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Review Article

IP_3 Receptors, Mitochondria, and Ca^{2+} Signaling: Implications for Aging

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The tight interplay between endoplasmic-reticulum-(ER-) and mitochondria-mediated Ca^{2+} signaling is a key determinant of cellular health and cellular fate through the control of apoptosis and autophagy. Proteins that prevent or promote apoptosis and autophagy can affect intracellular Ca^{2+} dynamics and homeostasis through binding and modulation of the intracellular Ca^{2+}-release and Ca^{2+}-uptake mechanisms. During aging, oxidative stress becomes an additional factor that affects ER and mitochondrial function and thus their role in Ca^{2+} signaling. Importantly, mitochondrial dysfunction and sustained mitochondrial damage are likely to underlie part of the aging process. In this paper, we will discuss the different mechanisms that control intracellular Ca^{2+} signaling with respect to apoptosis and autophagy and review how these processes are affected during aging through accumulation of reactive oxygen species.

1. Intracellular Ca^{2+} Signaling

Intracellular Ca^{2+} signaling is important in the regulation of multiple cellular processes, including development, proliferation, secretion, gene activation, and cell death. The formation of these Ca^{2+} signals is dependent on many cellular Ca^{2+}-binding and Ca^{2+}-transporting proteins, present in the various cell compartments of which the endoplasmic reticulum (ER) forms the main intracellular Ca^{2+} store [1]. The resting cytosolic $[Ca^{2+}]$ remains very low (~100 nM), through active extrusion of Ca^{2+} by pumps in the plasma membrane or in intracellular organelles, like the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump in the ER. Due to SERCA activity and intraluminal Ca^{2+}-binding proteins, the ER can accumulate Ca^{2+} in more than thousandfold excess compared to the cytosol [1, 2]. In the ER lumen, Ca^{2+} functions as an important cofactor for ER chaperones, thereby aiding in the proper folding of newly synthesized proteins [3]. Reciprocally, the Ca^{2+}-binding chaperones affect the Ca^{2+} capacity of the ER by buffering Ca^{2+} [2]. In addition, two tetrameric ER Ca^{2+}-release channels exist that, upon stimulation, release Ca^{2+} into the cytosol, thereby provoking Ca^{2+} signaling: the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) and the ryanodine receptor (RyR). They are similar in function and structure but differ in regulation, conductance, and expression profile [4, 5]. The rise in cytosolic $[Ca^{2+}]$ following its release from the ER results in various Ca^{2+}-dependent intracellular events. The exact cellular outcome depends on the spatiotemporal characteristics of the generated Ca^{2+} signal [6]. Since close contact sites between the ER and the mitochondria, involving direct molecular links with the IP_3R, exist (Figure 1), it is clear that ER-originating Ca^{2+} signals critically affect the mitochondrial function.

During aging, ER Ca^{2+} homeostasis alters and becomes dysregulated [7]. Most observations support a decline in ER $[Ca^{2+}]$ and in ER Ca^{2+} release (due to lower activity of SERCA, IP_3R, and RyR), but contradictory findings have been published, possibly related to the cell type under investigation (Figure 2). In addition, ER Ca^{2+} release and

