syntaxin 17 mutant brains [80].

The insulin/insulin-like growth factor (Igf) pathway modulates longevity in multiple species [177]. The first insights into the role of the insulin pathway in longevity came from *C. elegans*. Mutant worms with reduced insulin signaling (mutation in insulin/insulin like receptor (igf), *daf2*) live twice as long as wild-type ones [186]. The longevity effect of the daf2 gene mutation is mediated through *daf16*, the *C. elegans* homologue of transcriptional factor FOXO. The Igf pathway negatively regulates the downstream acting FOXO transcriptional factor [187]. Knocking down the expression of autophagy genes (atg5, atg12, or bec1) abolishes the longevity effect of reduced insulin signaling in *daf2* mutants. It is worth noting that deletion of bec1 also reduces life span in wild-type worms [188].

*Drosophila* mutants with decreased insulin signaling (mutation in Insulin like receptor (InR) or in insulin receptor substrate *chico*) exhibit slow ageing and increased life span [189, 190]. Similar to *C. elegans* Igf mutants, these mutants also require FOXO for life span extension [191, 192]. Phosphorylation of FOXO by activated Igf prevents its nuclear localization and leads to the transcriptional downregulation of FOXO target genes. FOXO mediates the activation of pathways that inhibit growth and promote stress response [193]. It has been shown that FOXO induces autophagy in *Drosophila* larvae [103]. Furthermore, specific activation of FOXO in head fat body increases life span and oxidative stress tolerance. This localized overexpression of FOXO decreases systemic insulin signaling and it is correlated with a decrease in expression of dilp 2 (insulin-like peptide 2) in neurons [193]. Further studies show that reduced insulin signaling causes transcriptional repression of dawdle, an activin-like ligand in the TGF-beta super family, through FOXO, which in turn activates autophagy, thereby maintaining protein homeostasis. This study also shows that overexpression of Atg8a in muscle is also sufficient for life span extension in *Drosophila* [194].

Progressive muscle degeneration is associated with ageing and this precedes other age-related pathologies across species. However, the mechanism underlying muscle ageing is not completely understood. Muscle degeneration is associated with the accumulation of ubiquitinated protein aggregates, which are also positive for Ref(2)P in *Drosophila*. Overexpression of FOXO, or its target 4E-BP, in muscle prevents protein accumulation and increases muscle function via autophagy in *Drosophila*. Overexpression of FOXO increases Atg gene expression in muscle. RNAi-mediated knockdown of Atg7 to about half in FOXO overexpression backgrounds partially increases protein accumulation, suggesting that the effects of FOXO overexpression require autophagy. Moreover, the increase in muscle function by FOXO/4E-BP overexpression is sufficient to extend life span. FOXO/4E-BP overexpression in muscles regulates organism-wide protein homeostasis by reducing feeding and also by decreasing the release of insulin-like growth factors from neurosecretory cells in the brain [195].

JNK signaling plays a major role in regulating ageing in *Drosophila*. Activation of JNK signaling increases tolerance to oxidative stress and extends life span [196]. Life span extension upon JNK activation is also mediated through FOXO. Flies with reduced FOXO activity fail to extend life span and exhibit reduced tolerance to oxidative stress even upon JNK activation. The JNK pathway antagonizes the ISS pathway and promotes the translocation of FOXO to the nucleus [197]. Nuclear translocation of FOXO results in the transcription of autophagy genes [103]. JNK/FOXO reduces Igf activity systemically by reducing dilp2 expression in neuroendocrine cells [197]. JNK-mediated protection from oxidative stress is abolished in flies with compromised autophagy, and the induction of JNK signaling may activate autophagy through FOXO [198].

Spermidine, a naturally occurring polyamine, increases life span in multiple species. Levels of polyamines have been shown to decrease during ageing [199]. Dietary supplementation of spermidine induces autophagy and extends life span in *Drosophila*, and spermidine-mediated longevity is abrogated in flies which lack Atg7 [199]. Moreover, spermidine triggered autophagy inhibits the age-associated cognitive impairment in *Drosophila* [200]. Spermidine regulates ageing most likely by epigenetically regulating autophagy. Spermidine inhibits histone acetyltransferases (HAT), which in turn cause a global deacetylation of histone H3 and activation of autophagy in yeast [199]. Interestingly, spermidine treatment may confer oxidative stress resistance both in autophagy-dependent and autophagy-independent ways in *Drosophila* [201].

The TOR pathway modulates ageing in multiple species. Decreased TOR signaling is associated with an increase in life span and increased tolerance to stress. Treatment of

*Drosophila* with rapamycin (an inhibitor of TOR) increases life span and tolerance to both nutrient starvation and oxidative stress. Rapamycin-mediated life span extension is abrogated in flies undergoing Atg5 RNAi [202]. Genetic inhibition of TOR also increases life span in flies [203]. This is likely due to the fact that TOR inhibition activates autophagy [5].

Dietary restriction (reduced food intake without malnutrition) has been shown to be an effective intervention to expand lifespan in multiple species, including *Drosophila* [174, 204]. Cellular pathways that mediate the longevity effect of dietary restriction are not fully understood. Studies in *C. elegans* show that autophagy is required for the longevity effect of dietary restriction. When autophagy is compromised (by deleting bec-1 and ce-atg7) in *eat-2* mutants (a genetic model for dietary restriction in *C. elegans*), longevity is blocked [205]. In fact, most longevity pathways have been suggested to converge on autophagy genes in worms [206].

*5.3. Autophagy and Neurodegeneration.* Neurodegenerative diseases encompass a group of progressive disorders characterised by memory loss, cognitive impartment, loss of sensation, and motor dysfunctions. The cellular hallmark of neurodegenerative disease is the presence of ubiquitinated protein aggregates and neuronal cell death [207]. Several lines of evidence connect autophagy with neurodegeneration. Autophagy maintains cellular homeostasis by removing aggregated proteins and damaged organelles. This process is the most critical in neurons, because neurons do not divide and cannot get rid of protein aggregates through self-replication or self-renewal [208].

One of the risk factors for neurodegenerative diseases is ageing. Ageing is associated with decreased autophagy [208]. The connection between autophagy, ageing, and neurodegeneration is described in detail in Section 5.2.

Several neurodegenerative disease models have been developed in *Drosophila*, based on overexpressing wild type or mutant versions of human disease proteins. These disease models also provide insights into the role of autophagy in the context of neurodegeneration [207].

The overexpression of a human huntingtin protein containing a 120-amino acid long polyQ expansion causes age-dependent degeneration in *Drosophila* compound eye [209]. Treatment of these flies with rapamycin reduces retinal degeneration in an autophagy-dependent manner, similar to results observed in mouse and cell culture models of HD [210]. Further studies showed that the beneficial effect of rapamycin was not restricted to huntingtin disease. Rapamycin treatment alleviates neurodegenerative phenotypes in *Drosophila* nonhuntingtin polyglutamine, polyalanine, and tau disease models [211]. Induction of autophagy by rapamycin is conserved from yeast to mammals. A high-throughput drug screen identified three novel drugs, which induce autophagy independent of TOR. These small molecules reduce the number of protein aggregates and cytotoxicity, both in cellular and *Drosophila* models of neurodegenerative disease [212, 213]. Overexpression of Rab5 also ameliorates huntingtin-induced cell death in *Drosophila*, potentially by the formation of a Rab5 complex with Beclin-1 and Vps34, leading to enhanced autophagosome formation [214].

An independent study documented that hyperactivation of the TOR pathway suppresses autophagy and leads to neuronal cell death. Overexpression of Rheb, an activator of TOR, causes age- and light-dependent degeneration in the *Drosophila* retina. This was likely due to autophagy suppression, as autophagy induction by Atg1 was sufficient to rescue retinal degeneration. Similarly, overexpression of Atg1 or genetic inhibition of TOR by overexpressing TSC1/2 alleviates the neurodegenerative phenotype in *Drosophila* HD and phospholipase C- (norpA-) mediated retinal degeneration models. This study suggests that neurodegenerative symptoms observed in these flies are due to TOR-dependent suppression of autophagy, and not due to the effect of TOR on cell growth [215].

Puromycin-sensitive aminopeptidase (PSA) is the only cytosolic enzyme capable of degrading polyQ sequences. PSA has been shown to be involved in neurodegeneration in *Drosophila*, mice, and cell culture models of poly Q diseases. Overexpression of PSA inhibits polyQ toxicity, whereas inhibiting PSA expression enhances poly Q toxicity in *Drosophila* models of poly Q diseases. PSA was suggested to reduce polyQ toxicity by activating autophagy and subsequent clearance of toxic aggregates, but how it may promote autophagy is still unknown [216].

Results of a genetic modifier screen aimed at the identification of genes involved in Ataxin3 toxicity in *Drosophila* found numerous candidates. A subset of the suppressors was proposed to act either by enhancing autophagy-mediated clearance of protein aggregates or by inhibiting autophagy to prevent autophagy-mediated cell loss. This study also pointed out that only the pathogenic form of ataxin3, and not wild type ataxin, induces autophagy [217].

Induction of autophagy does not rescue neurodegeneration caused by the polyglutamine-containing atrophin in *Drosophila* DRPLA (dentatorubropallidoluysian atrophy) model. The neurodegenerative phenotype is characterized by the accumulation of autophagic vacuoles in degenerating neurons and glia. Inhibiting autophagy by Atg5 RNAi or using an Atg1 null mutant enhances neurodegenerative phenotypes. However, both pharmaceutical and genetic inductions of autophagy failed to rescue neurodegeneration. Ultrastructural analysis showed the presence of abnormally large autolysosomes with impaired degradation of the contents. Thus, the beneficial effect of autophagy may be suppressed by lysosomal dysfunction in this case [218]. Transcriptional profiling identified that atrophin reduces the expression of fat, a tumor suppressor protein. Fat, and Hippo kinase acting downstream of it, may protect the neuron by activating autophagy [219]. Although the exact mechanisms of neuroprotection by the Fat/Hippo pathway are not fully understood, authors of these studies suggested two plausible mechanisms: (1) Hippo may activate autophagy by inhibiting TOR, or (2) Hippo might enhance autophagy through its interaction with Atg8a [220].

An immunoelectron microscopy study identified the accumulation of abnormal autophagic vacuoles (AV) in

human AD brain [221]. In line with that, overexpression of A$\beta$42 (the byproduct of APP proteolysis, a major component of Abeta inclusion in AD) results in age-dependent dysfunction of autophagy at a lysosomal stage in *Drosophila* [222]. This is characterised by the accumulation of abnormal autophagic vacuoles in the brain. The leakage of these vacuoles causes the acidification of cytosol, and further damage to membranes and organelles eventually leads to neuronal cell death. In contrast, overexpression of A$\beta$40, another byproduct of APP proteolysis, does not cause autophagy dysfunction or neuronal abnormality. This differential neurotoxicity raises the possibility that A$\beta$40 is degraded by autophagy. Interestingly, inhibition of autophagy partially rescues the neurodegenerative phenotype and activation of autophagy exuberates symptoms in A$\beta$42 *Drosophila* models. The authors of this study suggest that autophagy may act as a prosurvival pathway in early stages of the disease, and as a prodeath pathway in later stages [222].

Studies in *Drosophila* provide potential mechanistic links between UPS and autophagy. Autophagy is induced as a compensatory mechanism during proteasome dysfunction. This compensatory induction is dependent on histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase that interacts with polyubiquitinated proteins. Autophagy is induced in temperature sensitive proteasome mutant flies, and also in response to UPS impairment in *Drosophila* SBMA (spinobulbar muscular atrophy (SBMA)) models. Overexpression of HDAC6 was shown to rescue degenerative phenotypes associated with UPS dysfunction in an autophagy-dependent manner in these flies. Furthermore, HDAC6 overexpression rescues neurodegenerative phenotypes observed in *Drosophila* Ataxia and Abeta models. The rescuing effect of HDAC was again abolished in flies with impaired autophagy [223].

Studies in *Drosophila* have also contributed to our understanding of the link between endocytosis and neurodegeneration and its relation to autophagy. Mutations in the Endosomal Sorting Complex Required for Transport- (ESCRT-) III subunit CHMP2B are associated with FTD (frontotemporal dementia) and ALS (amyotrophic lateral sclerosis). These diseases are characterized by the presence of ubiquitinated protein aggregates, which are positive for p62/SQSTM1. The ESCRT complex is involved in the recognition and sorting of ubiquitinated endocytosed integral membrane proteins into the intraluminal vesicles of the multivesicular body (MVB) and is required for their subsequent degradation in lysosomes. Autophagic degradation is inhibited in cells overexpressing CHMP2B and in cells or *Drosophila* lacking ESCRT function. Reduced ESCRT function impairs the clearance of mutant huntingtin protein in cell and *Drosophila* models of HD diseases. These studies show that the functional MVB pathway is important for proper autophagic function [51, 224, 225].

## 6. Selective Autophagy in *Drosophila*

The Atg8 family proteins are required for the expansion of the phagophore membrane and also participate in cargo recognition and recruitment to the forming autophagosome. These ubiquitin-like (UBL) proteins are conjugated to phosphatidylethanolamine (PE) and are found both on the inner and outer sides of the autophagosome membrane. The Atg8 family proteins including LC3 (microtubule-associated protein 1 light chain 3) lie at the heart of selective autophagy, through their binding to selective autophagy receptors. Six receptors have been identified in mammals so far: p62/SQSTM1/SQSTM1, NBR1, NDP52, Nix, optineurin, and Stbd1 [226–228]. These proteins contain a LIR/LRS (LC3-interacting region/LC3 recognition sequence) motif and have been shown to interact with LC3 family proteins [198, 199].

*6.1. Selective Autophagy Receptors in Drosophila.* In *Drosophila*, only two selective autophagy receptors have been described so far: Ref(2)P, the homologue of mammalian p62/SQSTM1/SQSTM1, and blue cheese, the homologue of mammalian Alfy. p62/SQSTM1/SQSTM1 is the first and best understood selective autophagy cargo receptor. It is a multifunctional protein, performing a variety of functions in the cell [229, 230]. Human p62/SQSTM1 is 440 amino acids long and contains several functional motifs [229]. A Phox and Bemlp (PB1) domain is located at the N-terminus and is necessary for the multimerisation of the protein, as well as its interaction with a range of kinases (MEKK3, MEK5, ERK, PKC$\zeta$, PKC$\lambda$/t, and another autophagy receptor, NBR1) [229]. Following the PB1 domain is a ZZ zinc-finger domain, which interacts with the serine-threonine kinase receptor-interacting protein 1 (RIP1) [230]. Importantly, p62/SQSTM1 contains an LC3 interacting LIR/LRS motif, and a kelch-like ECH-associated protein 1 (KEAP1) interacting region (KIR) motif, which interacts with KEAP1 [231–233]. At its C-terminus, p62/SQSTM1 retains an ubiquitin-associated (UBA) domain, required for binding monomeric and multimeric ubiquitin [229].

p62/SQSTM1 binds to polyubiquitinated proteins and crosslinks these to the growing phagophore via Atg8/LC3 binding. A reduction in p62/SQSTM1 expression increases huntingtin-induced cell death in HD cell culture models [231, 234]. Autophagy deficient mice lacking p62/SQSTM1 failed to form ubiquitin positive aggregates, indicating that p62/SQSTM1 is important for aggregate formation [235]. The *Drosophila* p62/SQSTM1 homologue, Refractory to Sigma P (Ref(2)P), is 599 amino acids long and also contains an N-terminal PB1 domain, a ZZ-type zinc-finger domain, and a C-terminal UBA domain [236]. Similar to p62/SQSTM1, Ref(2)P is accumulated when autophagy is impaired and it has been found within protein aggregates in autophagy deficient *Drosophila* and in *Drosophila* neurodegenerative models [236] (Figure 4). It makes use of its PB1 domain to multimerise and is able to bind ubiquitin molecules via its UBA domain [237]. Ref(2)P also harbours a LIR motif between residues 451–458 (DPEWQLID) [237, 238], which fits well with the revised LIR motif sequence, proposed by Johansen and Lamark, which could be written as D/E-D/E-D/E-W/F/Y-X-X-L/I/V [229]. Ref(2)P has recently been established as a selective autophagy substrate in *Drosophila* as well [75]. Moreover, it has a putative KIR motif and

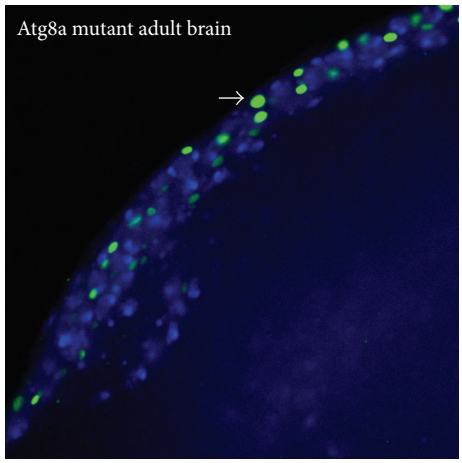


Figure 4: Ref(2)P accumulates in the brain of Atg8a mutant adult flies. Confocal micrograph of a mid-section of the optic lobe in the brain of an Atg8a mutant adult fly. The tissue is stained for Ref(2)P (green, arrow highlights an aggregate) and DNA (blue).

its interaction with both Keap1 and Atg8a appears to be conserved, too [73, 238, 239].

S6 kinase is a central regulator of autophagy and cell growth. TOR activation suppresses autophagy and leads to the phosphorylation of S6K. S6K was long considered as an autophagy inhibitor, a fact now contested, as S6K is found to be required for starvation-induced autophagy [62, 240]. Consistent with these observations, loss in S6K significantly increased the number (but not the size) of Ref(2)P aggregates in *Drosophila* larval fat body cells [57].

A novel role of Ref(2)P was reported in *Drosophila* haemocytes. Alongside Atg1, Ref(2)P-mediated selective autophagy was shown to be indispensable for cellular remodelling of the haemocyte cortex [241, 242]. Arresting autophagy with 3-methyladenine (3MA) or knocking down other Atg genes (Atg4, Atg6, Atg7, Atg8a, and Atg9) all produced a similar phenotype. Taken together, the above information demonstrates that Ref(2)P has a wide spectrum of cellular functions, like its human p62/SQSTM1 homologue, whose functions require further elucidation.

Loss of function mutation in *Drosophila* blue cheese gene (bchs) results in an age-dependent accumulation of ubiquitinated protein aggregates and amyloid precursor-like proteins and reduces life span. Abnormal central nervous system morphology and size were also documented in bchs mutants [243]. The ubiquitinated protein aggregates in bchs mutants are positive for Ref(2)P [244]. Alfy, the human homologue of *Drosophila* blue cheese, is involved in the selective disposal of ubiquitinated protein aggregates. Alfy is a large, 3527 amino acid long protein, which contains a variety of functional domains, including a FYVE domain suggesting an affinity for PI(3)-P rich endosomes. Instead, Alfy has been found to localise mostly to the nuclear envelope, but it translocates to autophagic membranes and ubiquitin-rich aggregates under strenuous cellular conditions [245]. Alfy-mediated aggrephagy makes use of p62/SQSTM1, the human homologue of *Drosophila* Ref(2)P. Alfy, together with p62/SQSTM1, may crosslink ubiquitinated protein aggregates with the core autophagy machinery for disposal, highlighting the importance of this so-called aggrephagy in neuronal homeostasis [246]. A genetic modifier screen based on the overexpression of blue cheese in *Drosophila* eye has linked lysosomal dysfunction to altered ubiquitin profiles and reduced life span and shows the genetic interaction between certain genes and blue cheese [247, 248]. Alfy has been shown to play a role in the removal of high polyQ-containing mutant huntingtin [246]. Blue cheese overexpression has been observed to rescue morphological and functional qualities in fly eyes expressing a polyQ127 transgene. Recent work by the Simonsen and Finley groups has established a link between overexpression of blue cheese C-terminal region and a general improvement of neurodegenerative phenotypes *in vivo* [246].

*6.2. Selective Autophagy and Chaperone Assisted Autophagy.* Chaperone-assisted autophagy (CAA) differs from macroautophagy in the method of cargo transport, which is mediated by chaperones in CAA, rather than via autophagosomes. However, there is a level of interplay between CAA chaperones and selective autophagy adaptor proteins, which uncovers a hybrid degradative solution, termed Chaperone-assisted selective autophagy (CASA). The *Drosophila* melanogaster cochaperone Starvin (Stv) interacts with ubiquitin adaptor Ref(2)P and ubiquitin ligase CHIP in order to coordinate the activity of Hsc70 and HspB8. This CASA complex is behind the selective degradation of damaged components in muscle Z disks. Loss of CASA function has been associated with progressive muscle weakness and general myopathies in flies, mice, and men [249, 250]. High molecular mass ubiquitin conjugates have been observed in mouse muscle tissue with a concomitant increase in the level of BAG-3 (mammalian ortholog of Starvin), as a result of repetitive tetanic contraction. These conjugates were observed to form microaggregates, which partially colocalised with LC3, suggesting an involvement of autophagosomal engulfment, as part of muscle protein degradation [249]. It is possible that selective macroautophagy and selective chaperone-assisted autophagy cooperate, in order to maintain a healthy protein landscape at tissue level.

*6.3. Mitophagy.* Mitophagy (selective autophagic degradation of damage impaired mitochondria) has been recently described in yeast and mammals [251]. Atg8/LC3 was observed to interact with mitochondrial membrane proteins via its LIR motif, such as the yeast Atg32 [252] and the mammalian NIP3-like protein NIX [253, 254]. The mechanism behind mitophagy is tightly connected to the fusion/fission behaviour of the mitochondrial network. A bioenergetically impaired mitochondrion is prevented from fusing back into the network, by the proteasomal degradation of the profusion factor mitofusin, Mfn, also known as marf in *Drosophila*. This behaviour is facilitated by the E3 ligase Parkin, recruited to the outer mitochondrial membrane (OMM) by PTEN-induced putative kinase protein I (PINK1) as a result of a loss in membrane potential [255, 256]. Parkin is thought to

target various OMM substrates such as Mfn: ubiquitinating them and targeting them for proteasomal degradation [257]. Fusion incompetent mitochondrial organelles are then removed by selective autophagy [251]. Mutations of Parkin and Pink1 are associated with familial forms of Parkinson's disease (PD). Most of our understanding of Pink1 and Parkin function comes from *Drosophila*. Pink1 or Parkin null mutants exhibit muscle degeneration, male sterility, reduced life span, and an abnormal mitochondrial morphology [258–260]. Overexpression of the mitochondrial fission inducer Drp1, or knocking down the expression of mitochondrial fusion inducers mfn or opa1 rescues the degenerative phenotypes in Pink1 and Parkin mutants. This suggests that Pink1 and Parkin maintain mitochondrial morphology at least in part by preventing mitochondrial fusion or by enhancing mitochondrial fission [261]. Pink1 and Parkin have been shown to be involved in mitophagy in mammalian cells [255]. Genetic analysis in *Drosophila* showed that Pink1 acts upstream of Parkin [258]. Recruitment of Parkin to mitochondria causes the ubiquitination of mfn in a Pink1-dependent manner. These studies indicate that both Pink1 and Parkin are involved in the removal of dysfunctional mitochondria, and loss of Pink1 or Parkin led to the accumulation of abnormal mitochondria, which causes oxidative stress and neurodegeneration [262, 263].

Recent work by Vincow et al. and colleagues suggests that mitophagy may be the result of an interplay between several processes [264]. Overall mitochondrial protein turnover in parkin null *Drosophila* was similar to that in Atg7 deficient mutants. By contrast, the turnover of respiratory chain (RC) subunits showed greater impairment with relation to parkin loss, than in Atg7 mutants. RC subunit turnover was also selectively impaired in PINK1 mutants [264]. Given the various degrees of mitochondrial protein turnover impairment in response to a deficit in either proteasom- associated factors or selective autophagy regulators, two theories attempt to pinpoint the pathways involved in mitophagy. One model revolves around the chaperone-mediated extraction of mitochondrial proteins [265]. Another possible model involves mitochondria-derived vesicles, which carry selected cargo directly to the lysosome, in an autophagy-independent manner [266]. The latter model has been observed experimentally, whereby vesicles were found to transport a membrane-bound complex IV subunit and contain inner mitochondrial membrane [267].

*6.4. Novel Selective Autophagy Regulators.* Protein ubiquitination is a widespread method for targeting molecules for selective autophagy, including bacteria, mitochondria, and aggregated proteins. As such, ubiquitinating proteins, such as the E1 Atg7, E2 Atg3, and E3 Atg12-Atg5-Atg16 are key regulators of autophagy [226]. Recent work has uncovered the first deubiquitinating enzyme of regulatory importance towards selective autophagy, Usp36 [268]. This protein inhibits selective autophagy in both *Drosophila* and in human cells, while promoting cell growth [269]. Despite phenotypic similarity, Usp36 is not actually part of the TOR pathway [268]. Loss of *Drosophila* Usp36 (*dUsp36*) accompanied the accumulation of aggregated histone H2B (known substrate of Usp36) in cell nuclei, reflecting profound defects of chromatin structure in *dUsp36* mutant cells. Knockdown of dUsp36 led to the accumulation of GFP-LC3 positive vesicles. Anti-LC3B antibody testing revealed an increase in both autophagosome and lysosome formation, inferring total autophagy flux activation in mutant cells and suggesting that USP36 inhibits upstream events of autophagosome initiation [268]. A link was established between p62/SQSTM1-mediated accumulation of ubiquitinated substrates following USP36 inactivation and subsequent induction of autophagy, providing a final piece of evidence that USP36 regulates selective autophagy by inactivating its cognate cargo via deubiquitination [268]. So far, USP36 is the only characterised deubiquitinating enzyme which has been linked to autophagy regulation. Recent studies have identified another two deubiquitinating enzymes, USP19 and USP24, both of which exert negative control on autophagy under normal nutritional conditions [270].

## 7. Conclusion and Future Direction

Studies on morphological aspects and the hormonal regulation of autophagy in insects including *Drosophila* have a long and successful history. More recently, molecular genetics has enabled the functional analysis of autophagy in this complete animal, in which all major tissue types and organs are found and function in many ways similar to our own body. Autophagy studies in *Drosophila* melanogaster have revealed that it has wide-ranging implications in sustaining homeostasis, with possible links to organism development, the immune response, and the removal of cellular damage and waste often associated with ageing and age-related diseases. From the presented literature, it is apparent that there are many unexplored avenues in the mechanisms and regulation of autophagic degradation in *Drosophila*. To better understand its molecular mechanisms, more efforts should be taken to identify selective autophagy receptors which are thought to govern the remarkable degradation specificity seen in certain settings. These studies will be facilitated by recently developed computer software to predict Atg8-family interacting proteins [271]. Manipulating selective autophagy influences the phenotype in a range of neurodegenerative disease models, such as Alzheimer's [272], Huntington's [273], and Parkinson's [274] diseases, which often revolves around the removal of molecules damaged by reactive oxygen species (ROS), or eliminating ROS synthesis sites such as impaired mitochondria. It would therefore be interesting to test whether upregulating autophagy can facilitate effective removal of proteins associated with neurodegenerative pathologies caused by the expression of hyperphosphorylated tau or high polyglutamine length huntingtin. It might be worth investigating the importance of mitophagy in maintaining a healthy cellular environment and resisting stress, particularly with regard to age-related myocardial degeneration, as this is a vastly underexamined area. Finally, the recent discovery of deubiquitinating enzymes as negative regulators of autophagy lays the ground for further study of a novel class of autophagy regulators.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Nitha C. Mulakkal, Peter Nagy, Szabolcs Takats and Radu Tusco are shared first authors. Gábor Juhász and Ioannis P. Nezis are shared senior authors.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 470482, 13 pages
http://dx.doi.org/10.1155/2014/470482

*Research Article*

# The Putative HORMA Domain Protein Atg101 Dimerizes and Is Required for Starvation-Induced and Selective Autophagy in *Drosophila*

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Received 17 January 2014; Accepted 11 March 2014; Published 8 May 2014

Academic Editor: Ioannis P. Nezis



The large-scale turnover of intracellular material including organelles is achieved by autophagy-mediated degradation in lysosomes. Initiation of autophagy is controlled by a protein kinase complex consisting of an Atg1-family kinase, Atg13, FIP200/Atg17, and the metazoan-specific subunit Atg101. Here we show that loss of Atg101 impairs both starvation-induced and basal autophagy in *Drosophila*. This leads to accumulation of protein aggregates containing the selective autophagy cargo ref(2)P/p62. Mapping experiments suggest that Atg101 binds to the N-terminal HORMA domain of Atg13 and may also interact with two unstructured regions of Atg1. Another HORMA domain-containing protein, Mad2, forms a conformational homodimer. We show that *Drosophila* Atg101 also dimerizes, and it is predicted to fold into a HORMA domain. Atg101 interacts with ref(2)P as well, similar to Atg13, Atg8a, Atg16, Atg18, Keap1, and RagC, a known regulator of Tor kinase which coordinates cell growth and autophagy. These results raise the possibility that the interactions and dimerization of the putative HORMA domain protein Atg101 play critical roles in starvation-induced autophagy and proteostasis, by promoting the formation of protein aggregate-containing autophagosomes.

## 1. Introduction

Autophagy ensures the lysosome-mediated degradation and recycling of cytoplasmic components including organelles. During the main pathway, a phagophore cistern (also called an isolation membrane) forms and captures cargo destined for breakdown into a double-membrane autophagosome. This vesicle then fuses with a late endosome or lysosome containing acidic hydrolases. Assembly of the phagophore is achieved by the action of Atg proteins. *Atg* genes were originally discovered in yeast, and the majority of them have clear orthologs in higher eukaryotes including animals [1, 2].

Initiation of autophagy usually begins with the activation of an Atg1 protein kinase complex in animal cells, which contains the serine/threonine kinase Atg1 (its orthologs are called UNC51 in worms and ULK1 and 2 in mammals), Atg13, FIP200/Atg17, and the metazoan-specific subunit Atg101 [3]. This complex directly binds to Tor (target of rapamycin) kinase, which is active when bound to digesting lysosomes and promotes cell growth and inhibits autophagy by phosphorylating Atg1 [4, 5]. Autophagy-inducing stimuli such as starvation rapidly lead to inactivation of Tor and induction of Atg1-dependent autophagy [3, 6]. This results in the removal of inhibitory phosphogroups from Atg1 by poorly characterized phosphatases, which may potentially include PP2A [7]. Atg1 then undergoes autophosphorylation on residues separate from those phosphorylated by Tor and also phosphorylates downstream targets including Atg13 [3, 8, 9]. Overexpression of Atg1 strongly promotes autophagy in *Drosopohila*, while expression of a kinase dead form suppresses starvation-induced autophagy [10].

It is largely unknown how the Atg1 kinase complex transmits its autophagy-inducing signal to downstream components, which include an autophagy-specific lipid kinase complex, phospholipid effectors such as Atg18, the transmembrane protein Atg9, and two protein conjugation systems that

include the ubiquitin-like Atg8 family proteins [1, 3]. Atg8 is conjugated to phosphatidylethanolamine, and thus it is bound to phagophore and autophagosome membranes [11, 12]. Atg8, and its mammalian homologs such as LC3, binds to cargo receptors including p62 to mediate the selective autophagic breakdown of ubiquitinated protein aggregates [13, 14]. Ref(2)P, the fly homolog of p62, is required for the formation of these protein aggregates in *Drosopohila* [15].

FIP200/Atg17 is thought to act as a scaffold protein in the Atg1 kinase complex, whereas the role of the metazoan-specific subunit Atg101 is poorly characterized [3]. Atg101 (also known as C12orf44) is a subunit of the Atg1/ULK complex in human cells, and shRNA depletion of *Atg101* impairs autophagy in mammalian cells [16, 17]. Moreover, the *Atg101* ortholog *epg-9* has recently been shown to be required for degradation of P granule aggregates in worm embryos as well [18]. As the precise role of Atg101 in autophagy is unclear, we decided to analyze its function in *Drosopohila*.

## 2. Materials and Methods

*2.1. Drosopohila Genetics.* Flies were reared on standard yeast-cornmeal-agar medium (fed), and mid-L3 stage larvae were transferred to a 20% sucrose solution for 3 h for starvation experiments. Fat body cell clones expressing RNAi constructs *Atg101[KK106176]* and *Atg101[HMS01349]* were generated spontaneously, as described in detail previously [19–22].

*2.2. Histology.* Dissected larval carcasses were incubated in a solution of LysoTracker Red (LTR) and DAPI as before [19–23]. Fixed samples were processed for indirect immunofluorescence using rat anti-Atg8a [9] and rabbit anti-ref(2)P [22] and imaged as described in detail previously [19, 20, 22]. Dissected fat body lobes containing *Atg101* RNAi clones were attached to poly-L-lysine-coated coverslips in PBS, photographed live to record the position of GFP-positive cells, fixed and embedded into Durcupan (Fluka), and then sectioned and analyzed by transmission electron microscopy as described [19, 21]. Statistical analysis was carried out as described [19, 20]. *N* refers to the number of animals, and multiple cells were evaluated from each animal.

*2.3. RT-PCR.* Total RNA was isolated from starved L3 stage larvae of the genotypes *w[1118]* (used as wild type), *Atg101[KK106176]/+*, *Act-Gal4/+*, and *Atg101[HMS01349]/Act-Gal4* using PureLink RNA Mini Kit (Invitrogen), followed by preparation of cDNAs using RevertAid First Strand cDNA Synthesis Kit (Thermo) and RNase free DNase I (Sigma). PCR reactions on cDNA samples were performed with the following primers: TACTCCTCCCGACACAAAGC, CTGGGTCATCTTCTCACGGT for *Actin5C* (23 amplification cycles), and ATGAACGCGCGTTCGCAG, TCACATTGCGAGCGTTTCCT for *Atg101* (25 amplification cycles). Parallel reactions were performed without adding reverse transcriptase to control for DNA contamination. As expected, no PCR products were obtained in these experiments.

*2.4. Cell Culture and Immunoprecipitations.* *Drosopohila* D.Mel-2 cells (Invitrogen) were used for transfection and immunoprecipitation experiments as recently described elsewhere [9, 19, 20]. The following constructs were used in coimmunoprecipitation experiments: an N-terminally truncated 3xFLAG-ref(2)P lacking the PB1 domain mediating self-aggregation, 3xHA-Keap1 [20], 3xHA-Atg18 [24], 3xHA-Atg101, full-length 3xHA-Atg13 and its N-terminal, middle, and C-terminal fragments [9], and kinase dead myc-Atg1 [8]. 3xHA-Atg8a, 3xHA-Atg16, 3xHA-RagC, 3xFLAG-Atg101, and Atg101-3xFLAG were generated by PCR amplifying the full-length coding sequences from a cDNA sample and cloning these into appropriate UAS vectors, respectively. GST coding sequence was PCR amplified and cloned into a UAS-3xHA vector downstream of the HA tag. 3xHA-GST-Atg1 constructs were generated by PCR amplifying and cloning the appropriate Atg1 fragments downstream of GST.

*2.5. Bioinformatics.* Sequence analysis and motif search were performed using the NCBI BLAST service as well as the Pfam (version 27.0) and Prosite (release 20.97) databases and their associated search tools. Alignments of orthologous sequences were extracted from OMA groups as cross-linked to the corresponding SwissProt entries [25]. For structure prediction, the I-TASSER and Phyre2 servers were used [26, 27]. Pairwise and multiple structural alignments were done with DaliLite and MAMMOTH-Mult, respectively [28, 29]. The ANCHOR and IUPred servers were used to predict unstructured regions and potential binding sites within these regions in fly Atg1, Atg13, and Atg101 [30, 31]. Structure visualization was performed using UCSF Chimera [32] and the Prosite HORMA logo was reproduced with WebLogo [33]. The alignment of human, fly, and worm Atg101 was generated using CLUSTALW and colored with TEXSHADE at the Biology Workbench server [34].

## 3. Results and Discussion

*3.1. Drosopohila Atg101 Is Required for Starvation-Induced Autophagy.* BLAST searches reveal that *Drosopohila* Atg101 shares 51% amino acid identity (111/218) and 71% similarity (156/218) with human Atg101 and 33% amino acid identity (81/244) and 46% similarity (113/244) with isoform a of worm Atg101/EPG-9, respectively (Figure 1), suggesting that it is an ortholog of Atg101 proteins. Two separate transgenic RNAi lines are available from public stock centers that allow inducible silencing of *Drosopohila Atg101*, both integrated into specific, nonrandom landing sites in the genome. *Atg101[KK]* is based on a long hairpin, while *Atg101[HMS]* contains a short, microRNA-based duplex RNA that targets a 22-nucleotide sequence in the 3′ untranslated region of the endogenous mRNA. LysoTracker Red (LTR) is widely used for the labeling of acidic autolysosomes in the larval *Drosophila* fat body, as this vital dye shows little to no punctate staining in fat body cells of well-fed larvae [9, 19, 23, 35]. Expression of either of the *Atg101* RNAi constructs in GFP-marked cell clones prevents starvation-induced LysoTracker Red- (LTR-) positive autolysosome formation (Figures 2(a),

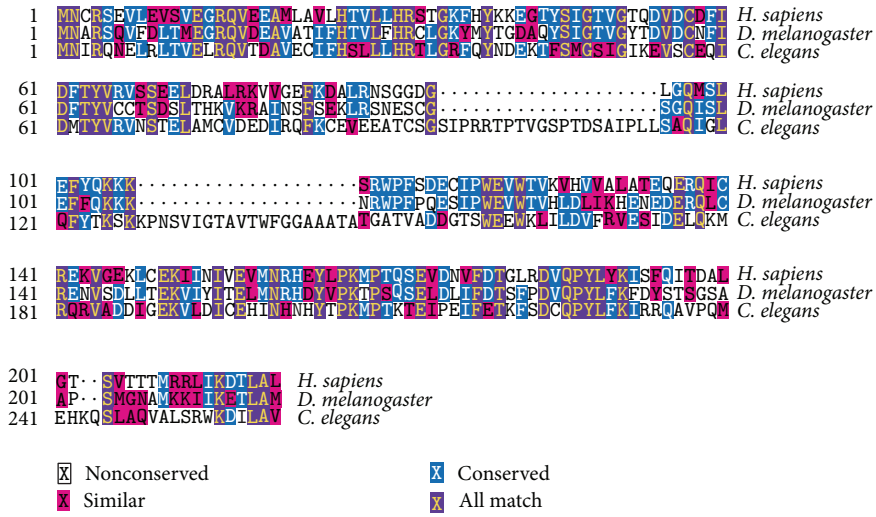


Figure 1: Multiple sequence alignments of human, fly, and worm Atg101 proteins.

2(b), and 2(c)). As expected, systemic expression of either of these transgenic RNAi lines strongly decreases endogenous *Atg101* mRNA levels (Figure 2(d)). Depletion of *Atg101* also impairs the distribution of endogenous Atg8a, as instead of the numerous small Atg8a-positive autophagosomes seen in control cells, fewer but larger Atg8a structures are observed in GFP-marked *Atg101* knockdown cells (Figures 3(a), 3(b), and 3(d)). This may not be simply due to incomplete gene silencing, as these aberrant Atg8a-positive structures are also seen upon simultaneous expression of both *Atg101* RNAi constructs (Figures 3(c) and 3(d)). This phenotype is very similar to the accumulation of Atg8/LGG-1 aggregates reported in *Atg101/epg-9* mutant worms [18]. The enlarged Atg8a structures observed in *Atg101* RNAi cells of starved animals colocalize with protein aggregates containing the specific autophagy cargo ref(2)P (Figure 3(e)). Although it is not possible to conclude that Atg101 is not required for Atg8a recruitment to ref(2)P aggregates solely based on RNAi experiments, this seems to be a possibility, which is also supported by data from mutant worms. Interestingly, of the core *Atg* genes, *Atg2* has also been shown to be dispensable for Atg8a recruitment to the phagophore assembly site and protein aggregates in worm, fly, and mammalian cells [19, 24, 36, 37]. To exclude the possibility that these Atg8a dots might represent autophagosomes, we carried out electron microscopy. Ultrastructural analysis indeed revealed that autophagosome formation is blocked in *Atg101* RNAi cells (Figure 4). A potential explanation for the presence of a few enlarged Atg8a-positive dots in *Atg101* loss-of-function cells but not in *FIP200* null mutants [9] may be that mammalian Atg8 homologs have been shown to directly bind to FIP200, Atg13, and Atg1/ULKs [38]. These Atg1 kinase subunits may facilitate the recruitment of Atg8a to aggregates containing ref(2)P and ubiquitinated proteins, which have been proposed to act as scaffolds for autophagosome biogenesis, both in mammals and *Drosophila* [9, 24, 39].

*3.2. Drosopohila Atg101 Is Required for Selective Basal Autophagy of Ref(2)P-Containing Protein Aggregates.* Ref(2)P appears to be selectively degraded by autophagy all the time, independent of the induction conditions such as starvation or developmental contexts, similar to its mammalian homolog p62 [24, 40]. Continuous basal autophagy is usually difficult to be visualized directly, due to its low levels. For this reason, assessing basal autophagic activity by looking at levels of ref(2)P has become a standard test, similar to mammals [14, 19, 22, 41, 42]. Expression of either of the two *Atg101* silencing constructs results in large-scale accumulation of ref(2)P aggregates in GFP-marked cells, when compared to surrounding wild-type tissue in well-fed larvae (Figures 5(a), 5(b), and 5(c)), indicating defects in selective basal autophagy.

*3.3. Atg101 Interacts with Atg1.* The Atg13, FIP200, and ULK1 subunits of the mammalian Atg1/ULK kinase complex have been shown to bind to Atg101 in coimmunoprecipitation experiments on the level of endogenous proteins [16]. Atg101 directly binds to Atg13 in mammals, and the Atg101 ortholog EPG-9 was recently proposed to directly interact with the Atg1 ortholog UNC-51 as well in *C. elegans* [16–18]. In line with these studies, we found that myc-tagged, kinase dead Atg1 coimmunoprecipitates with Atg101 in cultured *Drosopohila* cells (Figure 6(a)). We used the kinase dead form because wild-type Atg1 is expressed poorly in cultured cells, and it also reduces the expression of other transgenes, likely due to Atg1-mediated feedback inhibition of Tor-dependent translation [9, 10, 43]. To determine which regions of the 835 amino acid long Atg1 protein are involved in its interaction with Atg101, we generated four HA-GST-tagged constructs for different Atg1 fragments. The N-terminal kinase domain was not included in these experiments, as again it is expressed poorly and strongly impairs the expression of coexpressed constructs [9]. We decided to search for potential Atg101

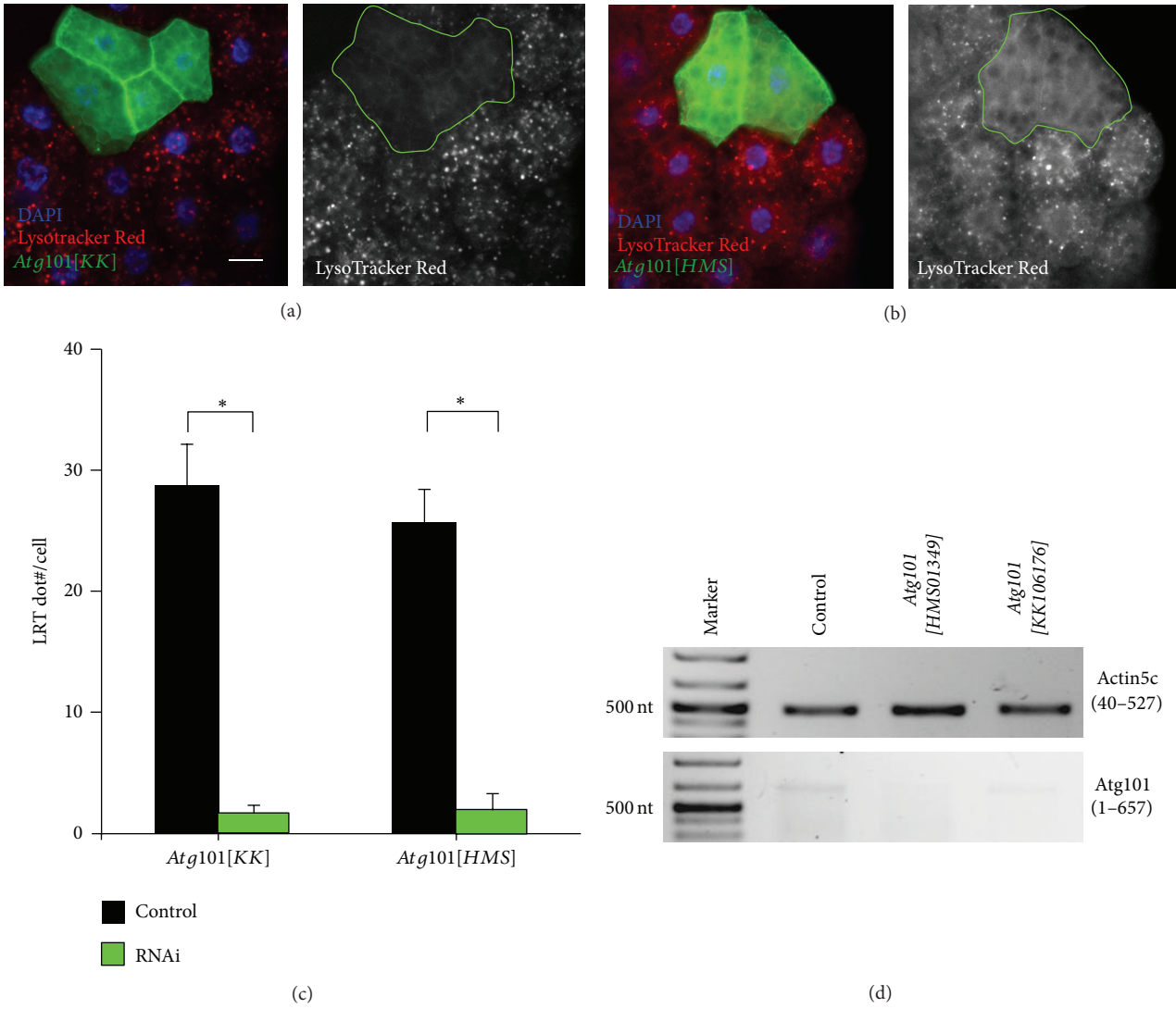


Figure 2: RNAi depletion of *Atg101* by expression of a long hairpin (a) or a microRNA-based silencing construct (b) in clones of cells marked by membrane-bound mCD8-GFP prevents starvation-induced punctate LysoTracker Red (LTR) staining, compared to surrounding control fat body cells of third instar larvae. (c) Quantification of data shown in (a) and (b), $N = 5$, $P < 0.001$, two-tailed, two-sample Student's *t*-tests. (d) Reverse Transcriptase-PCR analysis reveals that *Atg101* mRNA expression is strongly reduced upon expression of *Atg101[KK],* and it is undetectable in *Atg101[HMS]* expressing animals compared to wild type L3 stage larvae. Note that the expression level of *Atg101* is much lower than that of *Actin5C*, a commonly used control in such experiments. Numbers in parentheses indicate the amplified region of the coding sequence of these genes. Scalebar equals 20 $\mu$m for microscopic images.

binding sites in the middle region (amino acids 233–725) of Atg1, because the N-terminal kinase domain is the only known domain in this protein, and the C-terminal region is involved in its binding to Atg13 [44]. The rest of the protein is predicted to be mostly unstructured by the IUPRED server, and the ANCHOR method identifies numerous potential binding sites within these unstructured regions. We found that Atg101-FLAG coimmunoprecipitates with two Atg1 fragments containing either amino acids 233–360 or 565–725 but not with fragments containing amino acids 364–459 or 461–570 (Figures 6(b) and 6(c)). These results suggest that Atg101 interacts with multiple regions in Atg1 either directly or indirectly.

*3.4. Atg101 Binds to the HORMA Domain of Atg13, and It Can Dimerize.* Coimmunoprecipitations showed that full-length HA-Atg13 binds to Atg101-FLAG (Figure 7(b)), in line with data from mammals [16, 17]. Mapping experiments revealed that only those regions of Atg13 coprecipitate with Atg101 that contain amino acids 1–230, whereas the unstructured middle and C-terminal regions do not show binding (Figures 7(a) and 7(b)). The N-terminal part of Atg13 folds into a HORMA (Hop1, Rev1, and Mad2) domain similar to that of Mad2, a spindle checkpoint protein [45]. Mad2 forms a conformational homodimer, as its dimerization requires the binding of two different stable conformations to each other: open, O-Mad2, and closed, C-Mad2 [46]. Since the Atg13

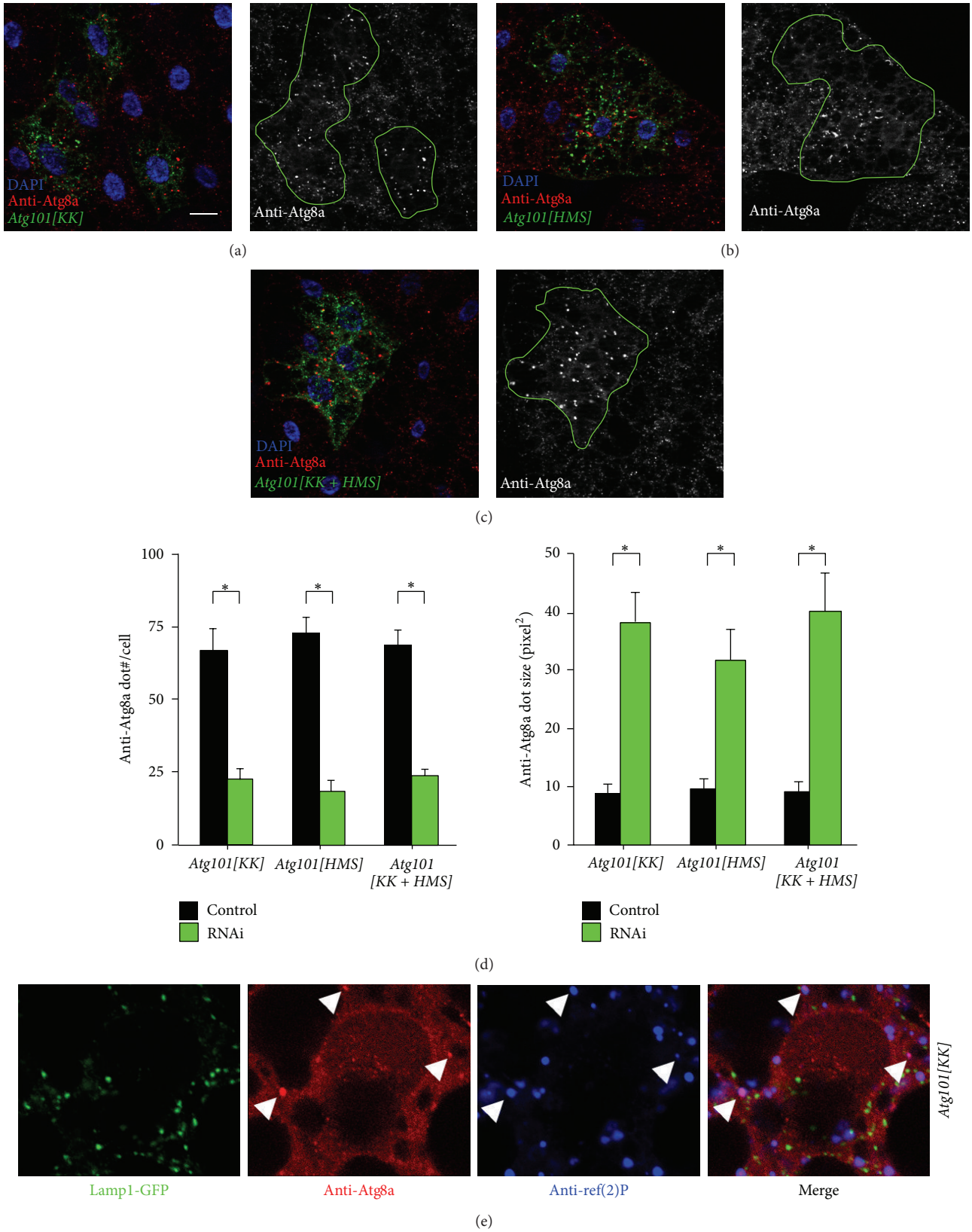


FIGURE 3: Knockdown of *Atg101* using separate RNAi lines ((a), (b)), or the combination of both (c), impairs the formation of Atg8a-positive autophagosomes upon starvation. Note that fewer but bigger Atg8a puncta are seen in Lamp1-GFP-marked RNAi cells than in neighboring control fat body cells. (d) Quantification of data shown in (a) to (c), $N = 5$, $P < 0.001$, two-tailed, two-sample Student's $t$-tests. (e) Overlapping Atg8a and ref(2)P structures are highlighted by arrowheads in *Atg101* depleted cells. Scalebar equals 20 $\mu$m.

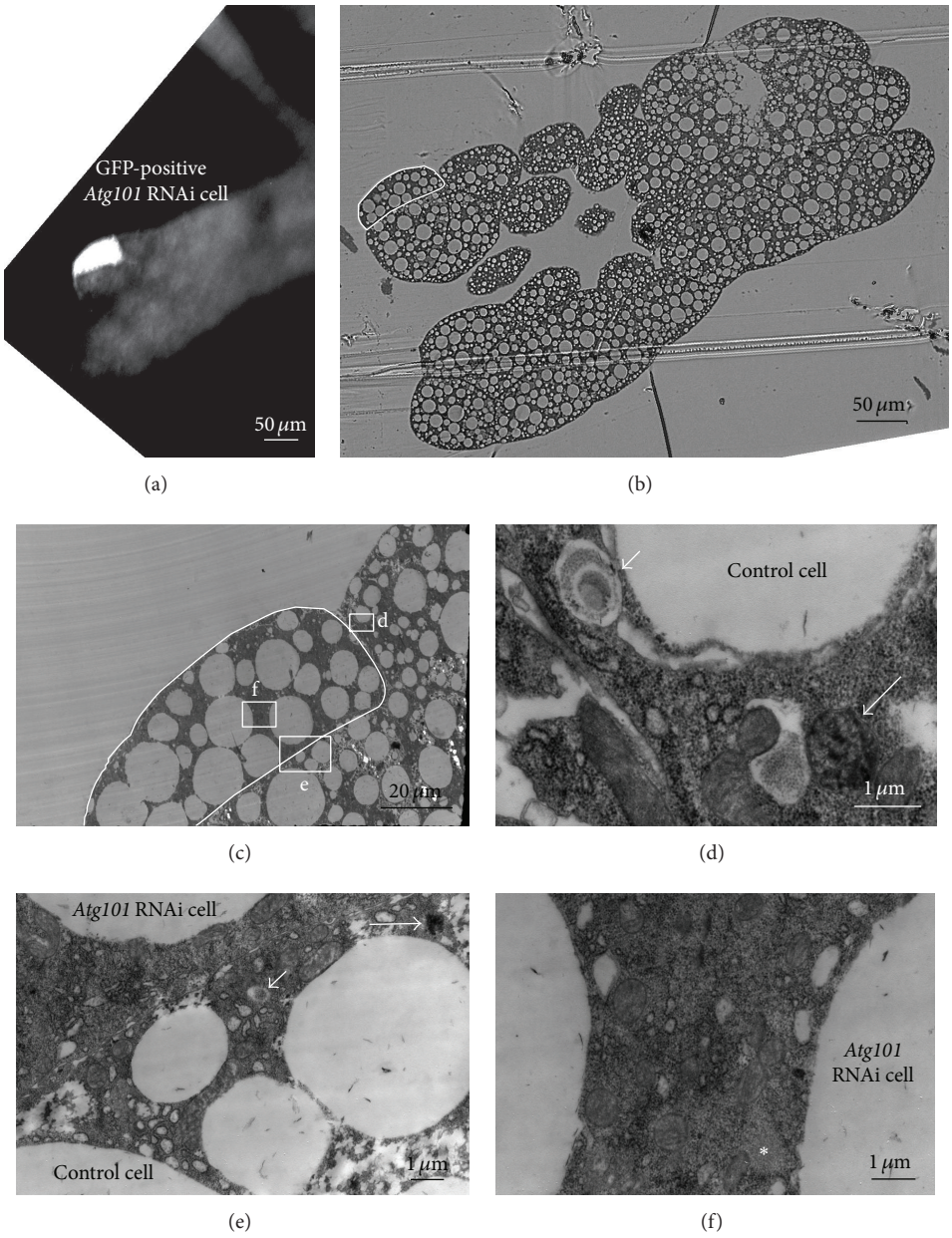


Figure 4: Ultrastructural analysis of *Atg101[KK+HMS]* RNAi cells. A fat body lobe containing a single GFP-marked RNAi cell (a) was embedded in plastic, and the same cell is highlighted in semithin (b) and low-magnification ultrastructural images (c). High magnification images show autophagosomes (short arrows) and autolysosomes (long arrows) in control cells ((d), and also in (e), bottom). The generation of such autophagic structures is inhibited in *Atg101* RNAi cells ((e), top, and (f)). Asterisk marks a potential cytoplasmic protein aggregate, which can be recognized by its homogenous appearance and exclusion of organelles and ribosomes.

HORMA domain is similar to C-Mad2 [45], we hypothesized that Atg101 may potentially be a HORMA domain protein as well, perhaps capable of forming either an O-Mad2 state or both O-Mad2 and C-Mad2 states. To study this model further, we first determined the interaction of Atg101 with itself. We found that Atg101-FLAG coprecipitates with HA-Atg101, and vice versa HA-Atg101 coprecipitates with Atg101 tagged by FLAG on either its N- or C-terminus (Figure 7(c)), suggesting that Atg101 dimerizes.