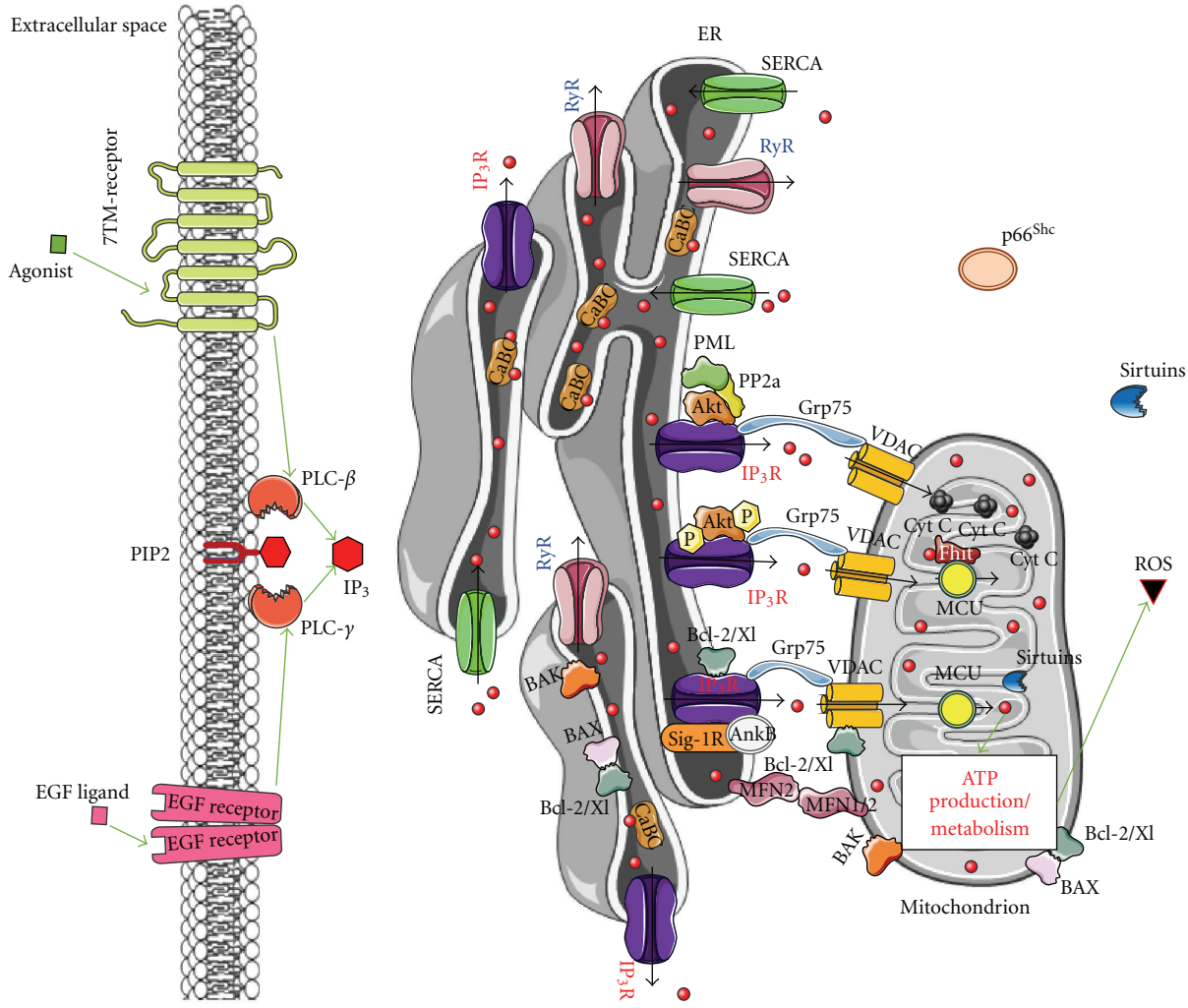

Figure 1: In a healthy cell, ER Ca^{2+}-handling components tightly regulate mitochondrial function and bioenergetics, representing the different key players involved in intracellular Ca^{2+} signalling with particular emphasis on the ER-mitochondria connections. The ER-Ca^{2+} content is regulated by channels and pumps (IP_3Rs, RyRs, SERCAs) and by Ca^{2+}-binding chaperones (CaBCs). IP_3 stimulates ER Ca^{2+} release and consequently the transfer of Ca^{2+} (red dots) from ER to mitochondria. Mitochondrial Ca^{2+}, transported via VDAC, is directly or indirectly involved in cellular energy metabolism and in the secondary production of reactive oxygen species (ROS). It is clear that IP_3R-mediated Ca^{2+} release ought to be tightly regulated to sustain mitochondrial activity and function. As a consequence, Ca^{2+}-flux properties of IP_3Rs are tightly and dynamically regulated by accessory proteins involved in cell death and survival, like Bcl-2, Bcl-Xl, PKB/Akt, Sigma-1 receptor (Sig-1R)/Ankyrin B (AnkB), and the recently identified PML. It is important to note that different regulatory mechanisms occur at the IP_3R, which may help cell survival (like Bcl-2, Bcl-Xl, PKB/Akt) or help to promote cell death (like PML). The latter is essential to prevent the survival of altered, damaged, or oncogenic cells. Thus, a tight balance between both outcomes is a requisite for cellular health and homeostasis, and a dynamic switch from prosurvival to prodeath is likely essential. In this paradigm, the production of ROS might contribute to the survival of cells by efficient detection of damaged/altered mitochondria and their removal by autophagy, while preventing excessive apoptosis. In addition, controlled apoptosis is likely to be important to eliminate cells, in which the removal of altered mitocondria by autophagy is not sufficient, thereby avoiding tumor genesis. In this process, the recently identified tumor suppressor PML may play a crucial role as it promotes IP_3R-mediated Ca^{2+} transfer from the ER into the mitochondria by dephosphorylating and suppressing PKB/Akt activity through PP2A. While PKB/Akt is known to suppress IP_3R-channel activity by phosphorylation of the IP_3R, the recruitment of PP2A via PML at the interorganellar ER/mitochondrial complex dephosphorylates and inactivates PKB/Akt. This suppresses PKB-dependent phosphorylation of IP_3R and thus promotes Ca^{2+} release through this channel and Ca^{2+} transfer into the mitochondria. At the mitochondrial level, the tumor suppressor Fhit has been shown to increase the affinity for the mitochondrial Ca^{2+} uniporter (MCU), thereby enhancing the uptake of mitochondrial Ca^{2+} at low and physiologically relevant levels of agonist-induced Ca^{2+} signals. Green arrows: stimulation; red lines: inhibition; black arrows: Ca^{2+} flux.