*3.5. Atg101 Is Potentially a HORMA Domain Protein.* Atg101 is classified as a single-domain protein with a domain of unknown function (DUF1649 in Pfam), with no trivially detectable relatives of known structure. Basic structural predictions suggest that it is a structured globular cytoplasmic protein. Results obtained with I-TASSER and Phyre2 indicate that the protein might have a HORMA domain fold. Both servers identified templates with HORMA fold and three of I-TASSER's 5 predicted structures have a fold similar to

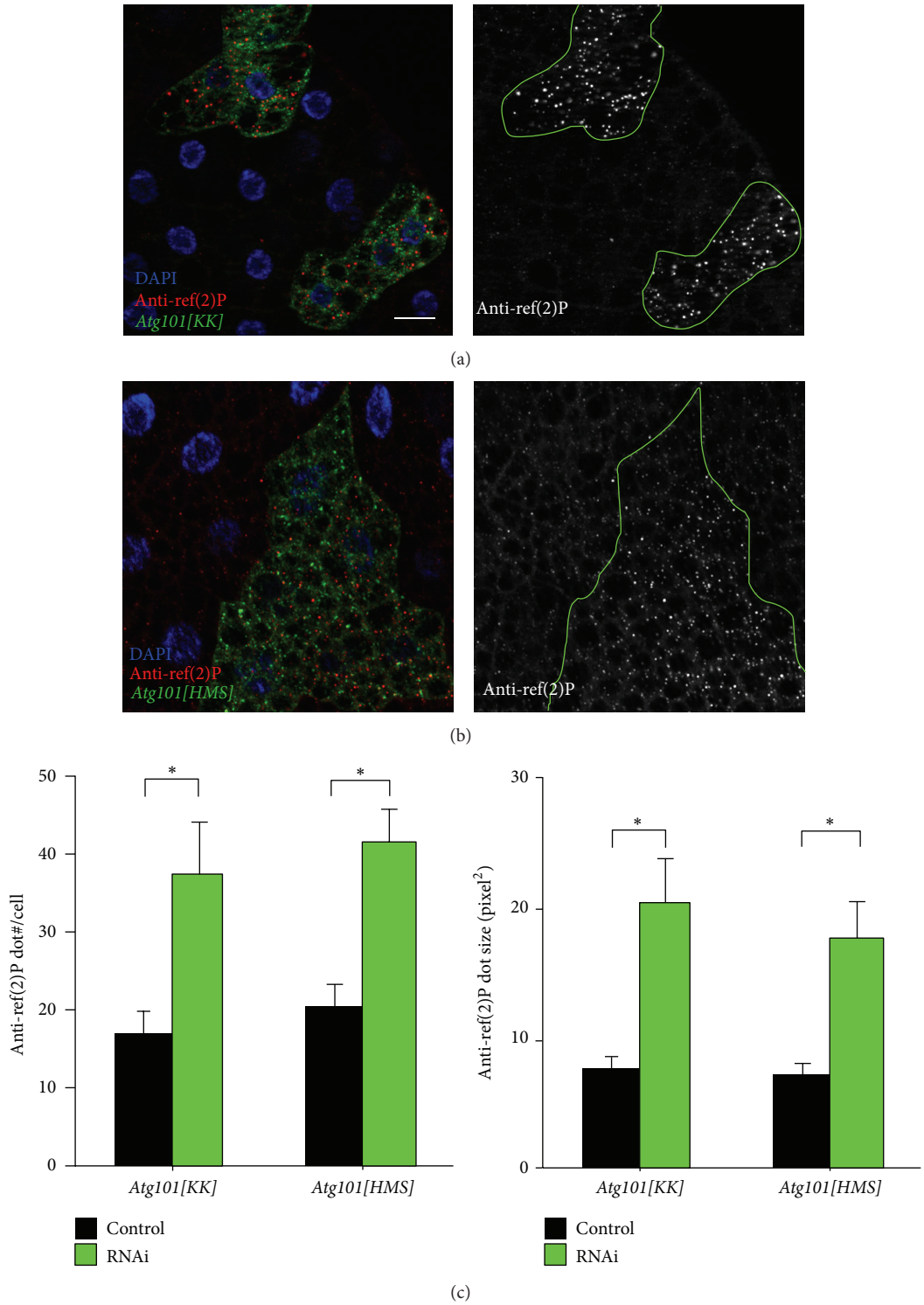


FIGURE 5: Depletion of *Atg101* in Lamp1-GFP-marked fat body cells of well-fed larvae results in the accumulation of ref(2)P aggregates ((a), (b)). (c) Quantification of data shown in (a) and (b), $N = 5$, $P < 0.001$, two-tailed, two-sample Student's $t$-tests. Scalebar equals 20 $\mu$m.

each other as well as the Atg13 and Mad2 HORMA domains (Figure 8). Moreover, the model predicted by Phyre2 also has a HORMA-like structural core (not shown). To assess the plausibility of this hypothesis, we created a multiple structure alignment of the best I-TASSER model of Atg101 with the known structures of human Mad2 [46] and yeast Atg13 [45], and manually compared it to the HORMA domain profile composed of 33 proteins in Prosite (entry PS50815). Our analysis reveals a number of corresponding sites showing conservation both in the HORMA domains in Prosite and

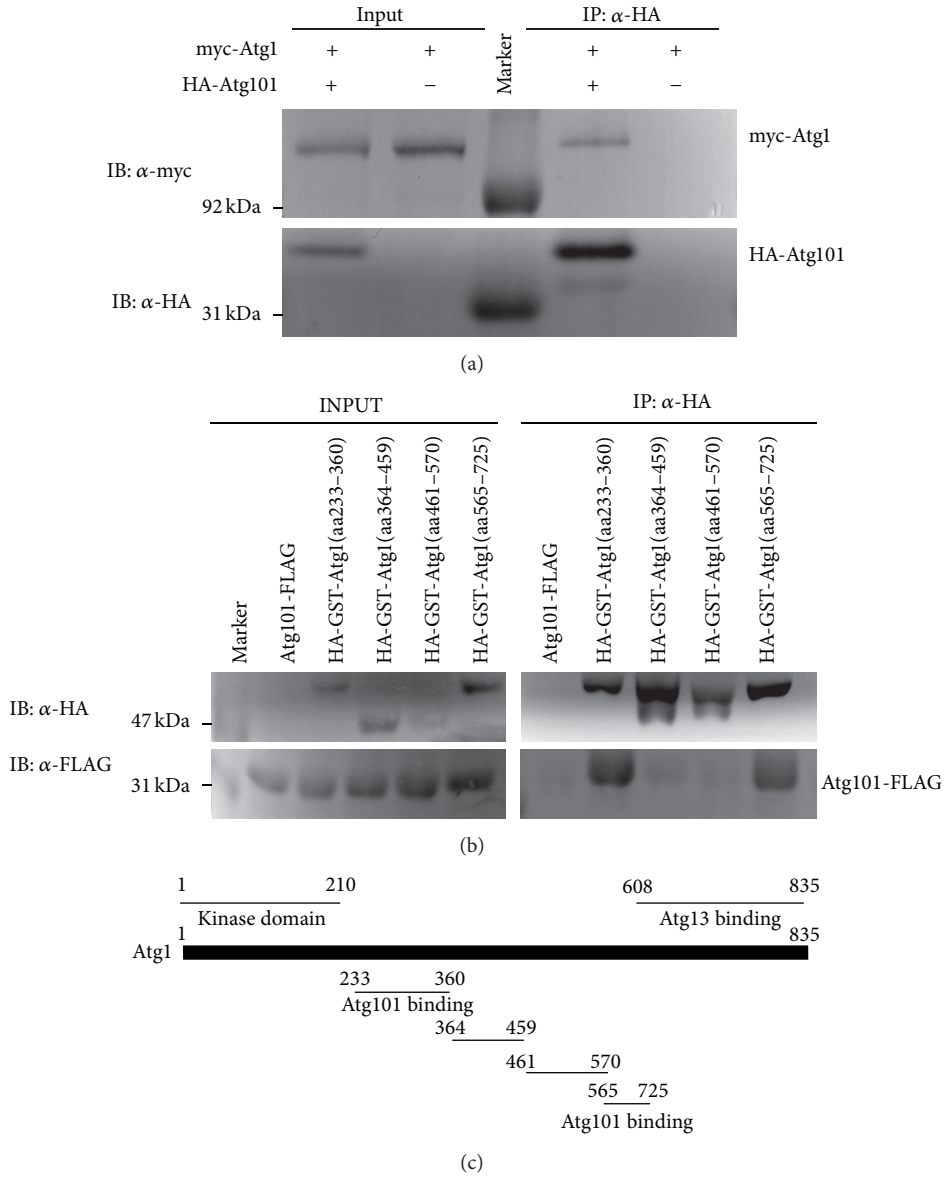


Figure 6: Full-length, kinase dead myc-Atg1 coprecipitates with HA-Atg101 but not with anti-HA beads (a). Atg101-FLAG coprecipitates with HA-GST-tagged Atg1 fragments 233–360 and 565–725 but not with 364–459 and 461–570 (b). Atg1 domain structure [9] and fragments used in mapping experiments (c).

within Atg101 homologs (OMA group 413828 containing Atg101 proteins from 66 different species). In addition, a characteristic pattern of largely conserved Leu/Ile/Val residues in Atg101 proteins and matching hydrophobic residues in the Prosite profile is apparent (Figure 9).

*3.6. Atg101 Interacts with Ref(2)P/p62.* *Drosopohila* Atg101 was suggested to bind to ref(2)P and also to several subunits of the lysosomal proton pump v-ATPase complex (Vha26, Vha36-1, Vha44, Vha55, and Vha68-2) in a large-scale proteomic study [47]. As we have recently found that the Atg1 kinase subunit FIP200 frequently localizes to ref(2)P aggregates near lysosomes [9], we wished to confirm the proposed interaction of Atg101 with ref(2)P. Indeed, FLAG-tagged ref(2)P coprecipitated with HA-Atg101 (Figure 10(a)). We used HA-Atg8a as a positive control in this experiment, as Atg8 homologs are established binding partners of p62 in mammalian cells, and fly Atg8a has been proposed to bind to ref(2)P through a conserved Atg8 interacting region as well [14, 15, 48]. These data raised the possibility that the entire Atg1 kinase complex may bind to aggregates of ref(2)P and ubiquitinated proteins. Indeed, we found that Atg13 also shows an interaction with ref(2)P, similar to Atg101 (Figure 10(b)). Positive controls that we used in these coimmunoprecipitations include Keap1 and Atg18, both of which have recently been shown to bind to ref(2)P [20, 24]. Ref(2)P coprecipitates with additional critical regulators

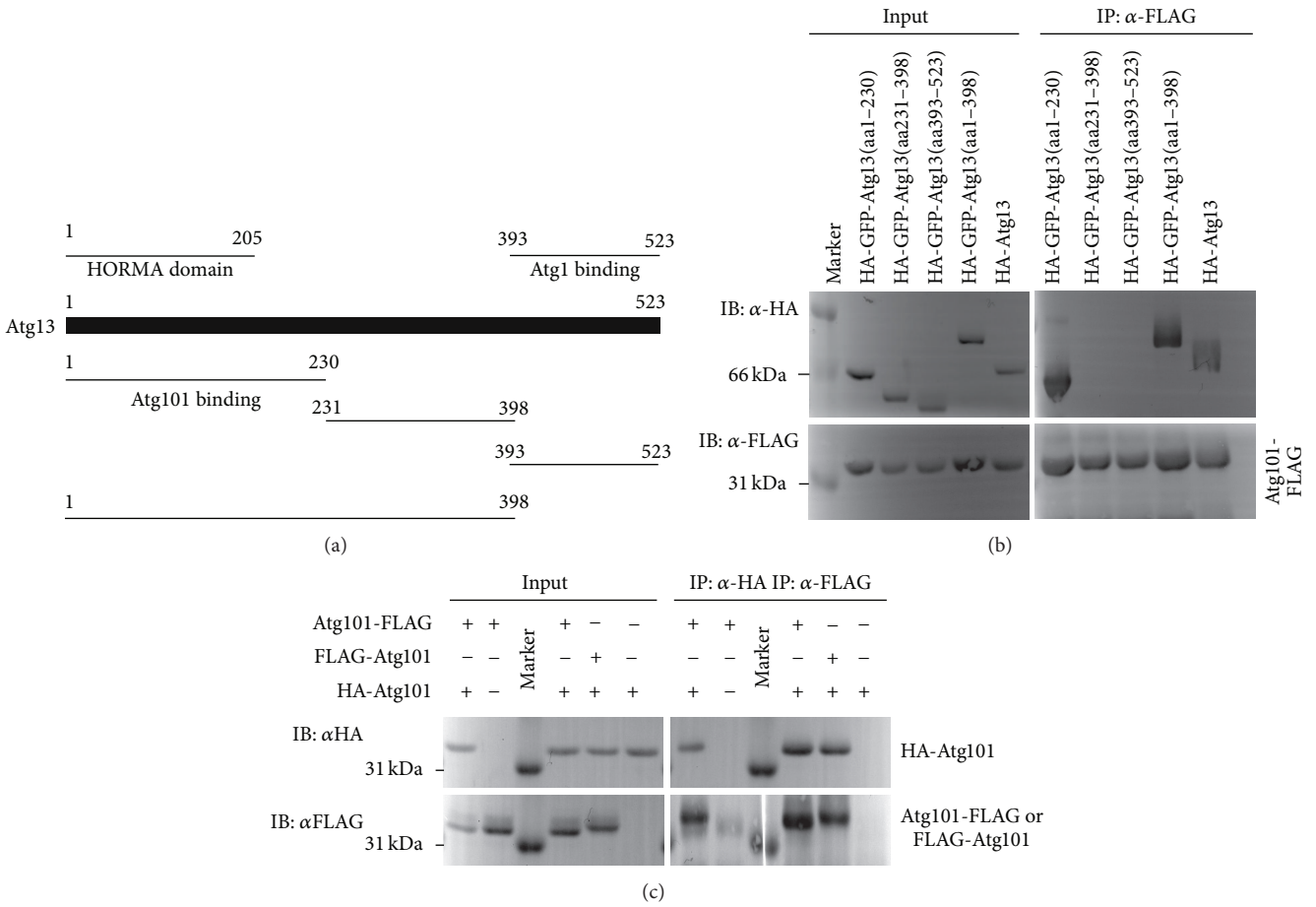


Figure 7: Atg13 domain structure and fragments used in mapping experiments [9] (a). Atg101-FLAG coprecipitates the full-length HA-Atg13, and also HA-GFP-Atg13 fragments containing the N-terminal HORMA domain: 1–230 and 1–398, but not the middle (231–398) or C-terminal (393–523) Atg13 fragments (b). Atg101-FLAG coprecipitates with HA-Atg101, and HA-Atg101 coprecipitates with both FLAG-Atg101 and Atg101-FLAG, but not with anti-HA or anti-FLAG beads alone, respectively (c).

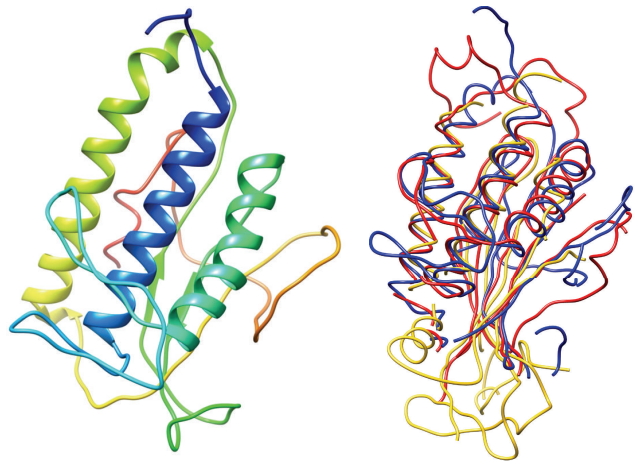


Figure 8: Ribbon representation of the best model of *Drosopohila* Atg101 obtained with I-TASSER (a). Atg101 structure alignment of the best model obtained with I-TASSER (blue), as well as human Mad2 (2V64, chain A, yellow) and Atg13 from the yeast *Lachancea thermotolerans* (4J2G, chain A, red) (b).

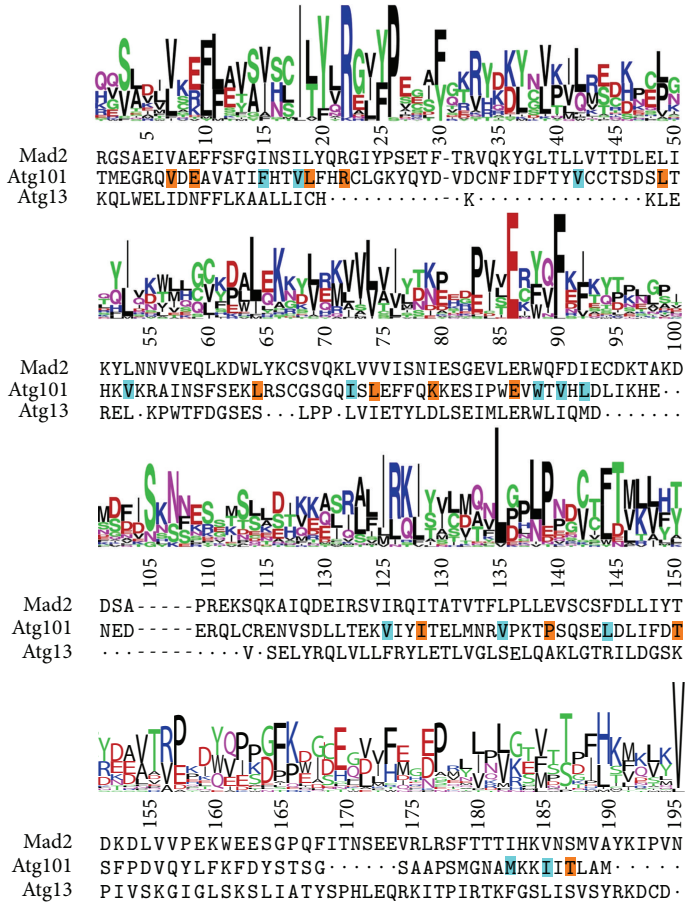


FIGURE 9: Comparison of the Prosite HORMA domain logo with the sequence alignment derived from the multiple structure alignment of the predicted Atg101 structure, Mad2 (2V64, chain A), and Atg13 (4J2G, chain A). Residues in *Drosopohila* Atg101 corresponding to the most frequent one in the Prosite logo and mostly conserved in other Atg101 proteins are highlighted with an orange background. Residues with a similar character to those in the logo and conserved in Atg101 proteins are highlighted with cyan background. Dots denote gaps arisen in the original structural alignment, dashes represent gaps relative to the Prosite logo. Note that the alignment is adjusted to match the full Prosite logo, so insertions relative to that have been removed from all sequences, and thus they do not correspond to the full native ones. In the Prosite logo, positively and negatively charged residues are colored blue and red, respectively; those with hydrophobic side chains are shown in black; Gln and Asn in magenta; other amino acids with hydrophilic side chains and glycine in green.

of autophagy as well: the ubiquitin-binding protein Atg16 [49] and the small GTPase RagC, a known Vha complex-associated regulator of Tor kinase [4], which shows a particularly strong interaction with ref(2)P as inferred from a large-scale proteomic study [47] (Figure 10(b)). These data are in line with the potential role of lysosome-associated protein aggregates as scaffolds for autophagosome biogenesis [9, 24, 39].

## 4. Conclusions

Our data establish that Atg101 is required for autophagy in *Drosopohila*, and that it is a subunit of the Atg1 kinase complex, similar to mammals and worms [16–18]. The potential binding of Atg101 to Atg1 may involve multiple regions in Atg1, whereas the HORMA domain of Atg13 appears to be the only region important for its interaction with Atg101. Atg101 is a small protein of 218 amino acids, and it is predicted to fold into a globular domain, potentially a HORMA domain. Proteins with such structure often have numerous binding partners, the identity of which determines the open or closed conformation in the case of Mad2 [46]. Our bioinformatics analysis predicts a possible HORMA domain structure for Atg101, and we show that it can indeed dimerize. If Atg101 functions similar to Mad2, then it may perhaps be capable of switching between open and closed conformations and form a conformational homodimer as well. This would represent an elegant way of determining which proteins can bind to Atg101 at one time. This model predicts that Atg101 binds to Atg13 through direct interaction of the two HORMA domains, whereas for some of its other partners (potentially including Atg1), certain oligopeptides located in unstructured regions may engage in an interaction with Atg101, as seen in the case of Mad2 as well [46].

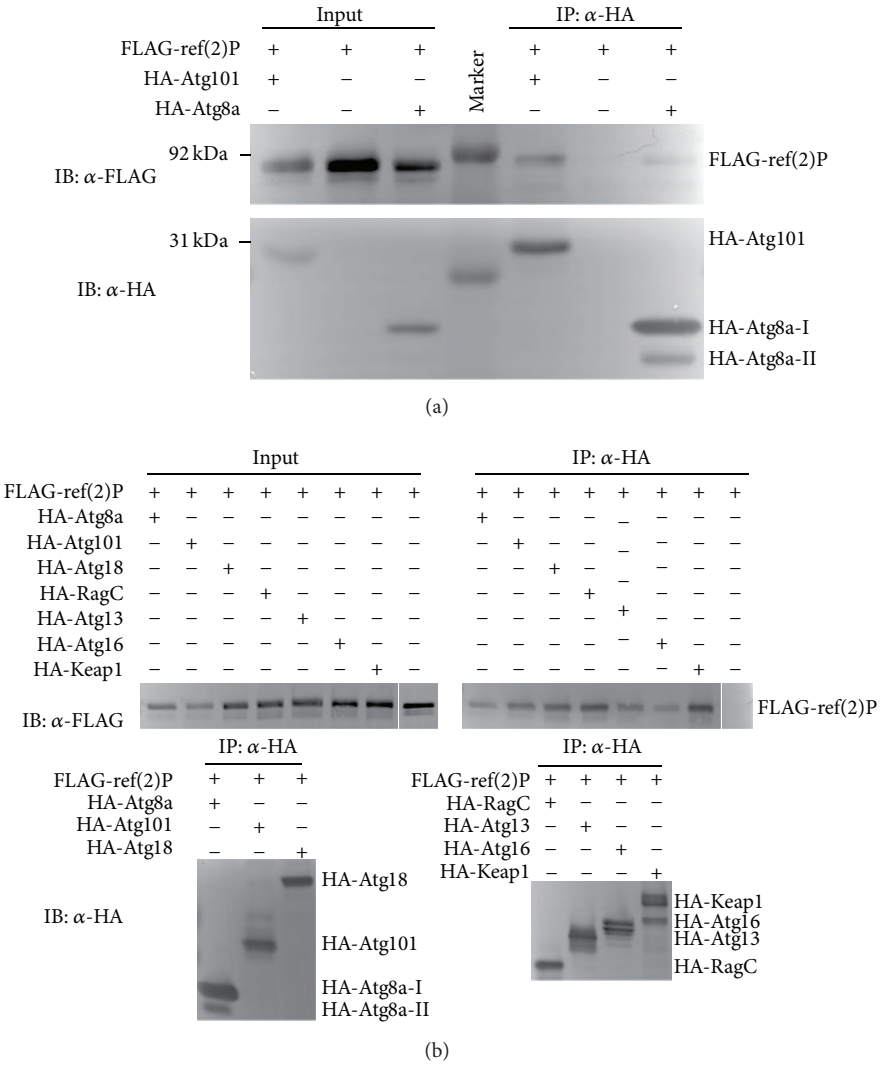


Figure 10: FLAG-tagged ref(2)P coprecipitates with both HA-Atg101 and HA-Atg8a, but not with anti-HA beads (a). Note that HA-Atg8a-II migrates faster than HA-Atg8a-I, due to the covalent attachment of the lipid moiety phosphatidylethanolamine to its C-terminus. (b) The selective autophagy cargo ref(2)P coprecipitates with Atg8a, Atg101, Atg18, RagC, Atg13, Atg16, and Keap1, but not with empty beads.

It is important to emphasize that the HORMA domain structure of Atg101 is not supported experimentally. Further analysis of Atg101 binding partners, fine mapping of the binding sites mediating the interaction of each partner with Atg101, and analysis of the Atg101 domain by structural biochemistry will be necessary to test our hypothetical model presented in this paper. This will be particularly important, as the absence of an Atg101 ortholog in yeast clearly indicates that a metazoan-specific regulatory mechanism has evolved to control the initiation step of autophagy in animal cells.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

The authors thank Sarolta Pálfia for her excellent technical assistance, László Nyitray, Viktor Kis, and Rita Pancsa for helpful discussions, and stock centers BDSC and VDRC for providing *Atg101* RNAi lines. This work was supported by the Wellcome Trust (087518/Z/08/Z), the Hungarian Scientific Research Fund (OTKA K83509), the European Union (TÁMOP-4.2.1.B-11/2/KMR-2011-0002), and the Hungarian Academy of Sciences (BO/00552/11).

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 382653, 16 pages
http://dx.doi.org/10.1155/2014/382653

*Research Article*

# Functional Interactions between 17$\beta$-Estradiol and Progesterone Regulate Autophagy during Acini Formation by Bovine Mammary Epithelial Cells in 3D Cultures

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Received 24 January 2014; Revised 26 March 2014; Accepted 9 April 2014; Published 7 May 2014

Academic Editor: Maria Vaccaro



Mammary gland epithelium forms a network of ducts and alveolar units under control of ovarian hormones: 17-beta-estradiol (E2) and progesterone (P4). Mammary epithelial cells (MECs) cultured on reconstituted basement membrane (rBM) form three-dimensional (3D) acini composed of polarized monolayers surrounding a lumen. Using the 3D culture of BME-UV1 bovine MECs we previously demonstrated that autophagy was induced in the centrally located cells of developing spheroids, and sex steroids increased this process. In the present study we showed that E2 and P4 enhanced the expression of *ATG3*, *ATG5*, and *BECN1* genes during acini formation, and this effect was accelerated in the presence of both hormones together. The stimulatory action of E2 and P4 was also reflected by increased levels of Atg5, Atg3, and LC3-II proteins. Additionally, the activity of kinases involved in autophagy regulation, Akt, ERK, AMPK, and mTOR, was examined. E2 + P4 slightly increased the level of phosphorylated AMPK but diminished phosphorylated Akt and mTOR on day 9 of 3D culture. Thus, the synergistic actions of E2 and P4 accelerate the development of bovine mammary acini, which may be connected with stimulation of *ATGs* expression, as well as regulation of signaling pathways (PI3K/Akt/mTOR; AMPK/mTOR) involved in autophagy induction.

## 1. Introduction

Functional development of the mammary gland is a process composed of a sequence of strictly regulated events that start early in the foetal life and end in mature females at the beginning of lactation. The first quiescent phase of mammary growth is characterized by the formation of rudimentary system of ducts from ectodermal epithelium during embryogenesis and after birth. More intensive phase of mammary development takes place at the onset of puberty and is manifested by the extension and branching of ducts, which is coincident with growth of mammary fat pad, but in the absence of alveolar structures. However, the majority of morphological and physiological development of the mammary gland takes place during pregnancy, when the parenchymal tissue undergoes extensive remodelling. Replacement of adipose tissue by parenchyma and condensation of stromal connective tissue to narrow bands are accompanied by alveologenesis formation of differentiated alveoli required for milk production [1]. The process of secretory epithelium division continues into the early stages of lactation and is terminated with the epithelial tissue regression occurring at involution. All stages of mammary gland development are under strict control of endocrinal factors: reproductive and metabolic hormones, as well as growth factors secreted locally in paracrine or autocrine manner [2].

*Sex Steroids*. 17$\beta$-Estradiol (E2) and progesterone (P4) are essential regulators of mammary gland development and are necessary to fulfill proper lobuloalveolar structure formation. While estradiol-mediated signaling plays a major role in ductal morphogenesis, progesterone is critical for development of lobuloalveolar structures [3]. 17$\beta$-Estradiol and progesterone mediate their biological responses mainly by their specific receptors (two forms of estradiol receptor, ER$\alpha$ and ER$\beta$, and progesterone receptor PR) via genomic

pathway. The expression and amount of these receptors in the mammary epithelial cells (MECs) undergo regulatory changes during mammary growth and involution [4]. The growing importance of sex steroids action in mammary gland development lies in their property of regulating crucial cellular processes such as growth, differentiation, apoptosis, and autophagy [3, 5–8].

Macroautophagy (herein referred to as autophagy) is a highly conserved cellular self-degradative pathway, activated under various stress conditions as an adaptation to nutrient deprivation, and used for energy and macromolecules generation to maintain cellular homeostasis [9]. We have previously reported that 17$\beta$-estradiol and progesterone positively regulate autophagy during formation of acinar structures by bovine mammary epithelial cells (MECs) cultured in three-dimensional (3D) system on reconstituted basement membrane (rBM), commercially available as Matrigel [8]. Bovine BME-UV1 mammary epithelial cells cultured on Matrigel form proper acinar structures within 16 days, and their development is enhanced in the presence of sex steroids: 17$\beta$-estradiol and progesterone. *In vitro* studies have demonstrated that autophagy is activated in the center of mammary acini developing on rBM. This process is preceded with polarization of cells that are in direct contact with extracellular matrix components but is induced prior to apoptosis, which is the main form of cell death responsible for lumen clearance, determining the proper development of mammary alveoli [10, 11]. Induction of autophagy in MECs is closely connected with the lack of contact with extracellular matrix and exposure to nutrient deprivation conditions, contributing to survival of cells during anoikis (cell detachment-induced apoptosis) [11]. Our research showed that in the presence of sex steroids, autophagy was augmented in the developing acinar structures. Therefore, in the present study we used 3D culture model to elucidate the mechanisms of autophagy regulation by E2 and P4 during formation of alveoli-like structures by bovine BME-UV1 mammary epithelial cells. We investigated the genomic effect of both sex steroids on the expression of chosen autophagy-related genes (*ATGs*): *ATG3, ATG5, BECN1, LC3B,* and their protein products. Additionally we examined activation of Bcl-2 and different kinases: Akt, Erk, AMPK, and mTOR, which are known to regulate autophagy, in order to determine whether the effect of E2 and P4 is also connected with the nongenomic actions of these steroid hormones.

## 2. Material and Methods

*2.1. Media and Reagents.* DME/F-12, RPMI-1640, NCTC 135, $\alpha$-lactose, lactalbumin hydrolysate, glutathione, bovine insulin, bovine holo-transferrin, hydrocortisone, L-ascorbic acid, insulin-like growth factor-I (IGF-I), 17$\beta$-estradiol (E2), PI3K inhibitor, LY294002, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless indicated differently. Antibodies against E-cadherin (sc-7870), $\beta$-actin (sc-47778), p-Akt (Ser 473; sc-7985-R), total Akt (sc-1618), p-ERK (Tyr204; sc-7383), total ERK (sc-94), p-AMPK (Thr172; sc-33524), and total AMPK (sc-25792), p-Bcl-2 (Ser70; sc-21864-R), p62/SQSTM1 (sc-25575), and beclin-1 (BECN1; sc 11427) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA., USA); most antibodies against autophagic proteins, Atg5 (NB110-53818), Atg3 (R-159-100), and LC3B (NB100-2220) were purchased from Novus Biologicals (Novus Biologicals, LLC, Littleton, CO, USA); anti-cleaved caspase-3 (cat. number 9661) and p-mTOR (Ser2448; cat. number 5536) were provided by Cell Signaling Technology Inc. (Danvers, MA, USA); anti-Ki-67-FITC conjugated antibody (cat. number 334711) and Alexa Fluor488 secondary antibody (cat. number A21441) were purchased from Life Technologies, Invitrogen (Carlsbad, CA, USA); secondary antibodies IRDye 680 or IRDye 800 CW used for proteins detection by Odyssey Infrared Imaging System (LI-COR Biosciences) were purchased from LI-COR Biosciences (Lincoln, NE, USA). Plastic cell culture plates and flasks were purchased from Corning Incorporated (Lowell, MA, USA). Sterile conical flasks, Lab-Tek Chamber Slides, and disposable pipettes were supplied by Nunc Inc. (Naperville, IL, USA). Growth factor reduced Matrigel was obtained from BD Biosciences (San Jose, CA, USA); each lot of the GFR-Matrigel contained 9–11 mg/mL protein concentration.

*2.2. Cell Culture.* Bovine BME-UV1 mammary epithelial cell line was purchased from the Cell Bank of The Lombardy and Emilia Romagna Experimental Zootechnic Institute, Italy. During routine culture cells were grown in monolayer, on plastic culture flasks, and in growth medium comprising DME/F-12, RPMI-1640, and NCTC 135 in proportions of 5 : 3 : 2 by vol. and enriched with $\alpha$-lactose (0.1%), glutathione (1.2 mM), bovine insulin (1.0 $\mu$g/mL), bovine holo-transferrin (5.0 $\mu$g/mL), hydrocortisone (1.0 $\mu$g/mL), L-ascorbic acid (10 $\mu$g/mL), 10% (v/v) heat-inactivated FBS, penicillin-streptomycin (50 IU/mL), fungizone (2.5 $\mu$g/mL), and gentamycin (50 $\mu$g/mL).

In order to begin 3D culture, plates (100 mm diameter) (Corning Inc., NY, USA) or 8-well, Lab-Tek Chamber Slides (Nunc Inc., Naperville, IL, USA) were coated with growth factor reduced Matrigel (BD Biosciences) and 400 $\mu$L of Matrigel was spread on the surface of culture plates or 25 $\mu$L (per well) on chamber slides. The plates and chamber slides were left at 37°C for 30 min for Matrigel to solidify. Confluent BME-UV1 cells grown in monolayer were trypsinized and resuspended in growth medium with addition of 2% Matrigel. The cells were plated at a concentration of 25000 cells/mL on culture plates or 5000 cell/mL on chamber slides. After 24 h the medium was replaced with a differentiation medium, containing DME/F-12, RPMI-1640 and NCTC 135 in proportions of 5 : 3 : 2 by vol., enriched with $\alpha$-lactose (0.1%), glutathione (1.2 mM), bovine insulin (1.0 $\mu$g/mL), bovine holo-transferrin (5.0 $\mu$g/mL), hydrocortisone (1.0 $\mu$g/mL), L-ascorbic acid (10 $\mu$g/mL), 2% (v/v) heat-inactivated FBS, penicillin-streptomycin (50 IU/mL), fungizone (2.5 $\mu$g/mL), gentamycin (50 $\mu$g/mL), and 2% GFR-Matrigel. Additionally in experimental conditions, differentiation medium was supplemented with E2 (1 nM), P4 (5 ng/mL), or combination of

E2 and P4. Medium was replaced every second day. Growth of the acinar structures formed by BME-UV1 cells was monitored daily using Olympus IX70 inverted phase contrast microscope (Olympus Optical Co., Hamburg, Germany).

*2.3. Confocal Microscopy.* BME-UV1 cells cultured on chamber slides coated with Matrigel were fixed in 3.7% paraformaldehyde (20 min) after 3, 6, 9, or 14 d of culture. Next cells were permeabilized with 0.5% Triton X100 in PBS (10 min), washed three times with PBS, and incubated overnight with primary antibody against E-cadherin, cleaved caspase-3, beclin-1, or p62 diluted 1 : 100 with PBS. After primary incubation the cells were washed three times with PBS and incubated with 1 : 500 Alexa Fluor488 secondary antibodies for 1 h at room temperature. After incubation with appropriate antibodies cells were incubated with 7-aminoactinomycin D (7-AAD), 5 $\mu$g/mL for 30 min to counterstain DNA. Finally coverslips were mounted on microscope slides using SlowFade Gold reagent (Life Technologies, Invitrogen). Cells were visualized using the confocal laser scanning microscope FV-500 system (Olympus Optical Co, Hamburg, Germany). The combination of excitation/emission were Argon 488 nm laser with 505–525 nm filter for Alexa Fluor 488 and HeNe 543 nm laser with 610 nm filter for 7AAD nucleus staining. Intensity of immunofluorescence staining of cleaved caspase-3 and p62 was also analyzed quantitatively using MicroImage analysis software (Olympus). Integrated optical density (IOD), which measures optical density of individual pixels in a chosen area, was used in the analysis. IOD of green fluorescence of cleaved caspase-3 or p62 was measured in the total area of each single mammosphere. In parallel IOD of red fluorescence of nuclei was measured in the same area. The data are presented as ratios of green fluorescence IOD to red fluorescence IOD for each analyzed mammosphere. Data were collected for each experimental replicate and underwent statistical analysis described in chapter 2.6. For each time point the presented images are representative of at least three independent experiments.

*2.4. Real-Time PCR.* BME-UV1 cells were grown on GFR-Matrigel-coated culture plates for 3, 6, 9, or 14 d in differentiation medium. Next, cells were pelleted by centrifugation, disrupted in 600 $\mu$L of RLT Buffer from the Qiagen RNeasy Mini Kit (cat. number 74104), and stored in −80°C until use. Total RNA was extracted from cells with the RNeasy Mini Kit according to the protocol supplied by the producer (Qiagen, Inc., Mississauga, Canada). RNA concentration and purity were determined spectrophotometrically, and quality was confirmed using microcapillary electrophoresis (Bioanalizer 2100, Agilent Technologies, Mississauga, Canada). During reverse transcription, 2 $\mu$g of isolated total RNA was converted to cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, United States), and the reaction was carried out in a Mastercycler pro (Eppendorf, Germany). Real-time PCR was performed in triplicate using SYBR Select Master Mix (Applied Biosystems). Each 10 $\mu$L reaction contained a final concentration of 0.5 $\mu$M each of forward and reverse primers, 1x master mix, and 1 $\mu$L cDNA (100 ng). Reaction was performed in Mx3005P QPCR machine (Stratagene, La Jolla, CA, USA). Cycling condition started with two initial phases at 50°C for 2 min and 95°C for 2 min, which were followed by 40 cycles: 95°C for 15 sec; 58°C for 15 sec; 72°C for 1 min each, respectively. Standard curves were run for each transcript to ensure exponential amplification, and "no RT" controls were run to exclude nonspecific amplification. *GAPDH* was used as a reference gene. Primers sequences are listed in Table 1. Comparative CT method [12] was used to calculate the fold change in gene expression normalized to reference *GAPDH* gene.

*2.5. Western Blot Analysis.* Cells were grown on GFR-Matrigel-coated culture plates for 3, 6, 9, or 14 d. Next cells were pelleted by centrifugation at 14000 rpm at 4°C for 5 min and frozen at −80°C until further analyses. Protein extracts from cultured cells were isolated by lysing the collected cell pellets with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, and 1 mM PMSF) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich) for 30 min at 4°C. Lysates were cleared for 20 min at 14000 rpm, and supernatants were collected. Protein concentration in the lysates was determined using Bio-Rad protein assay dye reagent according to the producer's instructions (Bio-Rad Laboratories Inc., Hercules, CA, USA). Proteins (50 $\mu$g) were resolved by SDS-PAGE and transferred onto PVDF membrane (Sigma-Aldrich). For immunostaining membranes were blocked with 5% nonfat dry milk in TBS (20 mM Tris-HCL, 500 mM NaCl) containing 0.5% Tween20. The membranes were incubated with chosen primary antibodies at a dilution range between 1 : 200 and 1 : 1000, depending on the antibody. Next the membranes were washed three times for 15 min and incubated with appropriate secondary antibodies conjugated with IR fluorophores: IRDye 680 or IRDye 800 CW (at 1 : 5000 dilution). Odyssey Infrared Imaging System (LI-COR Biosciences) was used to analyze the protein expression. Scan resolution of the instrument was set at 169 $\mu$m and the intensity at 4. Quantification of the integrated optical density (IOD) was performed with the analysis software provided with the Odyssey scanner (LI-COR Biosciences). Immunoblot analysis was performed on samples from three independent experiments. For the purpose of publication the color immunoblot images were converted into black and white images in the Odyssey software.

*2.6. Statistical Evaluation.* Statistical analysis was performed using GraphPad Prism version 5.00 software (GraphPad Software, Inc., La Jolla, CA, USA). In the case of analyzing the IOD results obtained for immunofluorescence staining of cleaved caspase-3 and p62 statistical significance of the mean IOD ratios was calculated using the one-way analysis of variance (ANOVA) and Tukey's multiple comparison posttest. The comparison was made between the treatments (control and different experimental conditions: E2, P4, and

Table 1: Primer sequences of examined *ATGs* and parameters of real-time PCR assay.

| Target gene | Nucleotide sequence | Real-time PCR |
|---|---|---|
| *ATG5* | FRD: 5′-TTT GAA TAT GAA GGC ACA CC-3′<br>REV: 5′-TGT AAA CCC ATC CAG AGT TG-3′ | SYBR Select Master Mix (catalogue number 4472908; Applied Biosystems)<br>(i) 50°C for 2 min<br>(ii) 95°C for 2 min<br>(iii) 40 cycles of<br>(a) 95°C for 15 sec<br>(b) 58°C for 15 sec<br>(c) 72°C for 1 min |
| *ATG3* | FRD: 5′-GGT TGT TCG GCT ATG ATG AG-3′<br>REV: 5′-GGG AGA TGA GGG TGA TTT TC-3′ | |
| *BECN1* | FRD: 5′-AGT TGA GAA AGG CGA GAC AC-3′<br>REV: 5′-GAT GGA ATA GGA ACC ACC AC-3′ | |
| *MAP1 LC3 B* | FRD: 5′-TTA TCC GAG AGC AGC ATC C-3′<br>REV: 5′-AGG CTT GAT TAG CAT TGA GC-3′ | |
| *GAPDH* | FRD: 5′-CTT CAA CAG CGA CAC TCA-3′<br>REV: 5′-CCA GGG ACC TTA CTC CTT-3′ | |

Primers were designed using Primer 3 software, on the basis of the bovine sequences from NCBI database and verified using Oligo Calc: Oligonucleotide Properties Calculator (free software available online, provided by Northwestern University) to exclude sequences showing self-complementarity, and BLAST (NCBI, U.S. National Library of Medicine) to exclude possible complementarity to other mRNA templates.

E2 + P4) at a specific time point (days 6, 9, and 12). Statistical significance of the mean IOD values for immunoblot bands was calculated using the two-way analysis of variance (two-way ANOVA) and Bonferroni multiple-comparison correction, when the comparison was made between the treatments (control and E2, P4, and E2 + P4) and duration of 3D culture (3, 6, 9, and 14 days). Since in most cases two-way analysis of variance did not show significance of the Western blot results obtained we also performed one-way ANOVA and Tukey's multiple-comparison posttest to analyze the potential differences between experimental conditions (control and E2, P4, and E2 + P4) at specific time point of the experiment (3, 6, 9, or 14 days). $P$ value of $\leq 0.05$ was considered statistically significant and $P \leq 0.01$ or $P \leq 0.001$ as highly significant.

## 3. Results

*3.1. Influence of 17β-Estradiol and Progesterone on Development of Acini Formed by Bovine MECs Cultured on rBM, Rate of Apoptosis, and Autophagy.* Our previous *in vitro* studies have shown that BME-UV1 bovine mammary epithelial cells cultured on rBM (Matrigel) form alveoli-like structures and their development occurs faster in the presence of sex steroids [8]. Since at the time of gestation both steroids are present in high concentrations, playing an important function during the final stages of mammary gland development, we decided to investigate the individual, as well as simultaneous effects of E2 (1 nM) and P4 (5 ng/mL) in the course of acini formation by bovine MECs cultured in 3D system. Confocal images of spherical structures formed by BME-UV1 cells confirmed our previous observations, showing that proper development of such acini was possible with and without sex steroids. Cells which were directly attached to extracellular matrix (ECM) underwent polarization, and the typical lateral localization of E-cadherin was noted in these cells in all tested conditions (control and with addition of sex steroids) (Figure 1).

When we analyzed the expression and localization of apoptotic marker, cleaved caspase-3, in developing acini we noted more immunofluorescence staining of active caspase-3 in the center of spherical structures formed in the presence of E2 and P4 than in control conditions. The differences were more evident in the second week of 3D culture, which is the time when the formation of hollow lumens occurs in these structures, as demonstrated in our previous study (Figure 2(a)) [8]. To confirm these observations we used MicroImage analysis software (Olympus) to measure the intensity of green fluorescence (IOD) related to cleaved caspase-3 immunostaining in each analysed acinus from days 6, 9, and 12 of 3D culture and compared it with red fluorescence of nuclei. The results, which are presented as ratios of green fluorescence IOD to red fluorescence IOD, confirmed the time dependent increase in cleaved caspase-3 expression within the developing acini. The highest mean IOD values were obtained in the spherical structures cultured in the presence of E2 and E2 + P4 (Figure 2(a)). However, the addition of both steroids simultaneously seems to create the optimal conditions for acini development, as the activity of caspase-3 in these conditions was significantly increased already on day 9 of 3D culture. Western blot analysis of the levels of cleaved caspase-3 in cell lysates also confirmed our observations, showing that addition of E2 and P4 together resulted in a significant increase in this apoptotic enzyme (Figure 2(b)).

Our previous findings demonstrated that 17$\beta$-estradiol and progesterone also enhanced autophagy in the center of developing alveoli formed by BME-UV1 cells, which was manifested by increased punctuated pattern of GFP-LC3-II [8]. To further confirm the role of both sex steroids in regulation of autophagy in bovine MECs in the course of acini development we investigated the expression of p62 in these structures. p62 (also known as sequestome 1-SQSTM1) is a ubiquitin-binding scaffold protein that directly binds both poly- or monoubiquitin via its ubiquitin-associated domain (UBA) and LC3 and links the ubiquitinated cargos to the autophagy machinery for autophagic degradation [13]. The protein itself is also degraded by autophagy. Since p62 accumulates when autophagy is inhibited and decreased levels can be observed when autophagy is induced, p62 may be used as

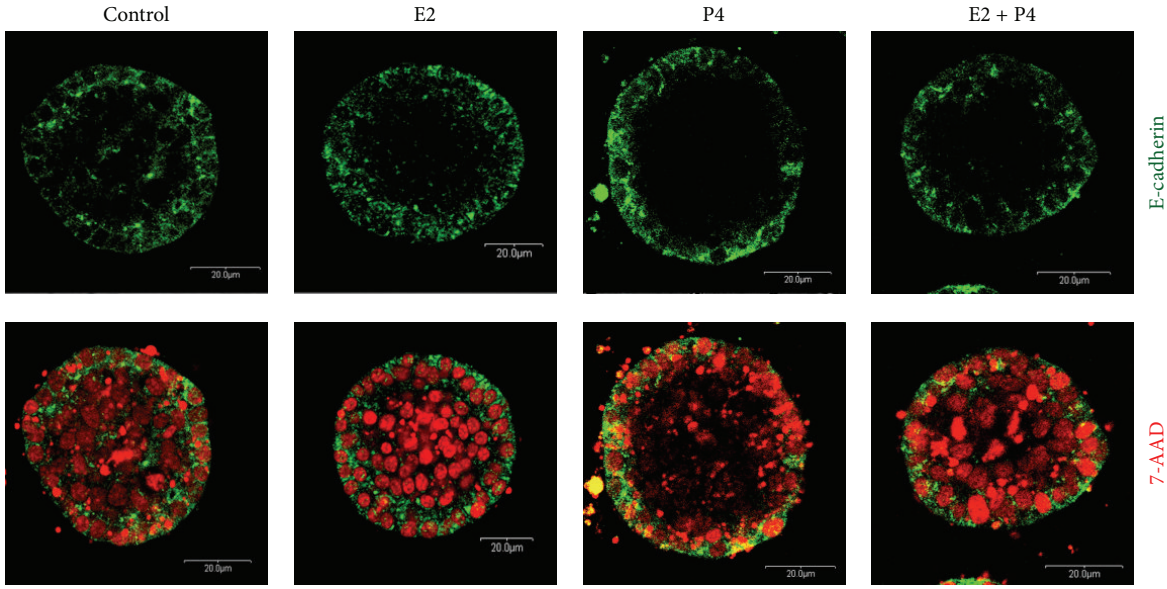
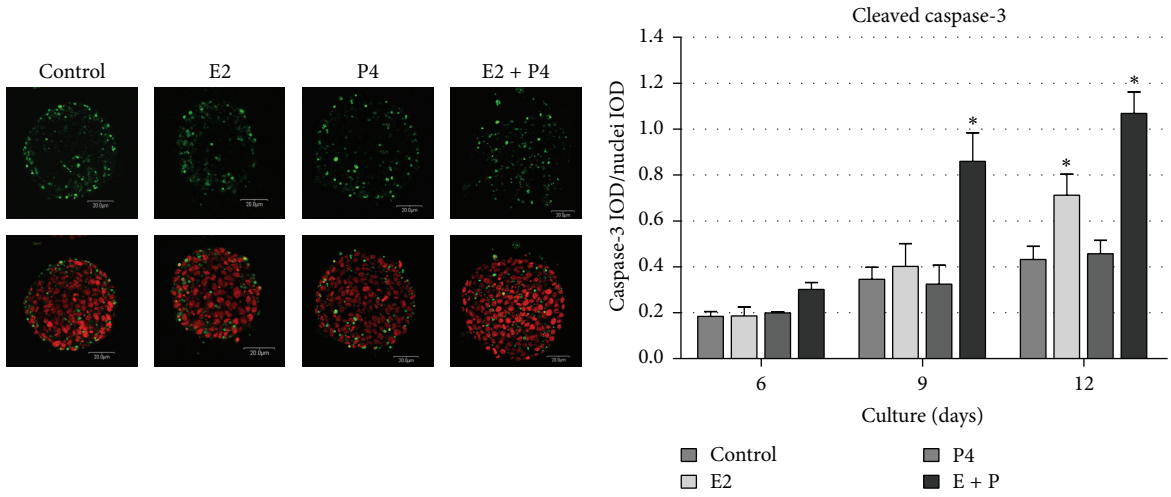


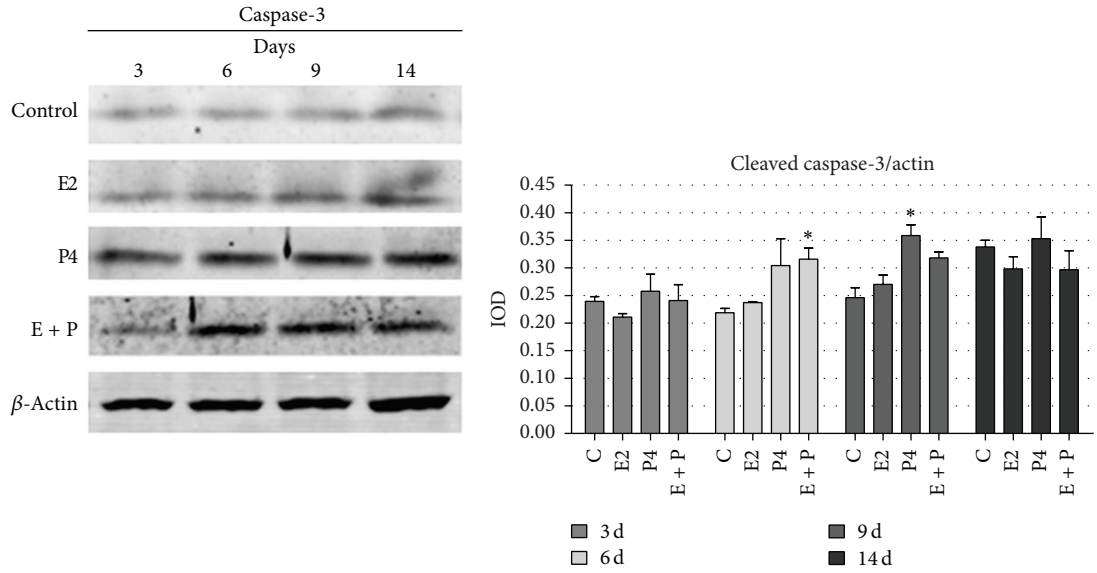

Figure 1: E-cadherin localization in acinar structures formed by BME-UV1 cells cultured on Matrigel for 12 days in differentiation medium (control), enriched with 17$\beta$-estradiol (E2, 1 nM), progesterone (P4, 5 ng/mL), or both (E2 + P4); E-cadherin (epithelial cell marker) was labeled with antibodies conjugated with Alexa Fluor 488 (green fluorescence) and DNA was counterstained with 7AAD (red fluorescence).

a marker to study autophagic flux [14]. Immunofluorescence staining of p62 revealed high expression of this protein mainly in the outer cells of the acini (Figure 3). In the first week of 3D culture (6 d) the levels of this protein were not high; however, increased expression could be noted in bovine MECs treated with P4 (Figures 3(a) and 3(b)). On day 9 of culture p62 positive staining was increased in all tested conditions, but in the presence of sex steroids the differentiation of two populations of cells with difference in p62 expression was more pronounced. This effect was especially clear after P4 treatment, and densitometric analysis done using MicroImage software confirmed the significant increase of p62 IOD in the presence of progesterone on days 6 and 9. However, these data represent the total fluorescence in the area of the acinar structures, without distinguishing the inner and outer population of cells. On day 14 spherical structures formed in the presence of sex steroids administered separately or together expressed p62 only in the outer cell population, whereas in control cells some green fluorescence could still be noted in the center. These results indicate that E2 and P4 increased the rate of autophagy in the centrally located cells of the acini, manifested by increased degradation of p62 (Figure 3).

*3.2. Regulation of Atg Proteins Expression in Bovine MECs Cultured on rBM.* In the next step of our study we investigated the potential influence of 17$\beta$-estradiol and progesterone (administered separately or together) on the level of autophagic proteins involved in autophagosomes formation. Western blot analysis was performed on cell lysates isolated from acini formed by BME-UV1 cells cultured on Matrigel in the presence of E2, P4, or E2 + P4 for 3, 6 9, and 14 days. In control conditions the levels Atg5 and Atg3 increased from day 9 of 3D culture (Figures 4(a) and 4(b)). Our results demonstrated that P4 markedly elevated the amount of Atg5 from the earliest time point (3 d), and this effect was also observed when steroids were added together (Figure 4(a)). In the case of E2, the increase in Atg5 level could also be noted, but from the 6th day of culture; however, densitometric analysis did not confirm these differences (Figure 4(a)). Similar tendencies were observed for Atg3 protein, in which an increase was observed faster in cultures supplemented with steroids (from day 6) than in control conditions (day 9) (Figure 4(b)). Also LC3 II, the active, cleaved form of LC3 protein, was detected from day 9 of 3D culture in the presence of steroid hormones in contrast to control conditions, in which a substantial increase in LC3-II did not appear until day 14 (Figure 4(c)). Interestingly, activation/cleavage of LC3 protein was the most pronounced when bovine MECs were treated with estrogen alone, or both steroids simultaneously, whereas progesterone did not exert such a clear effect. In the case of beclin-1 no distinct difference was observed between cells cultured under control conditions and those treated with E2, P4, and E2 + P4 together (Figure 4(d)). We have noted, however, differences in localization of beclin-1 in cells being in direct contact with rBM and those located in the center of developing acinar structures. Immunofluorescence staining of beclin-1 revealed that the most outer cells had perinuclear localization of beclin-1, whereas inner population of MECs showed cytoplasmic staining (Figure 4(f)). This pattern of staining was similar in all tested conditions. Since beclin-1 needs to be exported from the nucleus to the cytoplasm in order to play its function in autophagy induction our findings further support the hypothesis about the differences between the outer population of cells receiving direct signals from the substratum and the centrally located cells lacking




(a)




(b)

Figure 2: Cleaved caspase-3 expression in BME-UV1 cells cultured on Matrigel in differentiation medium (control), enriched with 17$\beta$-estradiol (E2, 1 nM), progesterone (P4, 5 ng/mL), or both (E2 + P4) for 3, 6, 9, 12, or 14 days; (a) images present immunofluorescence staining of cleaved caspase-3 (green fluorescence) and DNA counterstained with 7AAD (red fluorescence) and graph beside represents quantitative analysis of the intensity of green fluorescence of cleaved caspase-3 immunostaining, presented as the ratio of integrated optical density (IOD) of caspase-3 to IOD of nuclei in each acinus analysed; (b) Western blot analysis of the levels of cleaved caspase-3 in cell lysates: expression of $\beta$-actin was used as a loading control; graph beside the image represents the obtained results of densitometric analysis, in which IOD of each band was measured, and the values were normalized to IOD of $\beta$-actin; the IOD results are presented as means ± SEM from at least three separate experiments; statistically significant differences ($P < 0.05$) in comparison with control conditions were marked with $*$.

the contact with ECM, thus inducing autophagy and being prone to apoptotic cell death. Additionally, we investigated the level of Bcl-2 protein which forms complexes with beclin-1 inhibiting autophagy. Studies have shown that Bcl-2 is released from these complexes after phosphorylation (at Ser70) [15]; therefore, we analyzed the expression of the phosphorylated form of this protein. The results demonstrated that the levels of phos-Bcl-2 increase in time of 3D culture and sex steroids further elevated the level of this protein. The most pronounced effect was noted when bovine MECs were cultured in the presence of P4, and E2 + P4; however, densitometric analysis did not show statistical significance of these differences (Figure 4(f)).

*3.3. Regulation of ATGs Expression by 17β-Estradiol and Progesterone in Bovine MECs.* To investigate whether 17$\beta$-estradiol and progesterone regulate autophagy during spheroids formation via the classical genomic pathway we analyzed the influence of these steroids on the expression of genes involved in autophagy induction and formation

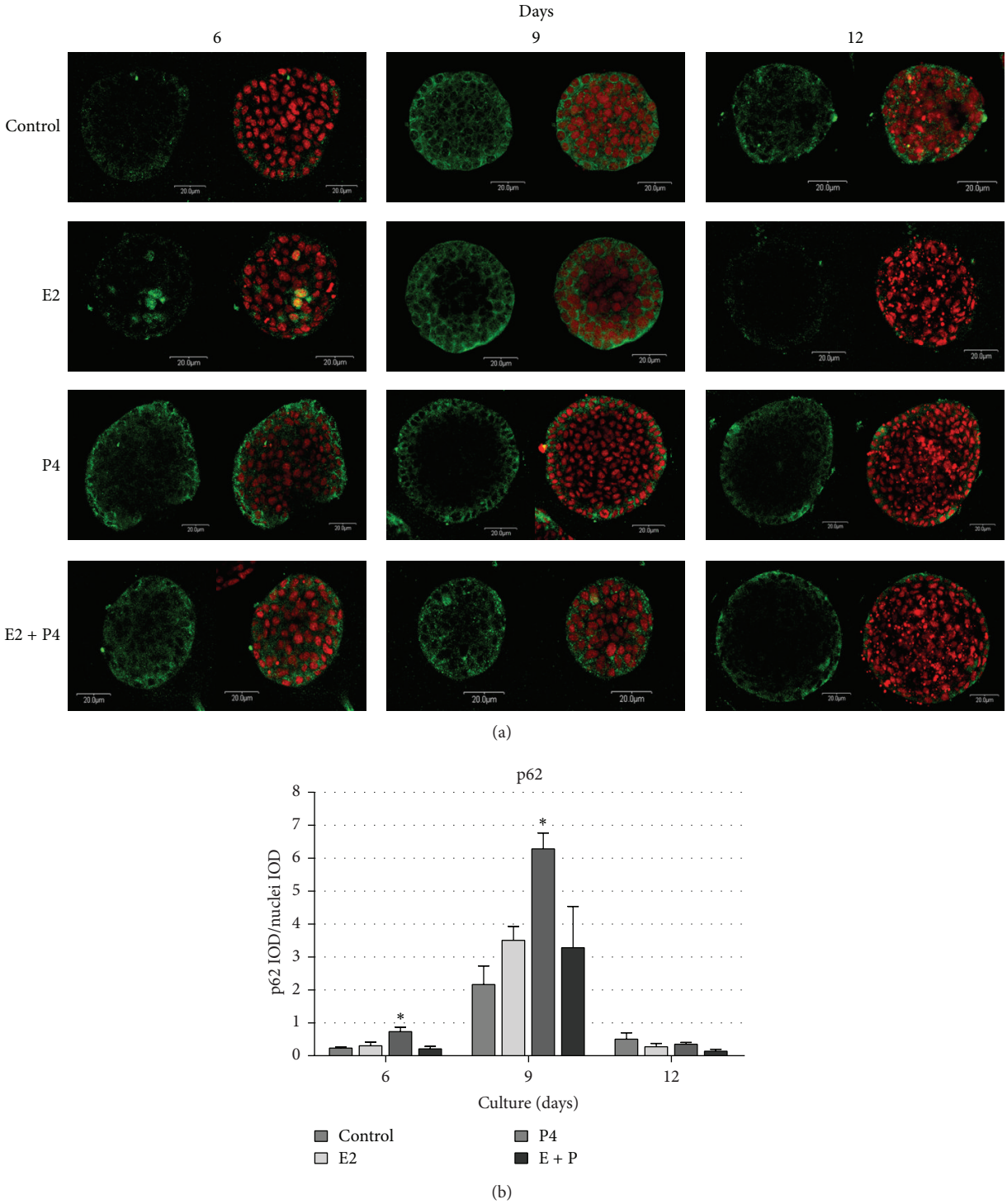



Figure 3: Confocal images of acinar structures formed by BME-UV1 cells cultured on Matrigel for 6, 9, or 12 days in differentiation medium (control), enriched with 17$\beta$-estradiol (E2, 1 nM), progesterone (P4, 5 ng/mL), or both (E2 + P4); (a) panel of images presents immunofluorescence staining of p62 (green fluorescence): DNA was counterstained with 7AAD (red fluorescence); (b) graph representing quantitative analysis of the intensity of green fluorescence of p62 immunostaining, presented as the ratio of integrated optical density (IOD) of p62 to IOD of nuclei in each acinar structure analysed; $^*$statistically significant difference ($P < 0.05$) in comparison with control conditions.