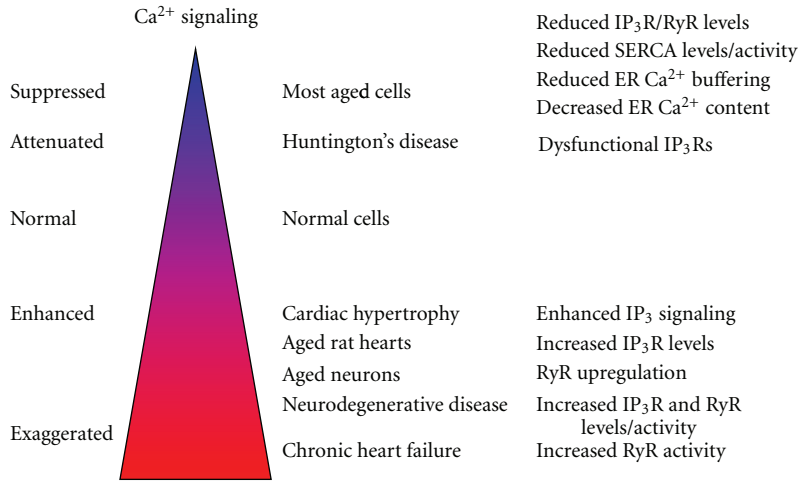

Figure 2: Altered Ca^{2+} signaling during aging and in age-related diseases. The Ca^{2+} dyshomeostasis during age is dependent on the cell type and the context. Most aged cells display decreased ER Ca^{2+} content and release, due to declined IP_3R or RyR levels, reduced SERCA activity, and decreased Ca^{2+} buffering by intraluminal Ca^{2+}-binding chaperones. However, in neurons and rat hearts, an enhanced Ca^{2+} signaling is found, caused by increasing IP_3R or RyR activity. Age-related diseases (neurodegeneration, cardiac hypertrophy, and chronic heart failure) are also characterized by enhanced Ca^{2+} signaling. However, this property may be disease dependent, since a mouse model for Huntington's disease displayed attenuated IP_3R1 activity due to impaired binding of Grp78 to IP_3R1. Hence, caution should be taken with general claims.

subsequent Ca^{2+} uptake by mitochondria regulate reactive oxygen species (ROS) production, autophagy, and cell death, processes implicated in aging.

In a previous review [8], we have focused on mechanisms regulating the Ca^{2+} content in the ER and its relevance for the development of physiological versus pathophysiological Ca^{2+} signalling. In the present review, we will focus on the subsequent step which is the mechanisms responsible for controlling Ca^{2+} transfer from the ER to the mitochondria. The Ca^{2+} level in the mitochondrial matrix plays an important role in the progression of apoptosis and autophagy [9, 10]. Here, we will especially analyze how the Ca^{2+} transfer to the mitochondria as well as apoptosis and autophagy are affected by the aging process in general and by reactive oxygen species in particular.

2. Mitochondrial Ca^{2+} Handling

In contrast with the role of the ER, the role of the mitochondria in physiological Ca^{2+} handling was underestimated or even ignored for a long time, but due to the seminal work of Rizzuto and his colleagues [11], this role is now generally accepted.

The electrochemical gradient ($\Delta\psi_m = -180\,\text{mV}$) between the inside and outside of energized mitochondria forms the driving force for the Ca^{2+} uptake in the mitochondrial matrix, which implies the transfer of Ca^{2+} ions over both the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM).

The Ca^{2+} ions taken up into the mitochondrial matrix stimulate the mitochondrial ATP production by regulating the activities of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and pyruvate dehydrogenase, three dehydrogenases of the Krebs cycle [12, 13]. Also other mitochondrial processes as fatty acid oxidation, amino acid catabolism, aspartate and glutamate carriers, the adenine-nucleotide translocase, Mn-superoxide dismutase, and F1-ATPase activity, are regulated by mitochondrial Ca^{2+} [12, 14, 15].

The ATP produced by the mitochondria is subsequently transferred to the cytoplasm; it will so especially regulate the activity of ATP-sensitive proteins localized in the close vicinity of the mitochondria. Two major proteins involved in Ca^{2+} transport, the SERCA, responsible for loading the ER, and the IP_3Rs, responsible for Ca^{2+} release from the ER, are stimulated by ATP. The bidirectional relation between Ca^{2+} release and ATP production allows for a positive feedback regulation between ER and mitochondria during increased energetic demand [16].

The uptake of Ca^{2+} in the mitochondria will also affect Ca^{2+} signaling. The local Ca^{2+} concentration near the mitochondria will depend on both the amount of Ca^{2+} released by the IP_3R and that taken up by the mitochondria. This will in turn depend on the efficiency of the coupling between both. Since both the SERCA pumps and the IP_3Rs are also regulated by Ca^{2+}, the local Ca^{2+} concentration in the vicinity of the mitochondria will determine the refilling of the ER and eventually the spatiotemporal characteristics of the subsequent Ca^{2+} signals. The way in which the Ca^{2+} signals are affected depends on the exact subcellular localization of the mitochondria, the production of ROS, the local Ca^{2+} concentration, the IP_3R isoform expressed, and may as well involve stimulation as inhibition of the signals [16–19]. Furthermore, the connection between mitochondria and the ER can be highly dynamic as the local Ca^{2+} concentration can also affect mitochondrial motility and ER-mitochondria associations in various ways [20].