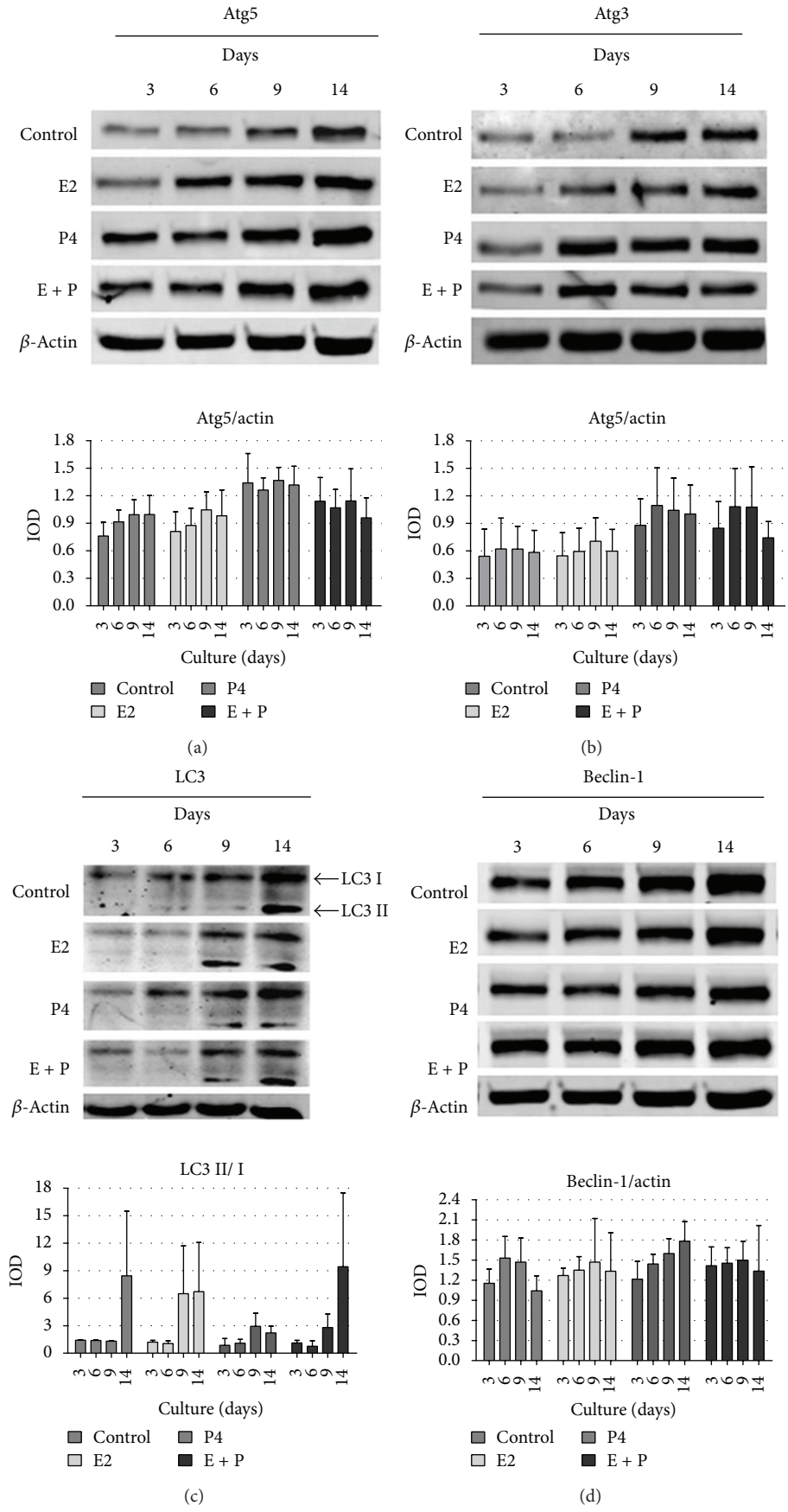



Figure 4: Continued.

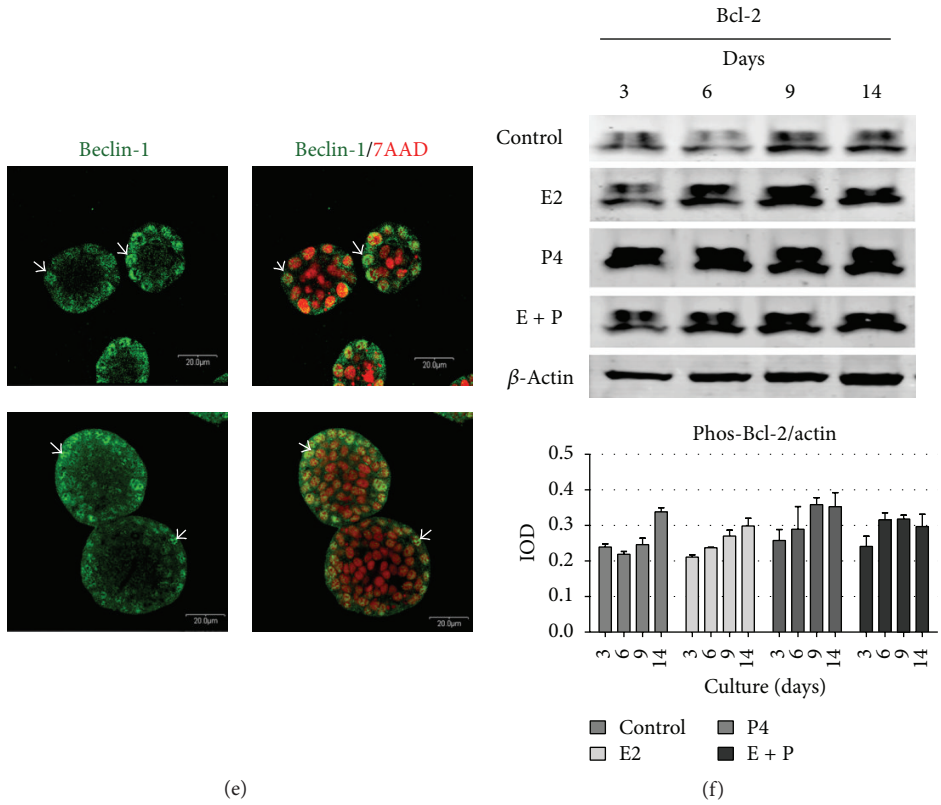



Figure 4: Expression of autophagic proteins (Atg5, Atg3, LC3, and beclin-1) and phosphorylated Bcl-2 (Ser70) in BME-UV1 cells forming acinar structures on Matrigel. Representative images of Western blot analysis of Atg5 (a), Atg3 (b), LC3 (c), beclin-1 (d), and phosphorylated Bcl-2 (Ser70) (f) in bovine MECs grown in 3D culture for 3, 6, 9, and 14 days in differentiation medium (control), enriched with 17$\beta$-estradiol (E2, 1 nM), progesterone (P4, 5 ng/mL), or both (E + P); expression of $\beta$-actin was used as a loading control; graphs below the images show the results of densitometric analysis, in which IOD of each band was measured, and the values were normalized to IOD of $\beta$-actin; the IOD results are presented as means ± SEM from at least three separate experiments. (e) Confocal images of immunofluorescence staining of beclin-1 (green fluorescence) in cells grown in 3D culture for 3 or 9 days in control conditions: DNA was counterstained with 7AAD (red fluorescence) and white arrows indicate the nuclear localization of beclin-1.

of autophagosomes in MECs. In order to choose candidate genes for real time RT-PCR we performed a preliminary computer analysis of potential binding sites for steroid receptors on promoters of bovine autophagy-related genes using MatInspector software (Genomatix). This analysis revealed that the promoter regions of bovine *ATG5* and *LC3B* genes potentially contain estrogen response element (ERE), whereas *ATG3* shows the presence of androgen response element (ARE). Additionally, other analyzed autophagic genes, such as beclin-1, contained many binding sites for AP1, Sp1, and CREB transcription factors, which are known to interact with steroids receptors, enabling an indirect activation of gene expression by sex steroids [16–19]. On the basis of these preliminary results of *in silico* analysis we chose to investigate the expression of four genes which play a key role in autophagy induction and autophagosomes formation (*BECN1*, *ATG5*, *ATG3*, and *LC3B*) and are potentially regulated by E2 and P4. We investigated the expression levels of these genes in BME-UV1 bovine mammary epithelial cells treated with E2, P4, and both steroid hormones combined in comparison with control conditions on 3, 6, 9, and 14 days of culture (Figure 2). The expression of each *ATG* under control conditions was appointed as 1 at each time point, and the levels of analyzed mRNA were normalized according to the relative *GAPDH* mRNA expression of each sample. A change in expression was considered significant when at least 1.5-fold increase was noted.

Real-time PCR results confirmed that *ATG5* was positively regulated by estrogen and progesterone. Over 2-fold increase was noted in cells treated either with E2 or P4, but only on day 9 of 3D culture (Figures 5(a) and 5(b)). It is worth noting, however, that at this time two distinct populations of cells could be distinguished within each acinus: the outer layer of polarized epithelium showing nuclear localization of beclin-1 and sustained expression of p62 protein and the inner cells undergoing stress connected with the gradual loss of contact with ECM. Interestingly, when BME-UV1 cells were treated with both steroids together a significant increase in *ATG5* was observed on the 6th day of culture, suggesting that under these conditions autophagy may be induced earlier (Figure 5(c)). Simultaneous presence of E2

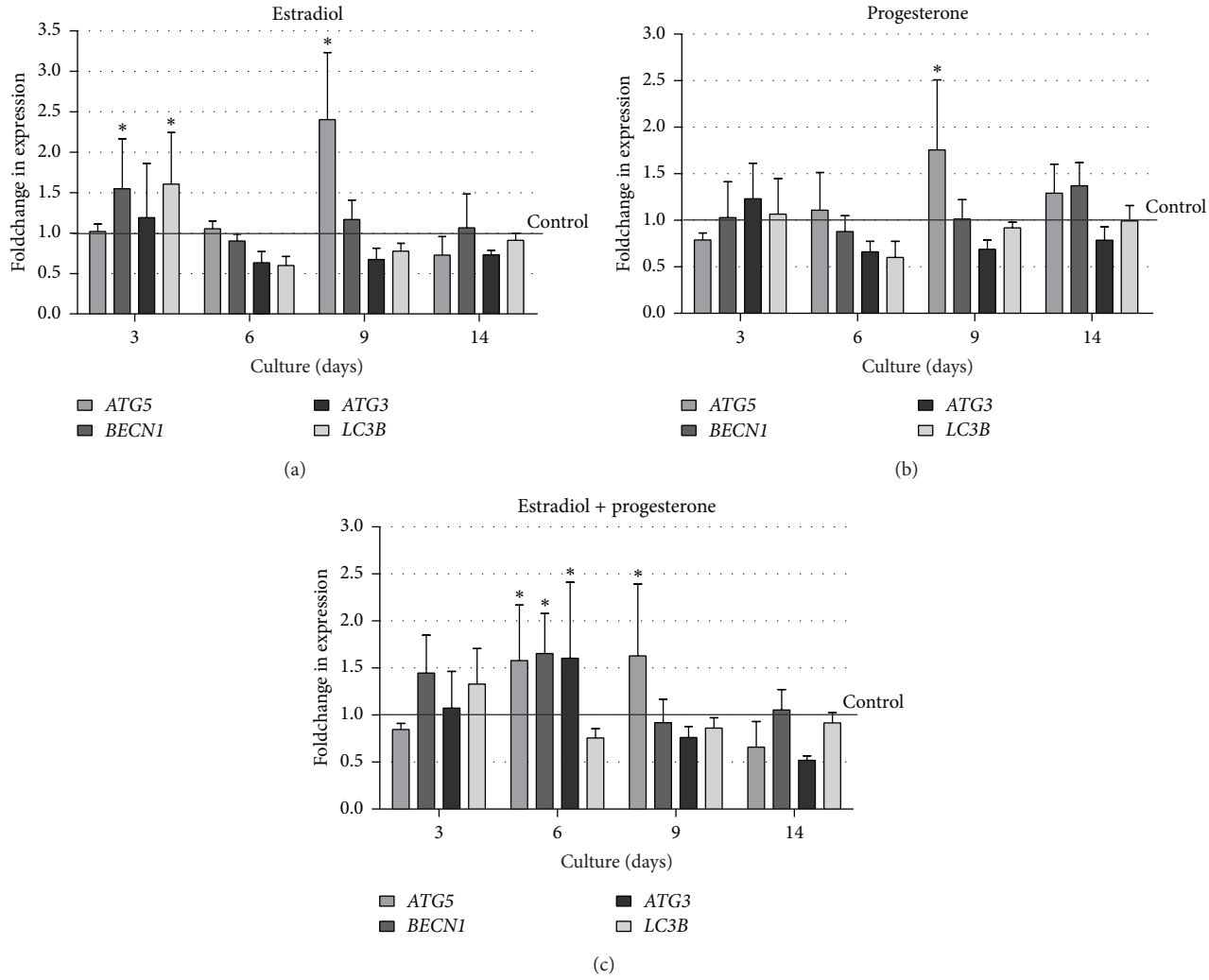



Figure 5: Real-time PCR analysis showing the effects of (a) 17$\beta$-estradiol (1 nM), (b) progesterone (5 ng/mL), or (c) both steroid hormones administered simultaneously on the expression of chosen autophagy-related genes: *ATG5*, *BECN1*, *ATG3*, and *LC3B* in BME-UV1 cells cultured on Matrigel for 3, 6, 9, and 14 days; the levels of analyzed mRNA were normalized according to the relative *GAPDH* mRNA expression of each sample; the expression of each *ATG* under control conditions was appointed as 1 at each time point; a change in expression was considered significant (*) when at least 1.5-fold increase was obtained.

and P4 in the culture medium also resulted in significantly increased expression of *ATG3* and *BECN1* on day 6, as well as augmented level of *BECN1* and *LC3B* on day 3 (Figure 5(c)), which points at important interactions between both steroid hormones in regulation of autophagy in bovine MECs. Increased *BECN1* and *LC3B* expression was also noted when BME-UV1 cells were treated only with estrogen (Figure 5(a)); however, these changes were significant only on day 3, whereas progesterone alone did not induce any changes in the levels of transcripts other than *ATG5*.

*3.4. Analysis of Kinases Activation after Addition of Sex Steroids during Acinar Structures Formation by Bovine MECs.* Estradiol and progesterone are known to mediate their biological actions through specific nuclear receptors: estrogen receptors (ER$\alpha$ and ER$\beta$) and progesterone receptor (PR). Nevertheless, these steroid hormones were also shown to rapidly recruit intracellular signaling pathways, and their action was linked to the transmembrane receptors: G protein-coupled membrane receptor GPR30 [20], membrane progestin receptors (mPR) [21], and progesterone receptor membrane components 1 and 2 (PGRMC) [22]. As a result of activating transmembrane receptors, E2 and P4 mediate their signals through the same cellular pathways as growth factors, mainly MAPK (especially ERK1/2 kinases) and PI3 K/Akt [23]. Downstream signaling pathways mediated through Akt, AMPK, or ERK1/2 kinases may regulate mTORC1 activity, which constitutes the major switch in autophagy induction [24]. Therefore, in our effort to explain regulation of autophagy by sex steroids, we also analyzed the activation pattern of main cellular kinases, which are known to be involved in autophagy induction. The levels of phosphorylated and total Akt, ERK1/2, AMPK, and mTOR kinases were determined by Western blot analysis on days 3, 6, 9, and 14

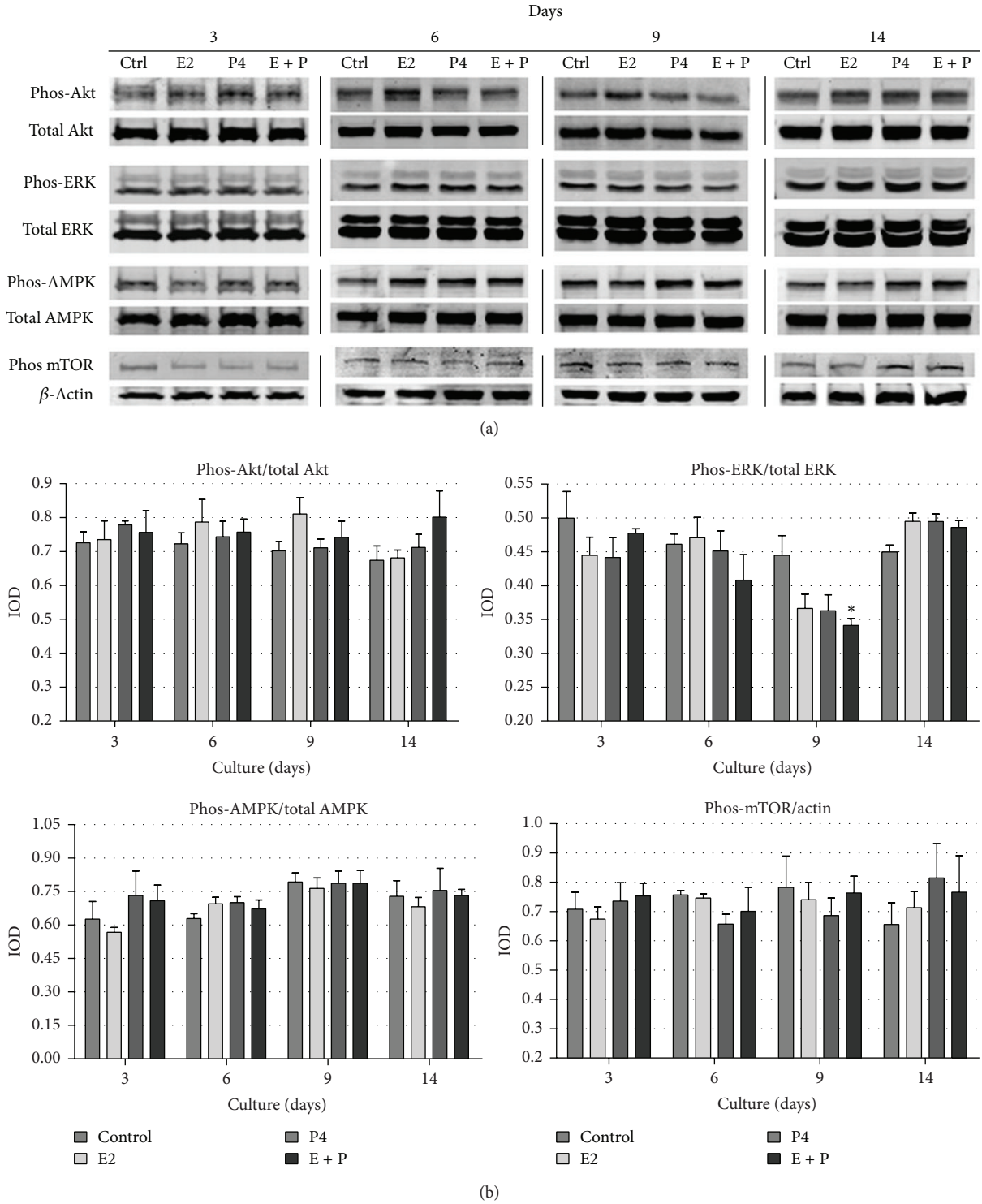



Figure 6: (a) Western blot analysis of the levels of chosen kinases (Akt, ERK, AMPK, and mTOR) involved in autophagy regulation, detected in BME-UV1 cells cultured on Matrigel for 3, 6, 9, and 14 days in differentiation medium (control), enriched with 17$\beta$-estradiol (E2, 1 nM), progesterone (P4, 5 ng/mL), or both (E + P): expression of $\beta$-actin was used as a loading control; (b) graphs represent the obtained results of densitometric analysis, in which IOD of each band was measured, and the IOD values for phosphorylated forms of kinases were normalized to the respective IOD of the total forms, with an exception of phos-mTOR, which was normalized to IOD of $\beta$-actin; results are presented as means ± SEM from at least three separate experiments; $^*$statistically significant difference ($P < 0.05$) in comparison with control conditions.

of 3D culture (Figure 6). In general, no significant changes in the activity of analyzed kinases were observed between the experimental conditions, especially when all performed replicates were analysed densitometrically (Figure 6). Only ERK1/2 activity was significantly reduced on day 9 of 3D culture in cells simultaneously treated with E2 and P4, when compared to control conditions. Steroids seemed to slightly attenuate ERK1/2 phosphorylation (Tyr202) between days 3 and 9 of acini formation by bovine MECs. Interestingly, on day 14 a higher activity of this kinase was noted in all tested conditions, but steroids added separately or together further increased ERK1/2 phosphorylation. Some tendencies could also be noted in the case of Akt kinase activation. At the early time point of 3D culture (day 3) both steroids stimulated phosphorylation of Akt (Ser473), whereas on days 6 and 9 17$\beta$-estradiol administered alone caused the most pronounced increase in the level of activated Akt in comparison with control, as well as other experimental conditions. This effect seemed to be suppressed by P4 because, in cells treated with both hormones simultaneously, the cellular level of phosphorylated Akt was lower than that observed under control conditions. In the case of AMPK phosphorylation (Thr172) increased levels were noted in the presence of sex steroids on days 3, 6, and 9, when compared with control conditions. The most pronounced effect (although not statistically significant) was observed on day 3 of 3D culture, when BME-UV1 cells were treated with P4 or E2 + P4. Between days 6 and 9 the activity of AMPK further increased in all tested conditions, with slightly higher level in the presence of steroid hormones, and this level was sustained also on day 14 of culture. Finally, E2 and P4 were shown to cause a small decrease in the activity of mTOR kinase (phosphorylation at Ser2448) (on days 3 and 9); however, it was statistically insignificant. It was surprising to find elevated activity of tested kinases on day 14 of 3D culture. This effect might have resulted from the fact that Western blots were performed on lysates from whole spherical structures, which, as mentioned earlier, are composed of two distinct populations of cells: outer population, which remains in favorable conditions thanks to direct interactions with ECM, and inner population, undergoing gradual apoptotic death. On 14th day of culture the number of centrally located cells was largely reduced due to ongoing process of lumen clearance; thus the high activity of kinases might have originated mostly from the remaining living cells forming acini.

## 4. Discussion

In the present study we examined the influence of 17$\beta$-estradiol (E2) and progesterone (P4) on the expression of autophagic genes and pathways regulating autophagy induction in bovine BME-UV1 mammary epithelial cells cultured on reconstituted basement membrane. The 3D culture model enables recreation of the conditions in which MECs form acinar structures morphologically similar to alveoli that develop during gestation forming the basic secretory units of functionally active mammary gland. The important role of sex steroids in mammary gland development is well documented. Ovariectomy of prepubertal dairy heifers curtails further mammary development, where the earlier in life the procedure is performed, the more drastic is the result [25]. Similarly, mice lacking estrogen receptor (ER) are unable to obtain full glandular development through adulthood, whereas knockout of progesterone receptor (PR) in rodents results in inability to undergo normal lobuloalveolar development [26, 27]. Up to date studies have shown that steroid hormones not only control proliferation of the mammary epithelium, but also are involved in regulation of many different processes, including development, differentiation, and death of mammary epithelium.

Our research demonstrated for the first time that E2 and P4 can regulate autophagy in bovine MECs [7, 8]. It was manifested with increased levels of LC3-II in Western blot analysis, as well as punctuated pattern of GFP-LC3II observed under confocal microscope. When BME-UV1 cells were cultured on rBM the lipidated form of LC3 (LC3-II) was shown to localize predominantly in the center of developing acini. Both E2 and P4 were shown to enhance the rate of spherical structures development by increasing the levels of LC3-II and cleaved caspase-3 [8]. In the present study we further confirmed these observations, showing that in the center of developing acini there is a marked decrease in the levels of p62, whereas cells in direct contact with rBM show sustained expression of this protein (Figure 3). We used p62 as the marker of autophagy, as this scaffold protein is involved in delivering cargo to autophagosomes binds with LC3 and becomes degraded in the course of active autophagy [14]. In parallel we observed central localization of cells expressing cleaved caspase-3 on day 12 of 3D culture (Figure 2). This supports the hypothesis on two distinct populations of cells, whose fate is determined by their localization within the developing mammary acinar structures. Polarized cells are found only in the outer layer because they are able to receive signals from ECM via integrin receptors found on their basal membrane [28, 29], whereas nonpolarized cells comprising the central inner region lack ECM contact and subsequently undergo apoptotic cell death leading to lumen formation [30, 31]. The inner cells also activate autophagy, which has been suggested to be induced due to loss of interactions with ECM [32]. In our study the differences between the two cell populations could also be noted on the basis of beclin-1 localization. The outer layer of bovine MECs expressed beclin-1 predominantly in the perinuclear region, whereas centrally located cells showed cytoplasmic localization of this autophagic protein (Figure 4(e)). Beclin-1 needs to be shuttled from the nucleus to the cytoplasm in order to form the PtdIns3K complex, which initiates nucleation and assembly of the isolating membrane of autophagosomes [13, 33]. Thus, our results demonstrate that only inner cells induce autophagy during acini development.

When BME-UV1 cells cultured on rBM were treated with sex steroids the rate of development of acinar structures was accelerated. We observed increased level of active caspase-3, especially in the presence of progesterone or both steroids together. Also, p62 seemed to be the most actively degraded in the centrally located cells of the acini formed by bovine

MECs treated with P4 or E2 + P4, since in these conditions no staining was detected in the inner cells on days 9 and 12 of 3D culture. This supports the general concept of the role of progesterone in alveologenesis. At the same time our findings point at possible important synergistic effects of E2 and P4 in the course of alveoli formation. The synergistic interactions between E2 and P4 signaling in regulation of mammary gland development have been indicated by several studies. Estrogen treatment alone or in combination with progesterone in the intact prepubertal heifers stimulated the proliferation of mammary epithelial and endothelial cells, whereas progesterone treatment alone had no effect on prepubertal mammary cell proliferation [34]. Furthermore, in nonpregnant, nonlactating cows, a combined estrogen and progesterone treatment stimulated MECs differentiation and lobuloalveolar development [35]. Both sex steroids were also shown to stimulate synthesis of glycosaminoglycans (GAGs), which are important ECM components regulating cell proliferation, migration, adhesion, differentiation, and cell-cell communication. A significant increase in total GAGs in the mammary gland of ovary-intact rats administered estradiol singly or in combination with progesterone indicated the stimulatory role of E2 and its synergism with P4 in augmenting total GAGs [36]. In the present study we have shown that simultaneous administration of E2 and P4 during 3D *in vitro* culture of bovine BME-UV1 mammary epithelial cells potentiates the rate of acini development by stimulation of apoptosis and autophagy inside the spherical structures. Since at the time of pregnancy both hormones are present in high concentrations in the mammary gland, it is possible that this coexistence is important for the proper rate of lobuloalveologenesis.