3. Transport Proteins Involved in the Transfer of Ca^{2+} between ER and Mitochondria

3.1. IP_3Rs. The first key player is the IP_3R, the main Ca^{2+}-release channel in the ER of most cell types. The IP_3R consists of 4 subunits of about 310 kDa each (i.e., about 2700 a.a.). In mammals, three different IP_3R isoforms are expressed (IP_3R1, IP_3R2, and IP_3R3) while diversity is increased by splicing and the formation of both homo- and heteromeric channels [4, 21, 22]. All IP_3R isoforms are activated by IP_3, though with varying affinity [23]. Low Ca^{2+} concentrations stimulate but high Ca^{2+} concentrations inhibit the IP_3Rs [24–27]. Further modulation of the IP_3Rs is performed by ATP, phosphorylation, and protein-protein interactions [4, 28–30].

For efficient Ca^{2+} transfer between ER and mitochondria, it is important that IP_3Rs are localized very close to the mitochondrial Ca^{2+}-uptake sites. As different IP_3R isoforms exist, an important point is whether interaction with the mitochondria is isoform specific [31]. In CHO cells, IP_3R3 is the least expressed isoform, but it demonstrated the highest degree of colocalization with the mitochondria and consequently its silencing had the most profound effects on mitochondrial Ca^{2+} signals [32]. However, this does not represent a general rule as, for example, in astrocytes IP_3R2 was found to preferentially colocalize within the mitochondria [33]. These differences in intracellular localization of the IP_3R isoforms may be due to differences in relative expression levels of the various IP_3R isoforms and in subcellular localization among different cell types [34]. Moreover, the physiological setting [35] and the differentiation status [36] determine the subcellular localization of the various IP_3R isoforms in a given cell type.

3.2. Voltage-Dependent Anion Channels: The Main Ca^{2+}-Transport System across the OMM. The Ca^{2+} fluxes through the OMM are mainly determined by voltage-dependent anion channels (VDAC). Of the 3 existing VDAC isoforms, VDAC1 is the most abundant in most cell types [37]. It was demonstrated that the transient overexpression of VDAC in various cell types led to an increased Ca^{2+} concentration in the mitochondria, leading to a higher susceptibility for ceramide-induced cell death [38].

VDAC, however, allows also the transport of other ions and metabolites, including ATP. It has therefore multiple functions in the cell and is a central player in the crosstalk between the cytoplasm and mitochondria. In this manner, VDAC is also implicated in the induction of apoptosis by various stimuli [15].

The permeabilization of the OMM is a crucial step in apoptosis, but how this is exactly performed is not yet clear. Proteins belonging to the B-cell CLL/lymphoma-2 (Bcl-2)-protein family appear anyway to be necessary [39, 40]. Several Bcl-2-family members can affect the permeability of the OMM, for example, by binding to VDAC and regulating its properties or by forming multimeric channel complexes. Independently of the mechanism by which the increase in permeability of the OMM is achieved, it allows the release of the apoptogenic factors present in the intermembrane space to the cytoplasm and the progression of apoptosis [15, 40–42].

3.3. Ca^{2+}-Transport Systems across the IMM. In contrast to the Ca^{2+}-transport system across the OMM, that of the IMM is not yet well characterized. For a long time, the main IMM Ca^{2+}-transport system was named the mitochondrial Ca^{2+} uniporter. Additionally, a so-called rapid mode of mitochondrial Ca^{2+} uptake was described, but the nature of neither was known [43].

Three different highly Ca^{2+}-selective channels that may contribute to this process were meanwhile characterized, that is, MiCa [44], mCa1, and mCa2 [45]. Two of these channels, MiCa and mCa1, have properties compatible with the former uniporter and may represent species- and/or cell-type-dependent variability [43]. At the molecular level, the mitochondrial Ca^{2+}-uptake channels are not yet identified, but evidence for a role of a number of proteins has been presented [46, 47]. Recently, a Ca^{2+}-binding protein, named MICU1, which appears essential for mitochondrial Ca^{2+} uptake, was described [48]. It is, however, not known whether it actually forms (part of) a Ca^{2+} channel or functions as Ca^{2+} buffer or Ca^{2+} sensor. Interestingly, the tumor suppressor protein Fhit (fragile histidine triad) seems to promote mitochondrial Ca^{2+} uptake by increasing the affinity of the mitochondrial Ca^{2+} uniporter at the ER/mitochondrial microdomain [49].