Enhanced autophagy induction in BME-UV1 cells treated with E2 and P4 is related to the ability of these hormones to regulate the expression of autophagy-related genes and their products. It is commonly known that both steroids exert their effect via their nuclear hormone receptors (ER and PR), which function as ligand-bound transcription factors regulating the expression of specific downstream targets [35]. Lu and coworkers [37] performed a microarray study to investigate the transcriptional changes within the mammary gland after administration of E2 or P4, individually and combined. Their results demonstrated that 60% of the differentially expressed genes required combined treatment with E2 and P4, confirming our hypothesis on the synergistic interactions between these sex steroids in regulation of autophagic genes during formation of acini by mammary epithelial cells [37]. However, so far there has been no data showing the possible genomic effect of estrogen and progesterone on the expression of *ATGs*. Our study seems to be the first indicating that both steroids singly as well as combined regulate the expression of *ATG5*, which was significantly increased in all tested conditions on day 9 of 3D culture (Figure 5). Other autophagic genes and their products were also upregulated by sex steroids; however, the effect varied depending on the conditions applied. 17$\beta$-Estradiol alone elevated the expression of *ATG5*, *BECN1,* and *LC3B* genes, but this effect was observed only at the early time of culture (day 3) and did not fully correspond with the results of Western blot analysis, which did not detect differences in the level of beclin-1 between the tested condition. However, E2 stimulated the formation of LC3-II protein which was increased between days 9 and 14 of the 3D culture of bovine MECs. These results are partially in agreement with the studies performed on other cell types. Yang et al. [38] demonstrated that E2 caused a significant increase in LC3, beclin-1, and ULK1 kinase expression in MC3T3-E1 osteoblastic cells. The promotion of autophagy in osteoblasts by E2 was blocked by ER antagonist, and upregulation of LC3 expression was suppressed by U0126 (ERK kinase inhibitor). The upregulation of *ATG5* and *LC3B* genes by estrogen confirmed the results of our *in silico* analysis, showing that the promoter regions of these genes in the cattle potentially contain estrogen response element (ERE). It was surprising to find, however, that the combination of E2 and P4 abrogated the genomic effect of E2 observed on day 3 but stimulated the expression of *ATG5, ATG3,* and *BECN1* on day 6, maintaining the high expression of *ATG5* also on day 9 of BME-UV1 cells culture. Administration of both steroids together resulted also in increased levels of Atg5 and Atg3 proteins, further supporting the important role of synergistic actions of E2 and P4 in autophagy regulation. It is possible that when E2 and P4 act together on bovine MECs, estrogen predominantly exerts its genomic function, whereas at the same time progesterone regulates the signaling pathways controlling autophagic machinery. Although little is known on the mechanisms of P4 nongenomic effect, some authors reported the rapid actions of this steroid on different cell types. Recent *in vitro* study on neurosteroids, steroid hormones synthesized in central and peripheral nervous system, revealed that progesterone as well as pregnenolone activated autophagy in astrocytes within a few hours after addition [39]. Progesterone activated LC3 protein, and this effect was inhibited by siRNA-mediated knockdown of beclin-1. The neurosteroids regulation of autophagy was proposed to be largely mTOR-independent, and the coexistent activation of mTOR and Akt by P4 probably constitutes a cytoprotective mechanism during autophagy induction in neural cells [39]. Our results also demonstrated increased phosphorylation of Bcl-2 (at Ser70) in the presence of progesterone (Figure 4(f)). Bcl-2 is an antiapoptotic protein, which also functions as autophagy inhibitor [40]. Under nutrient-rich conditions beclin-1 strongly interacts with Bcl-2 inhibiting formation of a multiprotein complex comprising PtdIns3K/Vps34 kinase and beclin-1 (as well as other proteins). The PtdIns3K complex is necessary to initiate autophagosome formation in the cells. In contrast, in starvation, when autophagic rates are high, the interaction between beclin-1 and Bcl-2 is weak. In these conditions Bcl-2 undergoes phosphorylation releasing beclin-1 [15, 41]. During formation of acini by bovine MECs the inner cells deprived of the contact with ECM undergo stress connected not only with the lack of integrin-mediated signaling, but also with undernourishment. In control conditions the levels of phosphorylated Bcl-2 gradually increased on the later days of 3D culture (d 9, 14). In the presence of P4 and E2 + P4 Bcl-2 phosphorylation was augmented already from day 3, suggesting earlier induction of autophagy.

Several other signaling pathways are orchestrated to regulate the dynamic process of autophagy. As a key cellular response activated to compensate extra- or intracellular stress, such as nutrient deprivation, hypoxia, and reduced levels of growth factors, autophagy is regulated by diverse signaling pathways, that induce or inhibit the activity of mTOR kinase [24]. Under normal conditions mTOR remains associated with other proteins in a complex known as mTORC1, leading to increased translation and synthesis of cell cycle regulating and ribosomal proteins [42]. Upon mTOR inhibition by stress, such as starvation, ULK1 and ULK2 (mammalian homologs of Atg1) become activated and phosphorylate Atg13 and FIP200, which are essential for autophagy induction [43]. The activity of mTORC1 complex is controlled by signals from two major sources, the phosphoinositide 3-kinase (PI3K) pathway, an important signaling pathway downstream of receptor tyrosine kinases, and the LKB1/AMPK pathway [44, 45]. PI3K/Akt pathway is induced by growth factors and hormones stimulation, leading to mTOR activation, whereas AMPK (AMP kinase) is a master regulator of cellular energy metabolism, sensing the ATP to AMP ratio and becoming activated when the ATP/AMP ratio is decreased. This in turn leads to inactivation of mTOR and induction of autophagy. The aforementioned pathways play also an important role in development of the mammary gland. PI3K/Akt signaling pathway is associated with induction of mammary epithelial cells' proliferation. Studies with the use of 3D culture system revealed that in the process of acini formation by MECs cultured on rBM lumen formation was associated with PI3K/Akt inhibition in the centrally located cells lacking contact with ECM components, which led to apoptotic cell death and lumen clearance [10]. As previously mentioned, these cells were also shown to induce autophagy [8, 11]. Furthermore, expressing a constitutively active variant of Akt or PI3K in MECs resulted in formation of large, misshapen spherical structures, which failed to elicit lumen [46, 47]. A similar effect was noted in bovine MECs cultured on Matrigel in the presence of IGF-I, which is a known mitogen, acting through PI3K/Akt pathway [8, 48]. Further studies revealed that estrogen can regulate the proper development of such IGF-I-stimulated spheroids, decreasing the activity of Akt kinase in the later stages of acini development, which facilitated lumen clearance [48]. Present research demonstrated that combination of E2 and P4 diminished the activity of Akt in the second week of 3D culture of bovine MECs, which was coincident with the induction of autophagy and initiation of lumen clearance in the acini (Figure 4). An opposite effect was noted on the 3rd day of 3D culture, when all cells showed proliferative activity and exhibited cell-cell contact via adherence junctions (results presented in previous report [49]). In this case 17$\beta$-estradiol alone or together with progesterone increased phosphorylation of Akt, which may be connected with known mitogenic actions of both steroids in the mammary epithelium.

So far, little is known about the involvement of AMPK-mediated pathway in the course of mammary alveoli formation. We showed that the activity of AMPK increased with the time of 3D culture (between days 3 and 9 in control conditions), indicating that the centrally located cells of developing spheroids undergo stress related to decreased energy levels due to diminished contact with nutrients and oxygen which are not able to easily penetrate inside the structures (Figure 6). Addition of P4 or E2 + P4 caused further increase in AMPK phosphorylation at these time points. Increased AMPK activation, corresponded with the slightly decreased levels of phosphorylated/activated mTOR, as well as diminished phosphorylation of Akt kinase on day 9 of 3D culture treated with both E2 and P4. It is worth noting that at that time phosphorylated ERK1/2 was also decreased, and this effect was significant in the case of simultaneous administration of both steroids. Although it has been demonstrated that estrogenic compounds are able to rapidly activate PI3K/Akt and MAPK pathways [50] via their nongenomic actions, the observed decrease in phosphorylation of both signaling pathways on the second week of acini formation by BME-UV1 cells may be correlated with the aforementioned changes in vitality of the centrally located cells and ongoing processes of autophagy and apoptosis. Surprisingly, we noted a renewed activation of Akt, ERK1/2 and mTOR on day 14 of culture on rBM. Phosphorylation of these kinases was augmented by sex steroids administered separately or together. This effect coincided with the time of final stage of lumen clearance, thus the protein lysates, in which kinases were detected by Western blot, might have originated mainly from the remaining population of polarized cells forming acinar structures.

## 5. Conclusions

In conclusion, we demonstrated that 17$\beta$-estradiol and progesterone enhance differentiation of bovine mammary epithelial cells forming alveoli-like structures in contact with rBM. Fully developed acini, showing hollow lumens in the center, were formed faster in the presence of both sex steroids simultaneously. This effect could be related to synergistic genomic actions of E2 and P4, which stimulated the expression of autophagy-related genes: *ATG3, ATG5*, and *BECN1,* as well as their protein products at the earlier time points of acini formation. Additionally, our results indicate that enhancement of autophagy in the presence of E2 and P4 can also be partially related to their ability to regulate signaling pathways (PI3K/Akt/mTOR; AMPK/mTOR) involved in autophagy induction.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

## Authors' Contribution

All authors were involved in the study presented in this paper. K. Zielniok and M. Gajewska designed the study, K. Zielniok performed the majority of the experiments, M. Gajewska supervised the performance of all experiments and performed confocal analysis, K. Zielniok and M. Gajewska analyzed the data and wrote the paper, and T. Motyl revised

the paper. All authors read and approved the final version of the paper.

## Acknowledgments

This work was supported by Grant no. N N311 515740 from the National Science Centre Poland and Grant no. IP2011 013271 from Ministry of Science and Higher Education.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 960803, 12 pages
http://dx.doi.org/10.1155/2014/960803

*Review Article*

# How to Take Autophagy and Endocytosis Up a Notch

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Received 23 January 2014; Accepted 12 March 2014; Published 22 April 2014

Academic Editor: Ioannis P. Nezis



The interconnection of the endocytic and autophagosomal trafficking routes has been recognized more than two decades ago with both pathways using a set of identical effector proteins and sharing the same ultimate lysosomal destination. More recent data sheds light onto how other pathways are intertwined into this network, and how degradation via the endosomal/autophagosomal system may affect signaling pathways in multicellular organisms. Here, we briefly review the common features of autophagy and endocytosis and discuss how other players enter this mix with particular respect to the Notch signaling pathway.

## 1. Introduction

In eukaryotes, degrading cytoplasmic components is vital to the cells, since this process removes potentially toxic organelle remainders and protein aggregates, protects organisms from invading pathogens, and provides cells with nutrients by recycling the degraded macromolecules in periods of scarce food or under stress conditions. Macroautophagy (referred to as autophagy hereafter; other types of autophagy are not discussed here) has been shown to be the major degradation pathway, where cytosolic material is engulfed by double membrane vesicles (autophagosomes) and subsequently degraded after fusion with lysosomes [1, 2]. Despite its role as a survival pathway, autophagy also acts as a death mechanism implicated in protecting against cancer and neurodegenerative diseases [1, 3].

Autophagy can be activated by a wide range of signals but most intersect at the central regulator of autophagy and the target of rapamycin (TOR) complex 1 (TORC1, see Figure 1(a)). TORC1 inhibits autophagy by phosphorylating the core autophagy proteins associated into the Atg1 (autophagy-related gene 1) complex. Upon autophagy induction, this inhibition is released, and the activation of the Atg1 complex is followed by the assembly of proteins and lipids at the sites of autophagosome formation. This vesicle nucleation requires the activation of a class III PI3K (phosphoinositide 3-kinase) complex containing Vps34 (vacuolar protein sorting 34), Vps15, and Atg6/Beclin. Once initiated, the expansion and closure of the autophagic vesicle is mediated by two ubiquitin-like systems, Atg5-Atg12 and Atg8 (see Figure 1(b)). After completion, the autophagosome can fuse with compartments of the endocytic pathway, such as early endosomes, multivesicular bodies (MVBs), or late endosomes prior to fusion with lysosomes. Eventually, the autophagosomal cargo gets degraded by the acidic hydrolases of the lysosome and essential molecules such as amino acids are recycled and reused for cellular processes (for review, see [4–7]).

The endocytic system functions to internalize nutrients, macromolecules and plasma membrane compartments into the cell from outside by membrane invaginations and the formation of vesicles. These vesicles fuse to form early endosomes, from which proteins can be either recycled back to the plasma membrane through so called recycling endosomes, transferred to the trans-Golgi-network, form late endosomes or accumulate to MVBs. Finally, MVBs fuse with lysosomes for digestion and degradation of their content to provide them for cellular use. Endocytic processes are regulated by various proteins, such as clathrins, SNAREs (soluble NSF attachment protein receptor), Rab GTPases, the

ESCRT (endosomal sorting complex required for transport), and HOPS (homotypic fusion and vacuolar protein sorting) complexes (reviewed in [8]) (see Figure 1(d)). In this respect, endocytosis acts as a complementary road to autophagy to provide the cell with amino acids and macromolecules.

Besides their common role in cellular nutrient supply and their shared terminal end point, the interconnection of both pathways is also demonstrated by the fact that autophagosomes fuse with early or late endosomes [9]. This collaboration in targeting vesicles for degradation may also be relevant for the regulation of cell signaling pathways. For example, epidermal growth factor receptor (EGFR) and Notch signaling were shown to require endosomal trafficking for activation, regulation, and degradation of the signal [10, 11] (Figure 1(c)). The evolutionary conserved Notch signaling pathway is fundamental in a wide range of cell types during several developmental and physiological processes, for example, by determining cell fate decisions required for neurogenesis and organogenesis or to control cell proliferation and cell death during development (reviewed in [12]). Given its pleiotropic function it is not surprising that dysregulation of Notch signaling leads to various diseases and forms of cancer (for a recent review see [13]). Thus, understanding the mechanisms regulating Notch signaling and the interconnection with other signaling pathways will be crucial to develop relevant therapeutic interventions.

## 2. Interconnection of Autophagy and Endocytosis

It has been postulated already in 1992 that endocytosis might be coupled to autophagy [14], and several studies have identified molecules that are used by both pathways for cellular degradation (for review, see [15]). We will shortly summarize the main common players present in both pathways and provide examples where autophagy and endocytosis are affecting each other.

*2.1. Autophagy and Endocytosis Share the Same Effector Molecules.* Given the fact that both autophagic and endocytic pathways are implicated in degrading cellular material, one would predict that autophagy and endocytosis partly use the same machinery. In fact, there is a striking conservation of regulatory factors between the two pathways, and many excellent reviews covering this aspect have been published (examples include [4, 15–18]). Here, we will focus on a subset of players with respect to recent publications demonstrating a link between endocytosis, autophagy, and the Notch signaling pathway (see below).

The nucleation of autophagosomes requires the activity of a class III PI3K complex, consisting of Vps34 (PI3K), Vps15, and Atg6/Beclin in yeast and higher eukaryotes [19, 20]. In mammals, the complex can be associated with other regulatory proteins, such as Atg14L/Barkor [21, 22], Rubicon (RUN domain and cysteine rich domain containing, Beclin1-interacting protein) [22], UVRAG (UV irradiation resistance-associated gene) [23], and Ambra1 (activating molecule in Beclin1-regulated autophagy) [24] to control the different functions of this complex. While the Ambra1-containing complex is required for the induction of autophagy [24], Atg14L/Barkor is thought to function in recruiting the Vps34-Vps15-Atg6 complex to the autophagosomal membrane to initiate autophagosome formation [25]. Rubicon, in contrast, negatively regulates autophagy and endocytosis by preventing the activation of Rab7, a protein that functions in lysosomal fusion and autophagosome maturation [22, 26, 27]. The role of UVRAG in autophagy remains controversial. Although it has been reported to positively regulate autophagy through overexpression in a cell line with monoallelic UVRAG deletion [23, 28], others could not detect a role for UVRAG in autophagy [21]. However, recent data supports a positive role of UVRAG by binding to BIF1 (also known as endophilin1), which was recently identified as a factor necessary for autophagosome formation [29]. UVRAG-associated complexes may promote autophagosome/endosome maturation, thus serving as a convergent point of both pathways [28]. Recently, it was shown that UVRAG influences later steps of autophagy by regulating the degradation of sequestered cargo in autolysosomes [30, 31]. Although orthologs of Atg14L and Rubicon exist in *Drosophila,* their function in the class III PI3K complex is not yet completely solved; however, recent data assigns dUVRAG a role in endocytosis [30–32].

In fact, the core class III PI3K complex containing Vps34, Vps15 and Atg6/Beclin is also found on early endosomes and is required for endocytosis (reviewed in [4, 8]). UVRAG, the accessory protein suspected to have a role in autophagosome maturation, has its primary role in the class III PI3K complex on endosomes, where it is necessary to activate the HOPS complex that mediates membrane tethering to fuse with other endosomes or lysosomes [21, 28]. UVRAG is normally bound and thereby inhibited by Rubicon. Upon activation, the small GTPase Rab7 competes for binding to Rubicon, which causes the release of UVRAG and its association with the HOPS complex involved in membrane tethering. The HOPS complex was shown to regulate tethering and fusion of endosomes to the vacuole in yeast as well as lysosomal delivery in *Drosophila* [33, 34]. Interestingly, the HOPS complex protein Dor was shown to control autophagosome maturation in *Drosophila* as well [35] (Figure 1). Thus, Rubicon and UVRAG may function both on endosomes and autophagosomes, while Atg14L and Ambra1 seem to be associated exclusively with the class III PI3K complex on autophagosomes.

However, a recent publication also links Atg14L to endocytic trafficking by demonstrating its binding to Snapin, a SNARE effector protein [36]. SNAREs are primarily involved in vesicle fusion events by assembling into trans-SNARE complexes with one v- (vesicle-) SNARE and three t- (target-) SNAREs on the donor and acceptor membrane, respectively. Several fusion events occur at different stages of autophagy to form autophagic structures, during maturation of autophagosomes and finally when autophagosomes unite with lysosomes. All these processes have been shown to involve the v- and t-SNARE proteins (for review, see [37]). Recently, autophagy was also assigned a role in secretion [38, 39], where SNAREs may mediate autophagosomal fusion with the plasma membrane in a similar way.

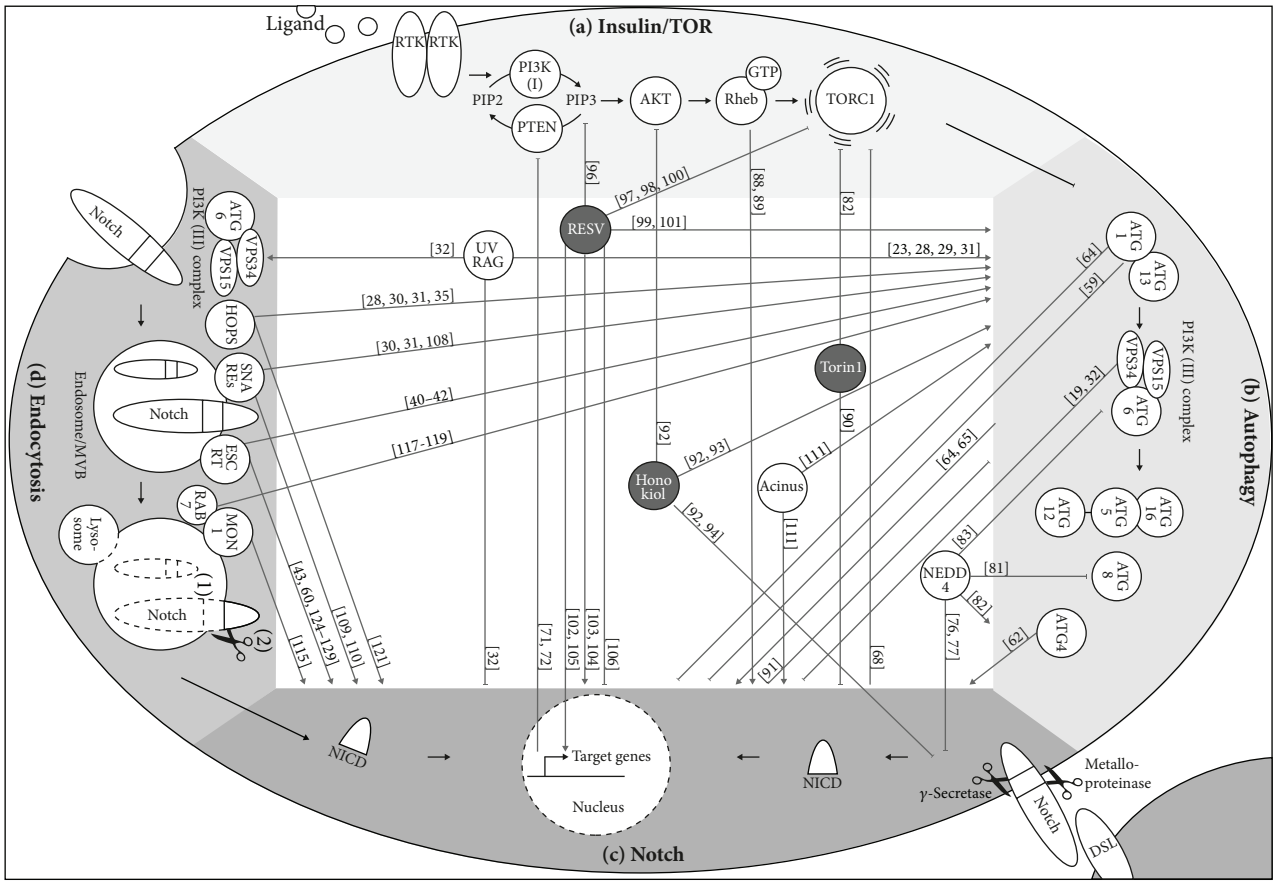


Figure 1: Network of the Insulin/TOR, autophagy, endocytosis, and Notch pathways. The Insulin/TOR pathway (a) normally inhibits the autophagic machinery (b). Notch signaling is induced by ligand (DSL) binding to the receptor and the cleaved NICD activates target gene expression (c). Alternatively, Notch can be activated ligand independently through receptor endocytosis ((d)2)), or the endocytosed receptor can be degraded to silence the signal ((d)1)). Connections of the pathways are shown between molecules directly or between molecules and the pathway in general (shaded areas). Bar-headed arrows indicate inhibition, arrows indicate activation. For simplicity, the trafficking routes are reduced to the processes and players discussed in the text. Numbers [] refer to publications cited in the text. Dark filled circles indicate drugs, whereas proteins are represented with white circles. RTK: receptor tyrosine kinase, RESV: resveratrol.

Finally, there is increasing evidence that another endosomal sorting complex is implicated in autophagy. ESCRT proteins were initially identified as major players in endosomal sorting and the formation of MVBs. Disruption of any of the 4 ESCRT complexes (0 to III) affects the fusion of late endosomes with autophagosomes, which has been studied extensively in models of neurodegenerative disorders (reviewed in [18]). Loss of function of the ESCRT machinery in *Drosophila* and mammals results in autophagosome accumulation and neurodegenerative diseases, most likely due to inhibited fusion with the endolysosomal system [40–42]. Interestingly, *Drosophila ESCRT* mutant cells show increased activation of JNK (c-Jun N-terminal kinase), a potent inducer of autophagy [43, 44]. Moreover, accumulation of autophagosomes and autolysosomes is also seen after knockdown of *ESCRT* components in mammalian cells [40, 41] (Figure 1). These findings in flies and mammals suggest that disrupting ESCRT function does not only inhibit autophagosomal-lysosomal fusion but may also induce autophagy.

ESCRTs have also been implicated in regulating other cellular functions, such as receptor signaling, cytokinesis, polarity, or migration. The sorting and degradation of components of various evolutionary conserved signaling pathways, such as EGFR, Hedgehog, or Wnt receptors, as well as Notch and its ligand Delta were shown to depend on the ESCRT machinery (reviewed in [45]).

*2.2. Endocytosis Affects Autophagy and Vice Versa.* Since the degradation of autophagosomal contents depends on lysosomes, the existence of a functional endocytotic pathway is fundamental to ensure the delivery of lysosomal hydrolases and the machinery to acidify vesicles to the lysosomal compartment. After digestion, the degradation products need to be able to exit the lysosome via specific transporters and permeases that are also provided by endocytosis [46]. Thus, endocytosis is a prerequisite for efficient autophagic flux.

On the other hand, there is increasing evidence that autophagy genes are required to ensure correct endocytic trafficking. *Drosophila Vps34* or *Atg6* (*Beclin*) mutant cells display defects in PI3P formation and fail to endocytose a fluid phase marker [19, 39]. This is in accordance with

data from other systems where *Beclin* mutants were shown to be defective in endocytosis [47, 48]. Moreover, the UVRAG-containing Atg6 (Beclin) complex has recently been implicated in endocytic degradation in mammalian cells [49]. Interestingly, fluid phase endocytosis occurs normally in *Drosophila* cells mutant for *Atg1*, the core component necessary for autophagy induction, indicating that the lack of endocytosis in *Atg6* mutant cells is not due to defects in autophagy *per se* [39]. Remarkably, deletion of *Atg1*, *Atg6,* and *Vps34* also disrupts protein secretion, illustrating another example how autophagy participates in membrane trafficking events [39].

Several recent studies highlight the interplay of autophagy and endocytosis in the degradation of internalized gap junctions (for review, see [50]). Gap junctions (GJ) plaques are removed from the plasma membrane by endocytosis to reduce cellular adhesion in situations when cells need to detach from neighboring cells. Remarkably, vesicles containing endocytosed GJ are subsequently degraded by autophagy [51–54]. This degradation pathway is induced under several physiological and pathological conditions, such as during starvation in mouse and human cell cultures [51, 54], in failing canine cardiac tissue [53] or to remove accumulations of defective gap junction proteins to prevent cataract formation [54]. These studies also show that the ubiquitin-binding autophagy adapter protein p62 targets internalized GJs for degradation via the autophagosomal route [51, 54]. Thus, autophagy is necessary to complete the endocytosis of internalized gap junctions.

Lastly, another interesting connection between autophagy and endocytosis concerns Atg14L/Barkor, a positive regulator of autophagy. Atg14L/Barkor was shown to be able to act as a switch to guide the class III PI3K complex from its endosomal localization to the sites of autophagosome formation [25]. Taken together, these findings indicate a tight interconnection of the endosomal and autophagosomal trafficking routes.

## 3. When Endocytosis and Autophagy Converge at Notch

Endosomal trafficking acts as an organizer and modulator of many signaling events between and within cells at multiple stages of animal development (for comprehensive reviews, see [55, 56]). As mentioned above, endocytotic events are necessary to ensure proper receptor signaling in various conserved cellular pathways. This was extensively studied for the Delta-Notch pathway, which is activated by the binding of a DSL ligand (Delta, Serrate, or Lag-2) from the signal-sending cell to the Notch receptor on the signal-receiving cell. In brief, this binding triggers two proteolytic cleavages, the first mediated by a metalloprotease in the Notch extracellular domain (NECD) and a second by a $\gamma$-secretase complex within the transmembrane domain, which are important for internalization of the intracellular domain of the Notch receptor (NICD) and subsequent translocation to the nucleus where the NICD activates the transcription of target genes important for several developmental steps and cell fate decisions (for a comprehensive review, see [12]; for a glance, see [57] and see Figure 1(c)). To enable this activation process as well as for silencing of the signal, endocytosis plays a pivotal role, and mutations in vesicular trafficking components, such as dynamin disrupt the Notch signaling pathway [58]. Several steps during the maturation, signaling, recycling, and degradation process of both the ligand and the receptor require vesicle trafficking and endocytosis (reviewed in [11]) (see Figure 1(d)). As autophagic and endocytic pathways are tightly connected and share many effector proteins (see Section 2), autophagy might also be an important player in Notch signaling regulation.

We have previously reported a hitherto unexpected function of autophagy in receptor activation of the Notch signaling pathway during egg development in *Drosophila*, where the loss of autophagy leads to a precocious activation of the Notch pathway in the ovarian follicle cells [59]. In fact, the retention of Notch in endosomal vesicles accelerates its intramembranous cleavage and intensifies Notch signaling [60]. Given the interplay of endocytosis and autophagy, we propose that the absence of autophagy might lead to a pause in the normally rapid endosomal processing of internalized Notch, which in turn leads to pronounced NICD cleavage and enhanced Notch activity [59].

Led by these findings, we became increasingly aware of other observations within the literature that indicate different possible intersections between the autophagic and the Notch pathway—some, but not all also related to endocytosis (Figure 1). Three possible mechanisms of interaction between both pathways could be found. In the first scenario, autophagy influences the Notch signaling pathway. Secondly, the Notch pathway affects autophagy and, finally, a "factor-X" impacts on both autophagy and Notch.

In the following sections we will give a short overview of these findings without discussing them in details, as the diversity of topics would go beyond the scope of this review. Instead, we hope to provide new aspects for further research regarding the interplay of endocytic trafficking, the autophagic, and the Notch signaling pathways.

*3.1. Influence of Autophagy on the Notch Signaling Pathway.* The Notch pathway is important during animal development, and defective signaling can result in malformed tissues such as the "notched-wing" phenotype in *Drosophila*, originating by impaired Notch signaling along the dorsoventral wing disc boundary [61]. The first direct observation of an *Atg* mutation impairing Notch signaling was made by Thumm and Kadowaki [62], who described that deletion of the cysteine protease Atg4 in *Drosophila* enhances the notched-wing phenotype induced by loss of function of components of the Notch pathway. However, the mechanism by which the lack of autophagy might influence Notch signaling remained unclear. In a more recent study the *Drosophila* ATPase Domino (Dom), a positive regulator of Notch signaling [63], was used to screen for modifiers of the Dom induced notched-wing phenotype [64]. Kwon and coworkers discovered that loss of *Atg1* restores normal development of the wing whereas overexpression of Atg1 enhances

the notched-wing phenotype [64]. Surprisingly, the downregulation of other *Atg* genes showed the opposite result and enhanced the notched-wing phenotype similarly to the results obtained for *Atg4* mutants [62], indicating a secondary function for Atg1 in addition to its general function in autophagy [64].

Dysregulated Notch signaling can also lead to wing-vein patterning defects [61], and downregulation of autophagy by Atg-RNAi in the *Drosophila* wing was shown to result in loss of vein tissue and/or ectopic vein patches [65]. In contrast, our results showed that expression of Atg1- and Atg5-RNAi in the wing did not induce any vein defects but led to slightly larger posterior wing compartments (unpublished data). However, these varying results might be due to different expression techniques and RNAi lines used, leading to variable levels of autophagic downregulation.

Notch signaling is also required during *Drosophila* oogenesis and egg chambers containing *Notch* mutant follicle cells display a wide range of developmental defects [66]. Similar defects were also found in egg chambers lacking autophagy, and mutations in *Atg1* or *Atg13* were found to induce precocious activation of the Notch pathway as shown by the expression of the Notch downstream effectors Cut and Hindsight (Hnt) [59].

Taken together, these results hint at a function for autophagy within the Notch pathway; however the underlying mechanisms are still obscure and unexplored. The fact that loss of autophagy seems to intervene in a positive as well as negative way with Notch activity renders a definite conclusion difficult. The variable effects seen might be due to secondary functions of specific *Atg* genes, specialized functions in different organs, tissues, or developmental stages. For example, Notch signaling in the *Drosophila* ovaries is activated by Delta expression in the germline within a defined time frame. Autophagy deletion affected Notch activity only during that short period of activation, most likely by regulating the processing of the endocytosed Notch receptor, which presumably can be compensated for by other degradation pathways in later stages of development [59].

*3.2. Influence of the Notch Pathway on Autophagy.* Interestingly, Notch signaling was also discovered to be important for regulating the autophagic pathway. A recent study resulted from work on the nematode *C. elegans*, a species with two *Notch* genes: *lin-12* and *glp-1* [67]. Mutants of *glp-1* were shown to exhibit decreased fat storage, defective germline proliferation, and increased autophagy levels [68–70]. Lapierre and coworkers suggest that autophagy in these mutants is induced at a transcriptional level, possibly via downregulation of TOR (Figure 1) [68]; however, so far no further investigations to confirm a link between the loss of Notch signaling activity and the upregulation of autophagy in *glp-1* mutants have been carried out.

In another study, Palomero et al. [71] investigated activating *Notch* mutations in T-cell leukemia (T-ALL) and were able to show that Notch regulates the expression of PTEN (phosphatase and tensin homolog; see Figure 1(a)) through activation of the Notch target gene *Hes1* (hairy and enhancer of split-1), which induces the activity of the class I PI3K-AKT signaling pathway in leukemic T cells, but also in normal thymocytes. Inhibition of Notch signaling within this system did indeed induce autophagy. PTEN downregulation by Notch-induced expression of Hes1 was also shown in DN3 thymocytes [72]; however, autophagy was not examined in this system. This regulatory link seems to be functionally conserved since class I PI3K-AKT activation is also necessary for Notch induced growth in *Drosophila* [71]. Despite the control via Hes1, other mechanisms for a regulation of class I PI3K through Notch seem to exist (for a review see [73]), but regulation of autophagy via the Notch—class I PI3K pathway has not been directly tested so far.

*3.3. "Factor X" Influences Both Autophagy and Notch.* Several studies identified factors that are intertwined between both pathways and either concomitantly regulate Notch and autophagy signaling or regulate one through the other by a yet unknown mechanism. One example is the E3 ubiquitin ligase Nedd4 (neural precursor cell-expressed developmentally downregulated 4) family known to regulate trafficking and endosomal degradation of multiple target substrates within different cellular environments (reviewed in [74]). It has been shown that both *Drosophila* Nedd4 ligases "suppressor of deltex" (SU (DX)) [75] and Nedd4 are capable of negatively regulating Notch mediated signaling by direct ubiquitination and subsequent degradation of Notch [76, 77]. Another E3 ubiquitin ligase, Mib1 (mindbomb homolog 1), acts on the Notch ligands Delta and Jagged to promote their endocytosis, leading to reduced Notch1 activity in mammals [78–80]. Recently, a proteomic analysis of the autophagy interaction network in human cells revealed that Nedd4 also associates with multiple Atg8 (LC3) proteins, possibly to target Atg8 for degradation [81]. In line with this, the interaction between Nedd4 and Atg8 orthologs was strongly reduced after treatment with Torin1, a TOR inhibitor [82], indicating that degradation of Atg8 by Nedd4 is decreased upon autophagy induction by inhibition of TOR. In addition, Nedd4 depletion led to an increase in autophagosome formation [81]. Furthermore, Nedd4 was also found to negatively control the stability of Beclin1, a protein important for the initiation of autophagy [83] (Figure 1). As discussed above and reviewed by Falk and coworkers [50], autophagy and endocytosis are concomitantly implicated in the degradation of internalized gap junctions. Notably, Nedd4 mediated ubiquitinylation seems to be required for the targeting of the autophagic machinery and subsequent autophagic degradation of gap junction connexins [51]. It therefore appears that shared ubiquitination proteins regulate both autophagy and Notch signaling. In this respect, it was shown that Wnt signaling is regulated by autophagy through specific degradation of its ligand dishevelled (Dvl). Ubiquitination of Dvl facilitates its binding to the autophagic receptor p62 which allows LC3-mediated autophagic degradation of Dvl to downregulate Wnt signaling [84]. A similar scenario could be envisioned for Notch and its ligand Delta, in which Delta is selectively ubiquitinated (e.g., by Mib1) and subsequently degraded by autophagy to downregulate Notch signaling.

The canonical Insulin/TOR pathway is well established as a regulator of growth and autophagic activity in various systems [85] and activation of this pathway by, for example, overexpression of the GTP-binding protein Rheb (Ras homolog enriched in brain) has been shown to activate growth and inhibit autophagy in multiple tissues in *Drosophila* [86, 87]. Surprisingly, two recent publications have indicated that the Insulin/TOR pathway might also be responsible for alterations in Notch signaling [88, 89]. Karbowniczek and coworkers found that Rheb overexpression in the asymmetrically dividing *Drosophila* external sensory organ produces a cell fate switch from neuronal development to the development of only hair and socket cells, a phenotype consistent with ectopic Notch activation [88]. Ma and coworkers [89] used mouse and human cells to also show that activated Rheb induced enhanced Notch signaling (Figure 1). TOR inhibitors were able to block Notch activation in these cells; however, this was not observed in *Drosophila* [88], hinting to a cell-type specific mechanism where Notch activation by Rheb is TOR independent. In addition, expression of Notch, but not the Notch target gene *Hes1*, was significantly reduced in mice tumor tissue treated with the TOR inhibitor Torin1 [90]. Furthermore, when the team of Ge and Ren [91] investigated the effects of alcohol intake on myocardial damage in aldehyde dehydrogenase-2 (Aldh2) overexpressing transgenic mice, they discovered that alcohol intake triggered myocardial autophagy and inhibited Notch signaling in wild type but not transgenic mice. The observation that Aldh2 seems to protect against alcohol induced inhibition of AKT, TOR, and STAT3 (signal transducer and activator of transcription 3) phosphorylation and the fact that additional inhibition of Notch signaling intensified autophagy lead the authors to the speculation that mTOR-STAT3 signaling and subsequent Notch activation inhibited autophagy induction [91] (Figure 1). These results show that autophagy and Notch signaling might—in some situations—both respond to changes in the activity of the Insulin/TOR pathway.

More evidence for a common regulatory mechanism for Notch and autophagic signaling emerges from a number of anticancer drug studies. The drug Honokiol, which was shown to increase autophagosome formation [92] and the expression of autophagic markers like Beclin1 and lipidated Atg8 [93], was also found to reduce Notch signaling, possibly by inhibition of the $\gamma$-secretase complex [92, 94]. Furthermore, Honokiol was also potent to inhibit AKT and TOR [92], suggesting again a role of Insulin/TOR signaling for the regulation of autophagy and Notch (Figure 1).

Another compound claimed to have protective effects on various diseases is resveratrol (for review, see [95]). It has been shown to negatively regulate TOR signaling by directly inhibiting class I PI3K [96] or AMPK (AMP-activated protein kinase) [97] but also TOR through the increase of its association to the inhibiting protein DEPTOR (DEP domain-containing mTOR-interacting protein) [98]. Resveratrol induced activation of autophagy via AMPK seems to depend on the cell type, as in human esophageal squamous cell carcinoma autophagy induction is independent of AMPK/TOR signaling [99] whereas in human lung carcinoma cells it is not [100]. However, the general potency of resveratrol to activate autophagy is strongly supported by various mechanisms, including Beclin-independent induction, regulation of AKT, mTOR, and AMPK activity, or though the activation of JNK or p62 pathways (for reviews, see [100, 101]). In addition, resveratrol was found in a high-throughput screen for the identification of Notch activating compounds using carcinoid cell lines [102], and treatment of thyroid carcinoma cells with resveratrol was shown to inhibit growth by arresting cell-cycle progression in the S-phase and induce Notch protein expression and signaling by transcriptional regulation [103, 104]. Furthermore, resveratrol also suppressed cellular growth and activated Notch expression in glioblastoma [105]. In contrast to most other studies, a microarray analysis to search for genes expressed in adipose tissue of obese men treated with resveratrol found genes of the Notch signaling pathway to be downregulated, whereas autophagy genes were found to be upregulated [106]. However, most studies agree on an activating effect of resveratrol on autophagy as well as on Notch signaling (Figure 1), which is probably interconnected with its growth inhibiting function that may as well depend on the cellular context.

As described above, the functionality of both autophagy and Notch signaling requires endocytic vesicle trafficking and fusion, the latter of which is often mediated by SNAREs (for a review, see [107]). SNAREs play a role in nearly all steps of autophagy, from the formation of the autophagosome, to its maturation and the autophagosome-lysosome fusion (reviewed in [37]). For example, loss of the SNARE protein Syntaxin 17 in *Drosophila* is followed by an accumulation of mature autophagosomes, indicating the requirement of Syntaxin 17 for the fusion with lysosomes [31, 108]. Interestingly, the interaction of Syntaxin 17 with the HOPS complex seems to be required for fusion of autophagosomes with lysosomes both in *Drosophila* and mammals [30, 31]. Evidence for a role of SNARE protein family members in Notch signaling was found in *Drosophila* by expressing mutant forms of Syntaxin in the developing wing, resulting in Notched wings indicative for a disruption of the Notch pathway [109]. In addition, trafficking defects were observed in mutants for Avalanche, another Syntaxin-family protein, leading to increased abundance of the Notch receptor at the cell surface and at peripheral structures [110] (Figure 1).

Another link between autophagy, endocytosis, and Notch is the *Drosophila* protein Acinus (dacn), which plays a role in endosomal trafficking of signaling receptors such as Notch and the EGF receptor. Loss of dacn inhibits autophagosome maturation into autolysosomes, thus blocking the autophagic pathway, but also destabilizes early endosomes to enhance the delivery of endocytosed receptors to lysosomes, which leads to an inhibition of Notch and EGFR signaling [111]. Thus, dacn might regulate the fusion of autophagosomes with endosomes, a step that is crucial for the maturation of autophagosomes, assigning dacn yet another role in the complex interplay between endocytosis and autophagy. The maturation of autophagosomes into autophagolysosomes presents another step in which endosomal and autophagosomal pathways may converge to modulate Notch signaling.

Reagents that elevate lysosomal pH and thus block lysosomal function (e.g., chloroquine or bafilomycin A) also inhibit autophagic degradation and are commonly used to determine autophagic activity [112]. Interestingly, the vacuolar proton pump responsible for acidification of intracellular compartments (V-ATPase) was shown to be required for Notch signaling and endocytic trafficking in *Drosophila*. Acidification of endocytic vesicles by V-ATPase enhances Notch degradation and signaling in endosomes, suggesting that the acidic endosomal environment is a prerequisite for efficient Notch activation in the signal-receiving cell [113, 114].

In Section 2.1, we describe the importance of the class III PI3K complex containing Vps34, Vps15, and Atg6/Beclin, and—depending on the function—also UVRAG or Atg14L and Ambra1, and how its members are involved in autophagy and endocytosis. Notably, *UVRAG* and *Vps34* mutant flies are both defective in autophagy and accumulate endosomal Notch [19, 32], which can lead to enhanced Notch activity [32]. However, the requirement of UVRAG in autophagy is most likely restricted to later steps of endocytic degradation, because autophagosomal-lysosomal fusion occurs normally in *UVRAG* mutant flies and *UVRAG* depleted mammalian cells [30, 31]. An accumulation of Notch in large endosomes could also be shown in flies mutant for Dmon1 (the *Drosophila* ortholog of Mon1/Sand1; [115], Figure 1), a protein essential for the exchange of Rab5 with Rab7 on maturing endosomes [116]. Rab7 and yeast Mon1 were both also found to be important for autophagy [117–119]; however, regulation of autophagosomes seems not to be affected in *Dmon1* mutant flies, nor was enhanced Notch signaling observed [115].

Rab7 also interacts with the heterohexamer membrane tethering complex HOPS to promote membrane fusion (for reviews, see [107, 120]) and HOPS is required for fusion of autophagosomes with the lysosome in *Drosophila* [35], and together with UVRAG for autophagosome maturation and endosomal fusion in humans [28]. Furthermore, *Drosophila* HOPS contributes to Notch signaling by delivering endocytosed Notch to the lysosome where internal parts are degraded, but extracellular parts can contribute to signaling [121] (Figure 1). Two recent papers demonstrate the requirement for HOPS in autophagosome-lysosome fusion [30, 31], and mutants of several subunits of the *Drosophila* HOPS complex accumulate Notch in the developing eye [31]. In the model proposed by Wilkin and coworkers [121], the NECD of the Notch receptor at the limiting membrane of late endosomes is removed by internal lysosomal proteases, whereas the NICD is released. This mechanism could also explain enhanced Notch activity seen in mutants defective for autophagy or endosomal trafficking [32, 59] where Notch could get similarly lodged due to discontinued degradation pathways.

Finally, the ESCRT complex, acting as cargo sequestering and sorting machinery for multivesicular bodies (MVBs), has well-established functions in autophagy (see Section 2 and for a review [18]), but also in the receptor downregulation of several cellular signaling events (reviewed in [122]). For silencing, transmembrane receptors like Notch are cleared from the membrane and delivered to endosomes, where they become ubiquitinated, and invaginated as MVBs before fusion with the lysosome and degradation. In ESCRT mutants, biogenesis of MVB cannot take place and receptors accumulate on enlarged endosomes (for a review on Notch activation, see [123]). Accumulation of the Notch receptor in endosomal compartments and ectopic signaling activity has been shown in cells mutant for components of the ESCRT-I, -II, or -III complex [43, 60, 124–129]. Increased Notch signaling activity in ESCRT mutant cells is presumably caused by the prolonged duration for which Notch and its ligand are trapped in endosomal compartments and are accessible for $\gamma$-secretase cleavage [43, 60, 126–128] (Figure 1); however, ligand independent activation of the pathway as described above [121] or ligand activation dependent of the ubiquitinylation status of Notch is also possible [125].

## 4. Summary

Several proteins are implicated in both autophagy and endosomal receptor sorting, and numerous intersections between the endosomal and autophagic pathways have been described. Despite the established role of endocytosis in sorting, recycling, and degradation of signaling receptors and their ligands, there is increasing evidence that autophagy is also involved in executing these events. Cells may regulate the trafficking of activated receptors to ensure adequate levels of signaling and to modulate the signal strength.

The activity of many pathways relies on correct processing of their receptors and/or ligands, as shown for EGFR and Notch, two evolutionary conserved key signaling pathways implicated in the development of higher eukaryotes. Given the fact that the dysregulation of Notch signaling contributes to tumor growth (reviewed in [13]), the described interconnections to the autophagy pathway will be of significant interest to exploit autophagic processes for cancer therapy.

By summarizing recent findings on the emerging network between autophagy, endocytosis, and the regulation of Notch signaling, we hope to establish novel starting points for further research in this area.

## List of Abbreviations

| | |
|---|---|
| Aldh2: | Aldehyde dehydrogenase-2 |
| Ambra1: | Activating molecule in Beclin1-regulated autophagy |
| AMPK: | AMP-activated protein kinase |
| ATG: | Autophagy related genes |
| DEPTOR: | DEP domain-containing mTOR-interacting protein |
| DSL: | Delta, serrate, or lag-2 |
| Dvl: | Dishevelled |
| EGFR: | Epidermal growth factor receptor |
| ESCRT: | Endosomal sorting complex required for transport |
| GJ: | Gap junctions |
| Hes1: | Hairy and enhancer of split-1 |
| Hnt: | Hindsight |
| HOPS: | Homotypic fusion and vacuolar protein sorting |

JNK: c-Jun N-terminal kinase
Mib1: Mindbomb homolog 1
MVB: Multivesicular bodies
NECD: Notch extracellular domain
Nedd4: Neural precursor cell-expressed developmentally downregulated 4
NICD: Notch intracellular domain
PIP2: Phosphatidylinositol 4,5-bisphosphate
PIP3: Phosphatidylinositol (3,4,5)-trisphosphate
PI3K: Phosphoinositide 3-kinase
PTEN: Phosphatase and tensin homolog
RESV: Resveratrol
Rheb: Ras homolog enriched in brain
RTK: Receptor tyrosine kinase
SNAREs: Soluble NSF attachment protein receptor
STAT3: Signal transducer and activator of transcription 3
SU (DX): Suppressor of deltex
Rubicon: RUN domain and cysteine-rich domain containing Beclin1-interacting protein
TOR: Target of rapamycin
UVRAG: UV-resistance associated gene
V-ATPase: Vacuolar $H^+$-ATPase
Vps: Vacuolar protein sorting.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 974393, 10 pages
http://dx.doi.org/10.1155/2014/974393

*Research Article*

# Association of CHMP4B and Autophagy with Micronuclei: Implications for Cataract Formation

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Received 11 December 2013; Accepted 29 January 2014; Published 11 March 2014

Academic Editor: Rodney J. Devenish



Autophagy is a mechanism of cellular self-degradation that is very important for cellular homeostasis and differentiation. Components of the endosomal sorting complex required for transport (ESCRT) machinery are required for endosomal sorting and also for autophagy and the completion of cytokinesis. Here we show that the ESCRT-III subunit CHMP4B not only localizes to normal cytokinetic bridges but also to chromosome bridges and micronuclei, the latter surrounded by lysosomes and autophagosomes. Moreover, CHMP4B can be co-immunoprecipitated with chromatin. Interestingly, a CHMP4B mutation associated with autosomal dominant posterior polar cataract abolishes the ability of CHMP4B to localize to micronuclei. We propose that CHMP4B, through its association with chromatin, may participate in the autophagolysosomal degradation of micronuclei and other extranuclear chromatin. This may have implications for DNA degradation during lens cell differentiation, thus potentially protecting lens cells from cataract development.

## 1. Introduction

Autophagy is an evolutionarily conserved process where the cells degrade their own cellular material. It is involved in protein and organelle degradation and plays an essential role in cellular and whole-animal homeostasis and differentiation. There are various types of autophagy such as macroautophagy, microautophagy, and chaperone-mediated autophagy (for a comprehensive review see [1]). During autophagy there is sequestration of cellular material into double-membrane vesicles called autophagosomes. The autophagosomes fuse with endocytic vesicles to form the amphisomes, which contain both endocytic and autophagic cargo. The autophagosomes and/or amphisomes are subsequently fused with the lysosomes where the sequestered cargoes are degraded by lysosomal hydrolases. The products of degradation are transported back into the cytoplasm through lysosomal membrane permeases and can be reused by the cell [1]. Autophagy serves as a cellular response in nutrient starvation but is also responsible for the removal of aggregated proteins and damaged organelles and therefore plays an important role in the quality control of proteins and organelles. Dysfunctional autophagy is implicated in ageing, neurodegeneration, infections, tumorigenesis, heart disease, liver and lung disease, myopathies, and cataract formation [2] and it is therefore important to characterize this process at the molecular level.

The endosomal sorting complex required for transport (ESCRT) machinery is required for multivesicular body (MVB) biogenesis, budding of HIV-1 and other enveloped viruses, macroautophagy, and cytokinesis [3, 4]. The ESCRT machinery consists of four complexes: ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III [3, 4]. ESCRT-III is specifically important for membrane scission events [5]. Based on electron microscopy studies, the ESCRT-III proteins CHMP4A and CHMP4B are able to assemble into filaments that curve and form circular arrays [6]. These membrane-associated

ESCRT-III polymers can delineate and generate vesicles within the lumen of MVB and participate in the membrane scission process [6]. This ability of ESCRT-III to catalyze membrane scission applies to its role in other processes as well, such as cytokinesis and viral budding. The ESCRT-III component CHMP4B has been found to play a very important role for the final step of abscission during cytokinesis [7–9].

Completion of cytokinesis by abscission depends on the complete clearance of chromatin from the intercellular bridge and can be significantly delayed by lagging or bridged chromosomes [10]. Such defects occur in about 1% of dividing somatic cells and at higher incidence in transformed cells [11, 12]. Chromosome bridges and micronuclei often occur during genotoxic events and chromosomal instability [13]. Chromosome bridges originate during anaphase, either due to defective separation of sister chromatids or due to dicentric chromosomes which are formed because of misrepair of DNA breaks and telomere end fusions [13]. Micronuclei originate during anaphase from lagging acentric chromosome or chromatid fragments which result from unrepaired or misrepaired DNA breaks [13]. Whole chromosomes that fail to be included in the daughter nuclei at the completion of telophase during mitosis can also lead to micronuclei formation [13]. Importantly, micronuclei can also arise from chromosome bridges [14]. Chromosomes in these bridges are usually prone to break into multiple fragments and often these fragments form micronuclei at the end of mitosis [14]. However, it is unclear how this process is regulated and what molecules are involved.

Cataract is a genetic disorder of the crystalline lens which leads to visual impairment [15]. In the eye lens, epithelial cells of the anterior surface of the lens differentiate into fiber cells in a process accompanied by changes in cell shape, expression of crystallines, and degradation of cellular organelles and DNA, which ensure the transparency of the lens. Degradation of DNA of lens epithelial cells during their terminal differentiation into fibre cells is not associated with cell division. DNA degradation in the lens requires DNase II-like acid DNase (DLAD), and DLAD-deficient mice are incapable of degrading DNA during lens differentiation. Since undigested DNA accumulates in the fiber cells, the DLAD deficient mice form cataract [15]. Interestingly, the gene that encodes CHMP4B protein, *CHMP4B*, is found mutated in autosomal dominant cataracts [16]. However, the molecular details of CHMP4B function during lens fiber cell differentiation and its association with cataract formation are not yet clarified.

Here, we provide evidence that CHMP4B is associated with both chromosome bridges and micronuclei and that autophagosomes and lysosomes accumulate around CHMP4B-positive micronuclei. This suggests that CHMP4B could mediate autophagolysosomal degradation of extranuclear chromatin. We also show that a cataract-associated mutation in CHMP4B abolishes its recruitment to micronuclei. This raises the possibility that impaired autophagolysosomal degradation of extranuclear chromatin could explain cataract formation in the absence of proper CHMP4B function.

## 2. Materials and Methods

*2.1. Cell Culture and Transfections.* Media and reagents for cell culture were purchased from Gibco. HeLa, Hep2, MCF7, and U2OS cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), 5 U $mL^{-1}$ penicillin, and 50 $\mu g\,mL^{-1}$ streptomycin. NIH3T3 cells were grown in Quantum 333 medium, containing 2% bovine serum and HLEB-3 cells were grown in Eagle's minimum essential medium (EMEM) containing 20% foetal bovine serum (FBS). Transfection of HeLa cells was performed as described previously [17].

*2.2. Confocal Fluorescence Microscopy.* Immunofluorescence microscopy was performed using HeLa, Hep2, MCF-7, U2OS, NIH3T3, and HLEB-3 cells as previously described [17]. Rabbit anti-human CHMP4B antibody was used in 1 : 1000 dilution and was synthesized as described before [17] and rabbit anti-human CHMP3 antibody synthesized in the same way was used in dilution 1 : 1000. Mouse anti-human Aurora B antibody, used in 1 : 200 dilution, mouse anti-human H2B antibody used in dilution 1 : 400, and mouse anti-human Lamin A antibody used in 1 : 200 dilution were purchased from Abcam. Mouse anti-human a-tubulin antibody used in dilution 1 : 1000 and mouse anti-human FLAG epitope M2 antibody used in dilution 1 : 150 were from Sigma-Aldrich, UK. Mouse anti-human Lamp1 antibody was from DSHB and used in dilution 1 : 200 and LC3 antibody was from Nanotools and used in dilution 1 : 200. The secondary antibodies used were Cy3-labelled goat anti-rabbit antibody in 1 : 500 dilution and Cy2-labelled goat anti-mouse antibody in 1 : 200 dilution and they were purchased from Jackson ImmunoResearch.

DNA was stained with Hoechst 33342 or DAPI at final concentration 1 $\mu$g/mL. For the scoring of micronuclei and nucleoplasmic bridges the following criteria were adopted from Fenech et al. [18]: (1) the diameter of the MNi should be less than one-third of the main nucleus; (2) MNi should have similar staining as the main nucleus; (3) MNi should be separated from or marginally overlap with the main nucleus and located in the cytoplasm; (4) nucleoplasmic bridges were considered to be nuclear remnants localized inside the cytokinetic bridge, with similar staining characteristics to nuclei.

*2.3. Co-Immunoprecipitation Analysis.* Rabbit antibody against CHMP4B or rabbit IgG (control) was rotated at RT (room temperature) with Protein A agarose beads for 1 h. The beads were washed two times with PBS and two times with 0.2 M triethanolamine, pH 8.2. Crosslinking was performed by rotating the beads in 0.2 M triethanolamine containing 3 mg/mL dimethyl pimelimidate at 4°C overnight. For the quenching of the unreacted beads, they were rotated with 10 mM ethanolamine, pH 8.2, at 4°C for 30 min. Then the beads were washed three times with PBS and were used for immunoprecipitation.

HeLa cells were grown confluent in 10-cm culture dishes and lysed in ice-cold lysis buffer (20 mM HEPES pH 7.2,

2 mM MgCl2, 100 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100) containing inhibitors [(*N*-ethylmaleimide, mammalian protease inhibitor mixture, phosphatase inhibitor cocktail I and II (Sigma-Aldrich))].The lysates were placed on ice and centrifuged at 10,000 g, 4°C, and the supernatant was added to the Protein A-coupled magnetic beads (Dynal, Invitrogen) which had been precoupled with rabbit antibody against CHMP4B or rabbit IgG as a control, in PBS Tween 20. Antibody coupled magnetic beads and cell lysates were gently mixed for 1 h at 4°C. The beads were then washed with lysis buffer, eluted in 4 × sample buffer plus 1 mM DTT at 95°C for 5 min. The eluted proteins were subsequently subjected to SDS-PAGE and immunoblotting as described previously [17].

*2.4. Plasmid Constructs.* All the CHMP4B-FLAG constructs were kindly provided by Phyllis I. Hanson [16].

*2.5. Quantification of the Colocalization of CHMP4B Wild Type and Mutant Constructs with Micronuclei.* For this experiment, cells were transfected with FLAG-CHMP4B full length construct wild type or FLAG-CHMP4B full length construct which contained the mutation D129V found in patients with cataract and in both cases, among the transfected cells, those that exhibit colocalization of the transfected construct with micronuclei or chromosome bridges (which were stained with Hoechst or DAPI) were quantified. In total, 651 transfected cells with FLAG-CHMP4B full length construct from 5 different experiments and 570 transfected cells with FLAG-CHMP4B-D129V full length construct from 5 different experiments were quantified. For the cells transfected with wild type FLAG-CHMP4B full length construct, in 553 cells (84.9%) there was colocalization between FLAG-CHMP4B and micronuclei or chromosome bridges, whereas for the mutant, 247 transfected cells (43.33%) showed colocalization of CHMP4B cataract mutant construct with micronuclei or chromosome bridges. The frequency of micronucleation in HeLa cells, deriving from quantification of 4506 control (untransfected) cells from 3 different experiments, was found to be 6.3%.

*2.6. Statistical Analysis.* Values are given as means and SD in all figures. The $P$ values are calculated based on $t$-test.

## 3. Results

*3.1. CHMP4B Localizes to Various Types of Intercellular Bridges in Interconnected Cells.* Previous studies have shown that CHMP4B localizes to the intercellular bridge adjacent to the midbody during cytokinesis in HeLa cells [7, 8, 17]. The availability of a sensitive and highly specific antibody against CHMP4B allowed us to perform a more detailed study of the localization of this protein by confocal microscopy. CHMP4B was found to localize to various types of intercellular bridges in interconnected HeLa cells. This occurred in a small proportion of cells (5.1 ± 2.3%) but was consistent in all the series of experiments performed ($n = 25$) (Figure 1(a)). This staining pattern was also observed in other cell types like Hep2, MCF-7, and U2Os and with similar frequency (3.2 ± 1.8% in Hep2 cells, 3.7 ± 1.9% in MCF-7 cells, and 4.2 ± 1.2% in U2Os cells) (Figures 1(b)–1(d)). Our immunofluorescence analysis showed that CHMP4B localizes to thin (Figures 1(a), 1(e), and 1(f)) or thick (Figure 1(g)) bridges and exhibits a long filamentous localization pattern which appears to interconnect the cells and to emanate from close proximity of the nucleus. Often, CHMP4B accumulates in large structures at the starting points of each bridge (Figures 1(a) and 1(g)). This staining pattern occurs independently of Aurora B staining suggesting that these intercellular bridges represent consequent events of incomplete or defective cytokinesis (Figures 1(e)–1(g)). In order to examine whether this staining pattern is exclusive for CHMP4B, we also tested the localization of other ESCRT-III components, such as CHMP3. We observed that CHMP3 also forms filamentous structures inside intercellular bridges (see Supplementary Figures 1(a) and 1(b) in Supplementary Material available online at http://dx.doi.org/10.1155/2014/974393). The above data show that ESCRT-III components CHMP4B and CHMP3, apart from their canonical staining pattern at the midbody during cytokinesis, exhibit an additional staining pattern in various types of intercellular bridges that connect dividing cells.