Finally, the permeabilization transition pore (PTP) is another channel of still unknown nature [50]. It is voltage and Ca^{2+} dependent and is sensitive to cyclosporine A. It is not selective for Ca^{2+} as the open conformation of the PTP has a high conductance for all ions, including Ca^{2+}, and for molecules up to 1500 Da [51]. Its long-time activation leads to the demise of the cell, either by apoptosis or else by necrosis, depending on whether PTP opening occurs in only a small part of the mitochondria or in all of them, respectively [51, 52].

In addition, Ca^{2+}/Na^+ and Ca^{2+}/H^+ exchangers are also present in the IMM. Their main function is probably to export Ca^{2+} from the matrix, but they may also contribute to Ca^{2+} uptake under certain conditions [43].

4. Structural and Regulatory Proteins Involved in the Control of Ca^{2+} Transfer between ER and Mitochondria

Mitochondria-associated ER membranes (MAMs) were originally described as sites for lipid synthesis and lipid transfer between ER and mitochondria [53]. These MAMs are, however, also ideally suited for Ca^{2+} exchange [14]. Several proteins may participate in the stabilization of those MAMs and, through this stabilization, affect Ca^{2+} transfer between ER and mitochondria. Other proteins may be directly involved in regulating the Ca^{2+}-transport proteins described above.

4.1. Glucose-Regulated Protein 75. Glucose-regulated protein 75 (Grp75) belongs to the Hsp70 family of chaperones but is not inducible by heat shock [54, 55]. Importantly, it can couple the IP_3R to VDAC1 and allows for a better transfer of the Ca^{2+} ions from the ER to the mitochondrial matrix [56]. The increased Ca^{2+} signals in the mitochondria were not due to an increased ER-mitochondria contact area. These results indicate that Grp75 is probably not the main determinant for the ER-mitochondrial linkage but regulates the Ca^{2+} flux between ER and mitochondria by controlling the interaction between the IP_3R and VDAC1.

4.2. Sigma-1 Receptor. The ER chaperone proteins known as sigma receptors are targets for certain neurosteroids. Based on their biochemical and pharmacological properties, two subclasses, sigma-1 and sigma-2 receptors, are distinguished but only the sigma-1 receptor was cloned and properly characterized [57, 58]. The sigma-1 receptor is involved in many physiological functions as well as in several pathological conditions [58].

Sigma-1 receptors are especially enriched at the MAMs [59]. A specific interaction between the Ca^{2+}-binding chaperone BiP and the sigma-1 receptor was described [59]. This interaction depends on the ER Ca^{2+} concentration: a decrease in ER Ca^{2+} concentration leads to their dissociation, whereby both proteins become active chaperones.

The sigma-1 receptor regulates several ion channels, including the IP_3Rs [58]. Agonists of sigma-1 receptors could so potentiate agonist-induced Ca^{2+} release in NG108 cells [60]. Hereby, an interaction between the sigma-1 receptor, cytoskeletal ankyrin B, and IP_3R3 was demonstrated [61]. In CHO cells, the sigma-1 receptor also interacted with IP_3R3, but here ankyrin was not observed in the complex. Finally, a specific role was found for the sigma-1 receptor stabilizing the IP_3R3 present at the MAMs, and so regulating Ca^{2+} transfer between ER and mitochondria [59].

4.3. Mitofusins. Mitofusin 1 and 2 are two dynamin-related GTPases acting on mitochondria. Mitofusin 2 is enriched at MAMs. The absence of mitofusin 2 not only affected ER and mitochondrial morphology but also reduced the number of contact points between ER and mitochondria by about 40% [62]. Mitofusin 2 on the ER appeared necessary for connecting the two organelles by directly interacting with either mitofusin 1 or mitofusin 2 on the OMM. Moreover, the diminished interaction observed in the absence of mitofusin 2 affected Ca^{2+} transfer between the ER and the mitochondria. A too strong ER-mitochondria interaction may also be detrimental as overexpression of mitofusin 2 led to apoptosis [63].

4.4. Bcl-2-Family Members. Bcl-2 is the prototype of a large family containing both anti- and proapoptotic proteins. The antiapoptotic members of this family, including Bcl-2 itself, are characterized by the presence of 4 Bcl-2-homology (BH) domains (BH1 to 4). The proapoptotic members either have 3 BH domains (BH1, BH2, and BH3) as, for example, Bax and Bak, or only a single BH3 domain, as for example, Bim, Bid, and Bad (the so-called BH3-only proteins) [39].

The BH1, BH2, and BH3 domains of the antiapoptotic proteins, as Bcl-2 and Bcl-Xl, form together a hydrophobic cleft that can bind the amphipathic α-helical BH3 domain of proapoptotic proteins. In this manner, the antiapoptotic Bcl-2 family members antagonize apoptosis at the level of the mitochondria by binding and neutralizing proapoptotic Bax and Bak [39, 64]. In addition to this mitochondrial function, antiapoptotic Bcl-2 family members also act on the ER Ca^{2+} homeostasis [65, 66]. The exact mechanism is, however, not yet clarified, and effects on several Ca^{2+}-binding or Ca^{2+}-transporting proteins were described, including on the IP_3R [67–69].

Although there is an agreement that the antiapoptotic proteins as Bcl-2 bind to the IP_3R, there is among the various studies a discrepancy with respect to the exact binding site and to the functional consequences. The results obtained are summarized here below.

Firstly, cells lacking Bax/Bak displayed a decreased ER Ca^{2+}-store content, which was associated with an increased (i) amount of Bcl-2 bound to the IP_3R, (ii) protein-kinase-A-(PKA-) dependent phosphorylation of the IP_3R, and (iii) Ca^{2+} leak rate from the ER. Hence, increasing the ratio of antiapoptotic over proapoptotic Bcl-2-family members seemed to decrease the ER Ca^{2+}-store content by promoting the Ca^{2+} leak via hyperphosphorylation and hyperactivation of the IP_3R [70].

Secondly, IP_3Rs were described to be activated by Bcl-Xl. Bcl-Xl bound to all three IP_3R isoforms, thereby sensitizing them to low IP_3 concentrations [71, 72]. The interaction site was demonstrated to be the C-terminal part of IP_3R1 [71]. The binding of Bcl-Xl to the IP_3Rs is important for the protection of cells against apoptotic stimuli, since the overexpression of Bcl-Xl in IP_3R triple-knockout (TKO) cells did not provoke resistance against apoptotic stimuli. By ectopically overexpressing the different IP_3R isoforms in the TKO cells, it was found that all IP_3R isoforms were sensitized by Bcl-Xl and so conferred resistance against apoptotic stimuli. However, a decline in steady-state ER Ca^{2+} levels was only found in TKO cells ectopically expressing IP_3R3 [72], suggesting that decreased ER Ca^{2+} levels are not a requisite for cellular protection against apoptosis. The antiapoptotic action may therefore be due to the enhanced Ca^{2+}-spiking activity resulting from the sensitization of the IP_3Rs, and be mediated either by increased mitochondrial bioenergetics or by modulation of transcriptional activity and gene expression [71, 72]. A similar mechanism was recently proposed for Bcl-2 and Mcl-1 [73].

Thirdly, an inhibition of the IP_3-induced Ca^{2+} release by Bcl-2 was also demonstrated [74]. In contrast to the work discussed above, the interaction site was mapped to the regulatory domain of IP_3R1; moreover, the interaction was mediated through the BH4 domain of Bcl-2, a domain which is not involved in the interaction with the C-terminus of the IP_3R [73, 75]. A peptide corresponding to the Bcl-2-binding site on IP_3R1 specifically disrupted this interaction and in this way counteracted the functional effects of Bcl-2 on the IP_3R [75, 76].

4.5. PKB/Akt and Promyelocytic Leukemia Protein. Another regulatory mechanism of the Ca^{2+}-flux properties of the IP_3R is its phosphorylation via PKB/Akt [29, 77, 78]. Upon prosurvival stimulation of cells, the prosurvival kinase PKB/Akt binds and phosphorylates the IP_3R, thereby reducing its Ca^{2+}-release activity. This mechanism underpins the increased resistance of cells towards apoptotic stimuli by inhibiting the Ca^{2+} flux into the mitochondria and may be perused by tumor cells, yielding a survival advantage. The latter has been shown to occur in glioblastoma cells that display hyperactive PKB/Akt, leading to IP_3R hyperphosphorylation and suppression of IP_3R-channel activity [77].

Very recently, extranuclear promyelocytic leukemia protein (PML) has been shown to be present at the ER and mitochondrial-associated membranes, thereby promoting ER Ca^{2+} release. At these microdomains, PML controls the Ca^{2+}-flux properties of the IP_3R by recruiting PP2A, which dephosphorylates PKB/Akt. The latter suppresses its kinase activity and thus the PKB/Akt-mediated phosphorylation of the IP_3R, resulting in increased IP_3R-mediated Ca^{2+} transfer into the mitochondria and thus OMM permeabilization [79, 80]. This mechanism supplements the other known functions of PML in the nucleus of higher eukaryotes. PML nuclear bodies seem to contribute to its tumor suppressive action by inhibiting cell cycle progression and promoting cell death [81].

5. The Transfer of Ca^{2+} between the IP_3R and Mitochondria in Apoptosis and Autophagy

From the previous it is clear that Ca^{2+} transfer from the ER to the mitochondrial matrix is crucial for regulating mitochondrial functions, including bioenergetics. The mitochondrial Ca^{2+} signal can, however, also control the choice between cell survival and cell death, as it can participate in the induction and progression of apoptosis and autophagy [9, 10].

5.1. IP_3Rs and Mitochondrial Ca^{2+} in Apoptosis and Necrosis. Different studies have placed the IP_3R as central player in the transfer of Ca^{2+} into the mitochondria. Many cell types display the propagation of agonist-induced Ca^{2+} signals into the interior of the mitochondria [11, 82].