*3.2. CHMP4B but Not CHMP3 Associates with DNA in the Chromosome Bridges and in Micronuclei.* During our immunofluorescence studies, we noticed that DNA is sometimes trapped inside intercellular bridges and forms chromosome bridges. In order to test the possible association of CHMP4B with the chromosomal bridges, we performed staining with CHMP4B and Hoechst or DAPI and we found that CHMP4B colocalizes strongly with DNA in the chromosome bridges (Figures 2(a)–2(d)). Interestingly, we observed that CHMP4B also colocalizes with micronuclei (Figures 2(d) and 2(e)). These structures often accumulate at the entry of the bridge suggesting that they can be derived from the chromosome bridge (Figure 2(d)). Importantly depletion of CHMP4A/B resulted in increased number of micronuclei (Supplementary Figure 2). Surprisingly, we observed that the ESCRT-III component CHMP3 did not associate with chromosome bridges or micronuclei (Supplementary Figures 1(c) and 1(d)). The above data show that the ESCRT-III component CHMP4B but not CHMP3 colocalizes with DNA found in chromosomal bridges and micronuclei.

*3.3. CHMP4B Co-Immunoprecipitates with Histone Protein H2B and the Inner Nuclear Membrane Protein Lamin A.* In order to further investigate the association of CHMP4B with DNA, we performed double stainings with CHMP4B and various chromatin-associated proteins, such as Histone 2B and Lamin A. We observed as expected that CHMP4B colocalized with Histone 2B and Lamin A at the micronuclei (Figures 3(a) and 3(b)), even though in some cases it did not seem to colocalize with Lamin A (Figure 3(c)). CHMP4B also strongly accumulated at Lamin A positive chromosome bridges (Figure 3(d)).

To investigate whether there is a physical association between CHMP4B and chromatin, we performed immunoprecipitation experiments between CHMP4B and Histone

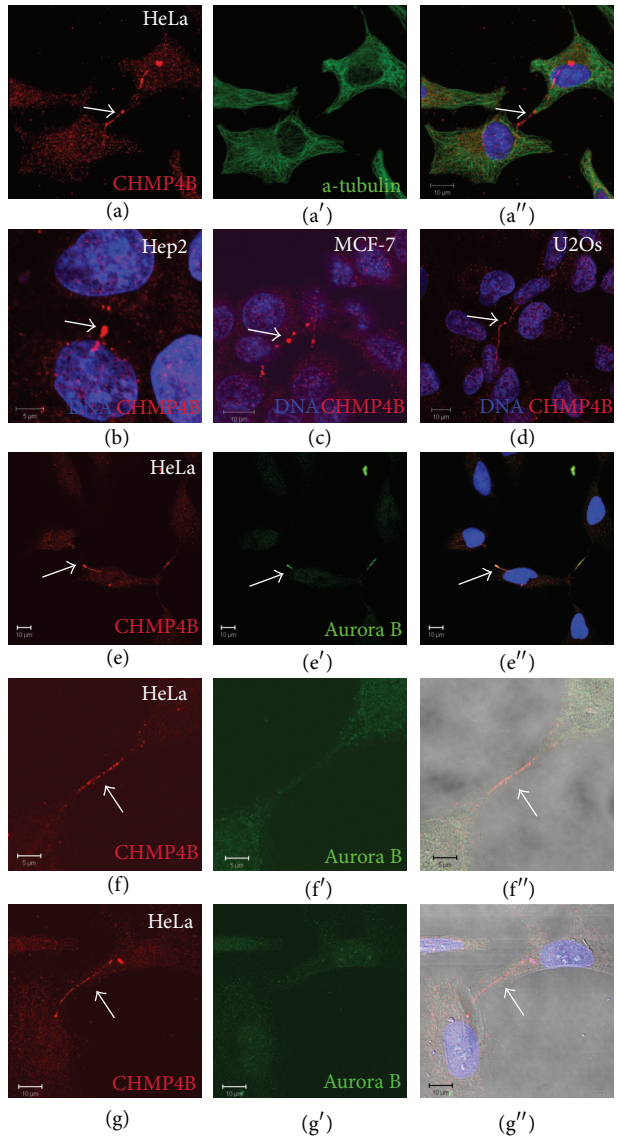


Figure 1: CHMP4B localizes to various types of intercellular bridges in interconnected cells. (a) Confocal micrographs of HeLa cells stained with CHMP4B, a-tubulin, and Hoechst. CHMP4B localizes to the intercellular bridge that links the cells (arrow). Scale bars: 10 $\mu$m. (b)–(d) Confocal micrographs of Hep2, MCF-7 and U2Os cells, stained with CHMP4B and Hoechst. CHMP4B localizes to the bridge between the cells in all the above cell lines (arrows). Scale bars: 10 $\mu$m. (e) Confocal micrographs of HeLa cells stained with CHMP4B, Aurora B and Hoechst. CHMP4B localizes to the intercellular bridge during cytokinesis (arrows). Scale bars: 10 $\mu$m. (f)-(g) Confocal micrographs of HeLa cells stained with CHMP4B, Aurora B and Hoechst. CHMP4B localizes to thin (scale bars: 5 $\mu$m) (f) and thick (scale bars: 10 $\mu$m) (g) bridges (arrows) independently of Aurora B localization.

2B or Lamin A. These experiments clearly showed that CHMP4B associates physically with Histone 2B and Lamin A (Figure 3(e)). Taken together the above results show that CHMP4B strongly associates with chromatin.

*3.4. Lamp1 and LC3 Localize Adjacent to Micronuclei.* CHMP4B was found mutated in autosomal dominant cataracts [16]. Cataract formation is associated with defective degradation of cellular organelles and DNA in the epithelial cells of the eye lens [15]. Since DNA degradation in the epithelial cells of eye lens was suggested to be mediated by the lysosomal machinery [19], we tested whether CHMP4B positive micronuclei are associated with lysosomal and autophagic markers. For this purpose, we co-stained HeLa cells with CHMP4B and the late endosomal/lysosomal marker Lamp1 (Figure 4(a)) as well as the autophagic marker LC3 (Figure 4(b)). We observed that both markers localize adjacent to CHMP4B positive micronuclei, suggesting that CHMP4B positive micronuclei may be degraded via the lysosomal machinery in HeLa cells (Figures 4(a) and 4(b)). Since this scenario fits with the proposed mechanism of DNA degradation during the differentiation of lens cells in the eye which protects them from the formation of cataract, we stained human epithelial lens cells HLE B-3 with CHMP4B to

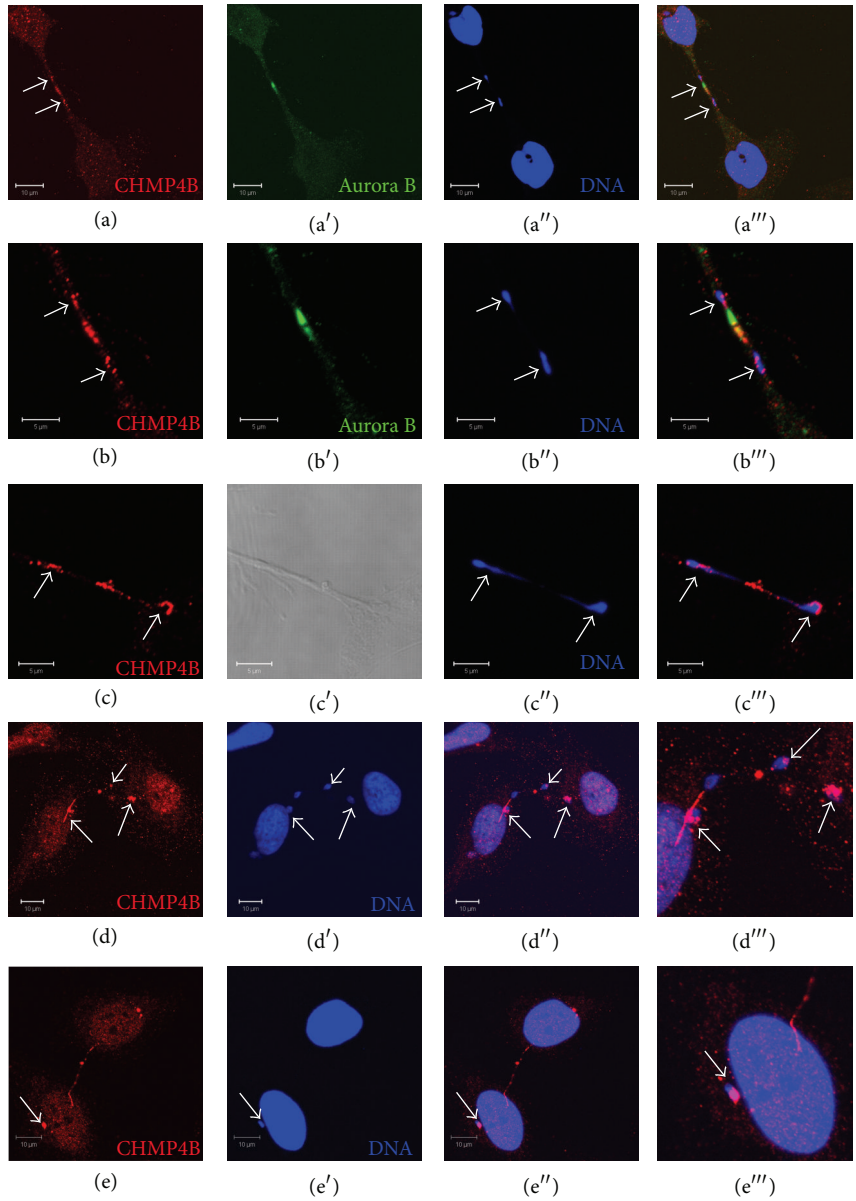


Figure 2: CHMP4B localizes to chromosome bridges and micronuclei. (a), (b) Confocal micrographs of HeLa cells stained with CHMP4B, Aurora B, and Hoechst. CHMP4B associates with chromosome bridges (arrows) (scale bars: 10 $\mu$m) (a) as also presented in higher magnification (arrows) (scale bars: 10 $\mu$m) (b). (c), (d) Confocal micrographs of HeLa cells, stained with CHMP4B and Hoechst. CHMP4B attaches to chromosome bridges (arrows) (scale bars: 5 $\mu$m) (c) and colocalizes with the DNA present in the chromosome bridge (arrows) (scale bars: 10 $\mu$m) (d). (e) Confocal micrographs of HeLa cells stained with CHMP4B and Hoechst. CHMP4B localizes to the micronucleus (arrow). Scale bars: 10 $\mu$m.

test its localization. We found that CHMP4B indeed localizes to micronuclei as well as to intercellular bridges in lens cells (Figures 4(c) and 4(d)). Additionally, the lysosomal/autophagic markers Lamp1 and LC3 were observed to localize adjacent to micronuclei in human epithelial lens cells HLE B-3, further supporting our findings in HeLa cells (Figures 4(e) and 4(f)).

*3.5. A CHMP4B Mutation Found in Cataract Abolishes Its Localization to Chromosome Bridges and Micronuclei.* In order to further investigate the functional role of CHMP4B in cataract formation, we asked whether there is any difference in the ability of association with chromatin between the wild type and cataract mutant CHMP4B forms. For this purpose, we transfected HeLa cells with FLAG-CHMP4B full length construct and measured the colocalization of FLAG-CHMP4B with DNA chromosome bridges and micronuclei (Figures 5(a), 5(b), and 5(e)). We repeated the experiment with a FLAG-CHMP4B full length construct which contained the mutation D129V found in patients with cataract

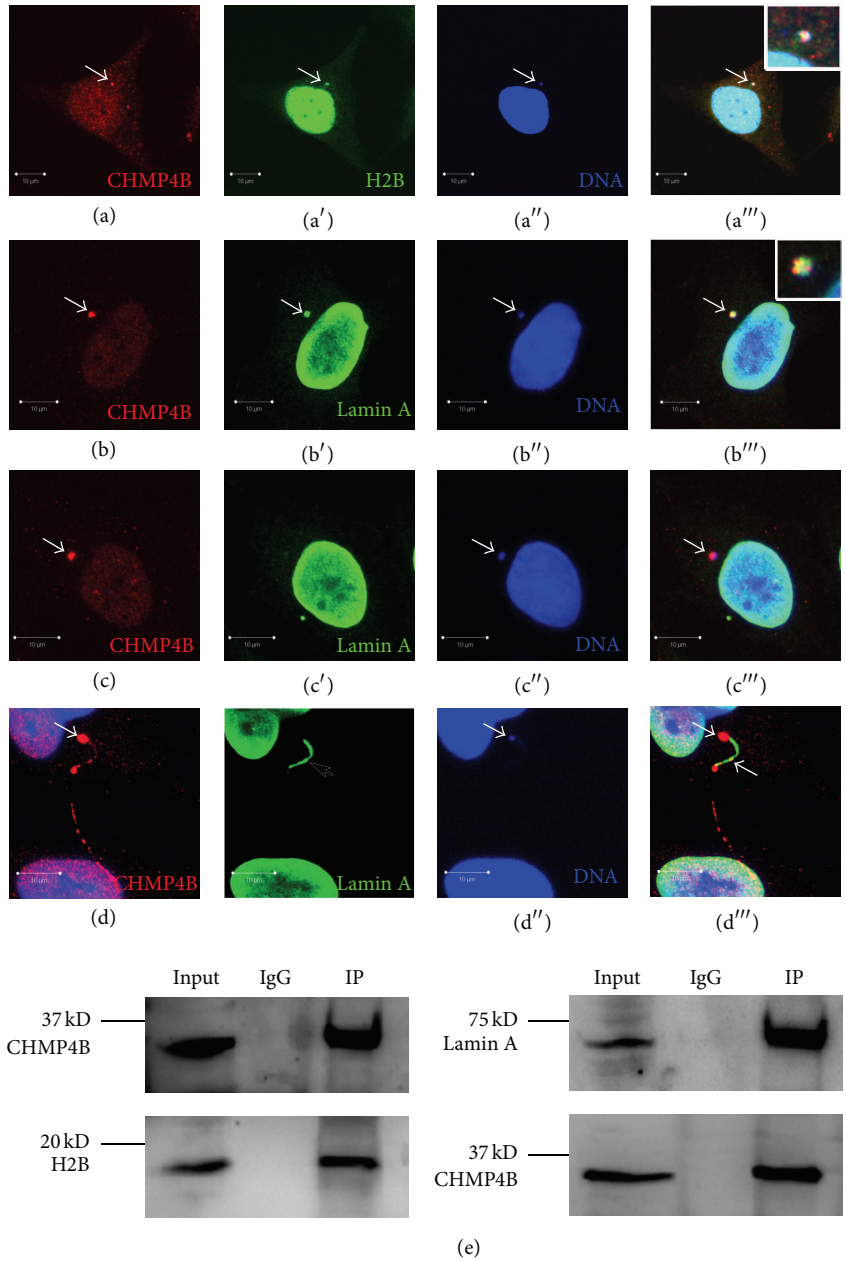


Figure 3: CHMP4B co-mmunoprecipitates with Histone 2B and Lamin A. (a) Confocal micrographs of HeLa cells stained with CHMP4B, Histone 2B, and DAPI. CHMP4B colocalizes with Histone 2B in the micronucleus (arrow). Magnification of the micronucleus is shown in the inset. Scale bars: 10 $\mu$m. (b)–(d) Confocal micrographs of HeLa cells stained with CHMP4B, Lamin A, and DAPI. CHMP4B colocalizes in some cases with Lamin A in the micronucleus (arrow) (b) and in some cases does not colocalize with Lamin A (arrow) (c) and is localizing to the chromosome bridge stained by Lamin A (arrows) (d). Scale bars: 10 $\mu$m. (e) HeLa cell lysates were subjected to immunoprecipitation (IP) with an antibody against CHMP4B. Immunoprecipitated proteins were detected by Western blotting, using anti-H2B, anti-Lamin A, and anti-CHMP4B antibodies.

(Figures 5(c), 5(d), and 5(e)). We found that in average of five separate experiments, 84.9% of wild-type FLAG-CHMP4B transfected cells showed strong colocalization between the transfected protein and chromosome bridges or micronuclei, whereas with the mutant construct the percentage was significantly reduced to 43.3% (Figure 5(e)). These results suggest that CHMP4B mutant protein found in cataract has a defective association with chromatin.

## 4. Discussion

The ESCRT-III subunit CHMP4B plays a crucial role in the final abscission step during cytokinesis by participating in the formation of helical filaments that support the constriction of the intercellular bridge and the final abscission [7–9]. Here, we demonstrate a novel localization pattern of CHMP4B to chromosome bridges and micronuclei in various cell lines.

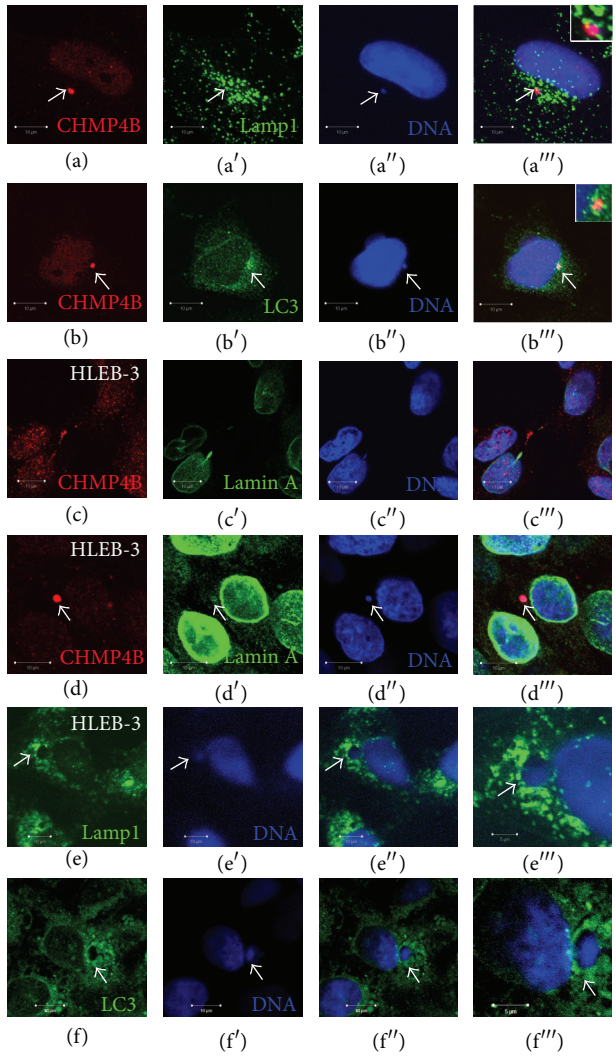


Figure 4: Lamp1 and LC3 localize adjacent to micronuclei in HeLa and HLEB-3 cells. (a) Confocal micrographs of HeLa cells stained with CHMP4B, Lamp1, and DAPI. Lamp1 positive lysosomes localize adjacent to CHMP4B positive micronucleus (arrow). Scale bars: 10 $\mu$m. Magnification of the micronucleus stained with CHMP4B, Lamp1, and DAPI is shown in the inset. (b) Confocal micrographs of HeLa cells stained with CHMP4B, LC3, and DAPI. CHMP4B positive micronucleus is adjacent to LC3 (arrow). Scale bars: 10 $\mu$m. Magnification of the micronuclei stained with CHMP4B, DAPI, and LC3 is shown in the inset. (c)-(d) Confocal micrographs of HLEB-3 cells stained with CHMP4B, Lamin A, and DAPI. CHMP4B is present on the intercellular bridge (c) and the micronucleus (d). Scale bars: 10 $\mu$m. (e)-(f) Confocal micrographs of HLEB-3 cells stained with Lamp1, LC3, and DAPI. Lamp1 positive and LC3 positive structures localize adjacent to the micronuclei (arrows). Scale bars: 10 $\mu$m.

This localization, together with our finding that lysosomes and autophagosomes accumulate around micronuclei, suggests the possibility that CHMP4B might mediate lysosomal degradation of extranuclear chromatin.

Micronuclei were shown to arise from chromosome bridges in cancer cell lines [14]. CHMP4B is the first nonnuclear protein to localize to both structures and thus connects failure of cytokinesis with micronuclei. The role of CHMP4B during this process will have to be addressed in detail in future studies, but it is interesting that ESCRT-III has previously been implicated in degradation of intracellular protein aggregates [20] suggesting a related mechanism for chromatin degradation.

Importantly, localization of CHMP4B to micronuclei was also observed in the HLEB-3 human epithelial lens cell line. The gene that encodes CHMP4B protein is found mutated in autosomal dominant cataract [16], a disease with unknown molecular mechanism, even though it is known that it is linked with unsuccessful degradation of cellular organelles and chromosomal DNA during lens cell differentiation from epithelial to fiber cells [15, 21]. Based on studies with mouse models, DNase II-like acid DNase (DLAD) has been shown to be responsible for the degradation of chromosomal DNA in the lens [19]. DLAD has been found to colocalize with the lysosomal marker Lamp1 [19], suggesting the possibility that degradation of DNA could occur via lysosomal degradation.

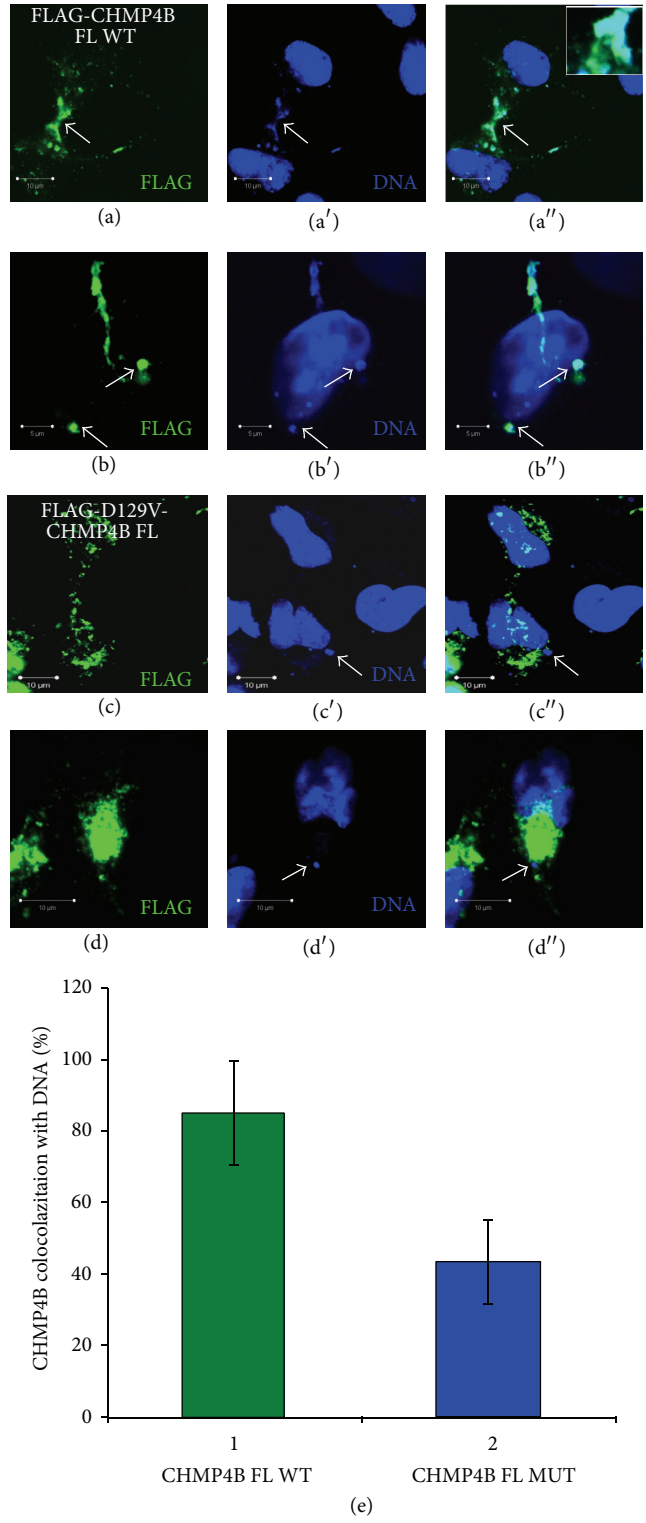


Figure 5: CHMP4B-mutant found in cataract shows reduced localization to chromosome bridges and micronuclei. (a)-(b) Confocal micrographs of HeLa cells transfected with FLAG-CHMP4B full length and stained with FLAG epitope M2 antibody and Hoechst. FLAG-CHMP4B full length associates strongly with chromosome bridges and micronuclei (a)-(b) (arrows). Scale bars: 10 $\mu$m and 5 $\mu$m. (c)-(d) Confocal micrographs of HeLa cells transfected with FLAG-D129V-CHMP4B full length and stained with FLAG epitope M2 antibody and Hoechst. FLAG-D129V-CHMP4B full length does not associate strongly with chromosome bridges and micronuclei (arrows). Scale bars: 10 $\mu$m. (e) Graphic presentation of quantification of %CHMP4B colocalization with DNA in transfected cells with FLAG-CHMP4B full length wild type versus FLAG-CHMP4B-D129V full length mutant. Error bars show mean ± SD. FLAG-CHMP4B full length: 5 independent experiments, $n = 651$ cells. FLAG-CHMP4B-D129V full length: 5 independent experiments, $n = 570$ cells. $P$ value for full length FLAG-constructs: 0.048. The $P$ values were derived from comparing means by independent samples $t$-test (SPSS, v.16.0).

Here we found that the lysosomal and autophagic markers Lamp1 and LC3 localize around CHMP4B positive micronuclei in HeLa and HLEB-3 cells, suggesting that micronuclei may be digested via lysosomal degradation.

Interestingly, the mutation in CHMP4B D129V found in cataract patients was shown to abolish its localization to micronuclei compared to the wild-type protein. This suggests that CHMP4B may have a role in mediating the degradation of micronuclei. We speculate that CHMP4B may facilitate the recruitment of lysosomes to micronuclei or the fusion of lysosomes with the micronuclear membrane. Since degradation of nuclei during lens cells differentiation has been associated with the lysosomal machinery [15, 19, 21, 22] we propose that CHMP4B participates in the lysosomal degradation of chromosomal DNA during lens cell differentiation, thus protecting from the formation of cataract. Organelle degradation during lens differentiation occurs independently of the canonical autophagy machinery, since it has been found to occur normally in ATG5 and PIK3C3/VPS34 deficient mice [23, 24]. In our experiments we have observed that autophagosomes localize adjacent to micronuclei. Autophagy may facilitate the degradation of small parts of the micronuclei. This observation is in agreement with the results reported by Nakahara et al. who showed that expression of the autophagy-related *atg3* and *atg4b* genes was significantly upregulated during fiber lens cells differentiation in mice [19]. Furthermore, it was recently shown that human lens expresses the full complement of genes required to carry out autophagy [25] and that these genes are expressed in both adult human lens epithelial cells and differentiating fiber cells. Additionally, ATG5-independent and PIK3C3/VPS34-independent autophagy have been reported to function in the autophagic elimination of organelles during erythrocyte differentiation and neuronal development [26, 27] raising the possibility that alternative non-canonical autophagy may participate also during lens differentiation. It has been also shown that mutations in the autophagy gene FYCO1 (FYVE and coiled coil domain containing 1) cause autosomal recessive congenital human cataract [28] and that autophagy and mitophagy have a role in ocular lens organelle degradation [28, 29]. Finally Rello-Varona and colleagues recently reported that micronuclei can be subjected to autophagic degradation [30]. These reports together with our present data suggest that autophagy is important for human lens cells differentiation.

Defects in cytokinesis may also cause cataract. There are reports showing that cell cycle arrest by kynurenine and expression of phosphorylation-compromised vimentin may play a role in cataract formation [31, 32]. Thus, given the role of CHMP4B in cytokinesis, we cannot exclude the possibility that defective CHMP4B function in lens cells might cause cataract due to cytokinesis failure.

In conclusion, in the present study we demonstrate that CHMP4B is a novel common component of chromosome bridges and micronuclei. We propose that CHMP4B may participate in the autophagolysosomal degradation of micronuclei, and this may have implications in DNA degradation during lens cell differentiation and cataract formation. Future studies will hopefully shed more light on the molecular details of these processes.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Authors' Contribution

Antonia P. Sagona, Ioannis P. Nezis, and Harald Stenmark conceived and designed the experiments. Antonia P. Sagona and Ioannis P. Nezis performed the experiments. Antonia P. Sagona and Ioannis P. Nezis analyzed the data. Antonia P. Sagona, Ioannis P. Nezis, and Harald Stenmark wrote the paper.

## Acknowledgments

The authors thank Alan Shiels and Phyllis I. Hanson for the CHMP4B constructs. Antonia P. Sagona was supported by the Centre for Cancer Biomedicine and by FUGE Program of the Research Council of Norway. Ioannis P. Nezis was supported by the European Research Council. This work was supported by an Advanced Grant from the European Research Council (to Harald Stenmark). This work was partly supported by the Research Council of Norway through its Centres of Excellence funding scheme, Project no. 179571.

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