Ca^{2+} uptake in the mitochondria is crucial for multiple important cellular functions, but the risk of mitochondrial Ca^{2+} overload exists, which may result in the induction of cell death. At a high concentration, mitochondrial Ca^{2+} supports opening of the PTP in the IMM [51, 83]. This opening leads to the release of ions (including Ca^{2+}) and molecules (including ATP), mitochondrial depolarization, ROS production, cessation of oxidative phosphorylation followed by ATP hydrolysis, matrix swelling by osmotic forces, remodeling of the IMM, and eventually rupture of the OMM [52]. Subsequently various apoptogenic factors, including cytochrome C (CytC), apoptosis-inducing factor, Smac/Diablo, HtrA2/Omi, and endonuclease G, are released from the mitochondria [40]. These apoptogenic factors will activate effector caspases, as caspase-3 and caspase-7, and lead the cell into the execution phase of apoptosis. Permeabilization of the OMM is therefore considered as the decisive event in the development of cell death [84]. Given the proximity of IP_3Rs to the mitochondrial Ca^{2+}-entry sites, IP_3-induced Ca^{2+} spikes appear ideally suited for the stimulation of apoptosis [85], while the knockdown of the IP_3R by siRNA led to the suppression of the Ca^{2+} transfer to the mitochondria.

In addition to this canonical pathway, the group of Mikoshiba recently showed that not only excessive IP_3R-mediated Ca^{2+} release and the concomitant mitochondrial Ca^{2+} overload but also the loss of IP_3R function may lead to apoptosis by lowering the mitochondrial membrane potential [86]. In this study, it was shown that ER stress in neuronal cell leads to attenuation of IP_3R function by impairing the positive regulation of IP_3R1 by the ER chaperone Grp78, which acts as a major regulator of the unfolded protein response and thus prevents ER stress. The loss of Grp78 binding to the luminal domain of the IP_3R1 leads to impaired subunit assembly and thus dysfunctional channels. This property seems selective for IP_3R1, since Grp78 knockdown attenuated IP_3R1-mediated Ca^{2+} release but did not affect IP_3R2- or IP_3R3-mediated Ca^{2+} release. Hence, it is interesting to note that Ca^{2+} transfer from the ER to mitochondria requires a fine-tuned regulation, in which both suppressed and excessive Ca^{2+} transfer leads to apoptosis.

While a severe impairment of IP_3R1 function and attenuated Ca^{2+} release lead to mitochondrial apoptosis, low-level Ca^{2+} signaling from ER to mitochondria or enhancing ER-originating Ca^{2+} oscillations elicits a prosurvival action by stimulating the mitochondrial energy production or by inducing transcription of specific genes [9, 31, 67, 69, 87]. In this paradigm, Bcl-Xl has been proposed to promote cell survival through its direct action on the IP_3R by enhancing prosurvival Ca^{2+} signaling, increasing mitochondrial bioenergetics and activation of signaling via nuclear factor of activated T cells [71, 72].

Mitochondrial Ca^{2+} is a central factor in several neurodegenerative diseases as Alzheimer's disease, Parkinson's disease, and Huntington's disease [88]. The inhibition of cell death by preventing mitochondrial Ca^{2+} overload or by preventing the collapse of the mitochondrial membrane potential is likely therapeutically relevant for the treatment of these diseases. In contrast, enhancement of mitochondrial Ca^{2+} overload can lead to inhibition of tumor cell growth. Stimulation of the Ca^{2+} transfer between ER and mitochondria could lead to increased apoptosis and in this way inhibit uncontrolled cellular proliferation [89]. In this concept, it is not surprising that many tumor suppressor proteins emerge as regulators of the transfer of Ca^{2+} from the ER to the mitochondria, like Fhit and PML. Fhit acts at the mitochondrial level by increasing the affinity of the mitochondrial Ca^{2+} uniporter, thereby promoting mitochondrial Ca^{2+} elevations at low levels of agonist-induced Ca^{2+} signaling [49]. PML acts at the level of the ER, where it is recruited by the IP_3R via a phosphorylation-dependent process involving Akt and PP2A, thereby promoting Ca^{2+} transfer between the ER and the mitochondria and inducing cell death [79, 80]. Mutations or ablation of proteins, like Fhit

and PML, which may involve attenuated ER/mitochondrial Ca^{2+} transfers, has been associated with the development of tumors.

5.2. IP_3Rs and Mitochondrial Ca^{2+} in Autophagy. Autophagy is a delivery pathway used for the lysosomal degradation of long-lived proteins, protein aggregates, damaged organelles, and foreign pathogens. In stress situations (e.g., nutrient starvation), this process offers the cell a fresh pool of building blocks and has thus a prosurvival function [90]. Cells in those conditions have to make the decision between survival (autophagy) and death (apoptosis). Important crosstalks exist between these two pathways [91, 92]. Interestingly, Ca^{2+} and IP_3Rs have been implicated in both apoptosis and autophagy, although the role of Ca^{2+} in autophagy only recently emerged [9, 10, 93]. Nonetheless, Ca^{2+}/IP_3Rs may represent key players in the apoptosis-autophagy decision.

The first results on Ca^{2+} in autophagy even appeared contradictory. On the one hand, autophagy was activated by an increase of the cytosolic Ca^{2+} concentration [94–96]. On the other hand, autophagy was also activated by conditions that all would lead to a decrease of the IP_3R activity and/or cytosolic Ca^{2+} concentration and therefore potentially of the mitochondrial Ca^{2+} concentration [97–100]. In a recent report, it was shown that IP_3R activity is necessary to provide for a basal Ca^{2+} signal to the mitochondria, in order to control mitochondrial bioenergetics. IP_3R knockdown or inhibition will blunt these Ca^{2+} signals, thereby compromising mitochondrial ATP production. The resulting increase in AMP/ATP ratio will subsequently activate autophagy via AMP-activated protein kinase (AMPK) [87].

Other results indicate that IP_3Rs could inhibit autophagy through a scaffold function, via binding of both Bcl-2 and Beclin-1 (an essential autophagy protein), thereby promoting the anti-autophagic interaction between these two proteins. Treatment of HeLa cells with the IP_3R inhibitor xestospongin B promoted the release of Beclin-1 from the IP_3R-Bcl-2 complex, leading to autophagy activation [101].

So far, the data on Ca^{2+}-stimulated autophagy concern the Ca^{2+} in the cytosol [94–96] or ER [102, 103]. It is not yet clear whether the IP_3R is hereby involved, although treatment with an IP_3R inhibitor did blunt cadmium-induced autophagy stimulation [95]. The exact mechanism by which Ca^{2+} promotes autophagy is also still under debate. AMPK-dependent [94], AMPK-independent [96], or ERK-dependent pathways [95] are all possible.

Taken together, these data indicate that a specific, low-intensity Ca^{2+} transfer from ER to mitochondria is necessary to inhibit autophagy, while an increase of the cytosolic Ca^{2+} concentration would activate autophagy.

6. Implications of Ca^{2+} Signaling in Aging

6.1. Aging: A Process of Disorganization. All biological processes involved in the transformation of a fertilized egg into a mature individual capable of reproduction are driven by a purposeful genetic program. Through evolution, natural selection has favored individuals that are reproductively successful [104, 105]. Biological systems, like everything else in the universe, change as a result of entropic changes. Entropy is the tendency for concentrated energy to disperse when unhindered. Natural selection has resulted in sufficient relative strengths of the chemical bonds in our molecules to prevent entropic changes and also installed repair and replacement mechanisms. Evolution has therefore kept the biomolecules in a functional state until reproductive maturation.

After sexual maturation, there is no longer a species-survival benefit for indefinitely maintaining these energy states and, hence, the fidelity in most molecules. As we grow older, stochastic or random events not driven by a genetic program cause energy loss resulting in biologically inactive or malfunctioning molecules. Aging is therefore characterized by increasing entropy. The intrinsic thermodynamic instability of the molecules whose precise three-dimensional structures are no longer maintained leads to covalent modifications such as glycation, conformational changes, aggregation and precipitation, amyloid formation, altered protein degradation, synthesis rates, and nuclear and mitochondrial DNA damage and alterations. When the loss of structure and, hence, function ultimately exceeds repair and turnover capacity, vulnerability to pathology and age-associated diseases increases. Because of the randomness of the molecular disorder underlying aging, the loss of molecular fidelity varies within the body. The weakest links in this system will be the first that lead to disease, like in the vascular system and in cells with a high tendency for cancer development. The very heterogeneous aging process contrasts with the virtually identical stages of development until adulthood [106]. In this respect, we will here focus on the age-related disorganization in the Ca^{2+} signaling machinery, ROS production, and autophagy.

6.2. Mechanism Involved in Aging: ROS, Mitochondria, and Autophagy. The role of ROS accumulation and subsequent macromolecular damage in age-related degeneration has been supported by a plethora of cellular and biological data from various model systems and organisms [107]. Antioxidants act as ROS scavengers and protect against the detrimental effects of cellular ROS exposure. Genetically, genes that extend lifespan were clustered in the IGF-1/insulin-like signaling pathway in a variety of model systems [108]. Nongenetic mechanisms to extend lifespan in different organisms are achieved by caloric restriction and/or by physical activity [109–113]. The composition of the diet during caloric restriction is important; addition of antioxidants (like vitamins, flavonoids), minerals (like Zn and Se), and other compounds such as caffeine, omega 3, and fatty acids has been shown to enhance lifespan [114]. It should be noted, however, that most studies concerning these mechanisms were performed in yeast and animal models, but not yet in humans [115].

Here, we will discuss the molecular mechanisms of ROS underlying aging. First, we will discuss the remodeling of Ca^{2+} signaling during aging. This is important since

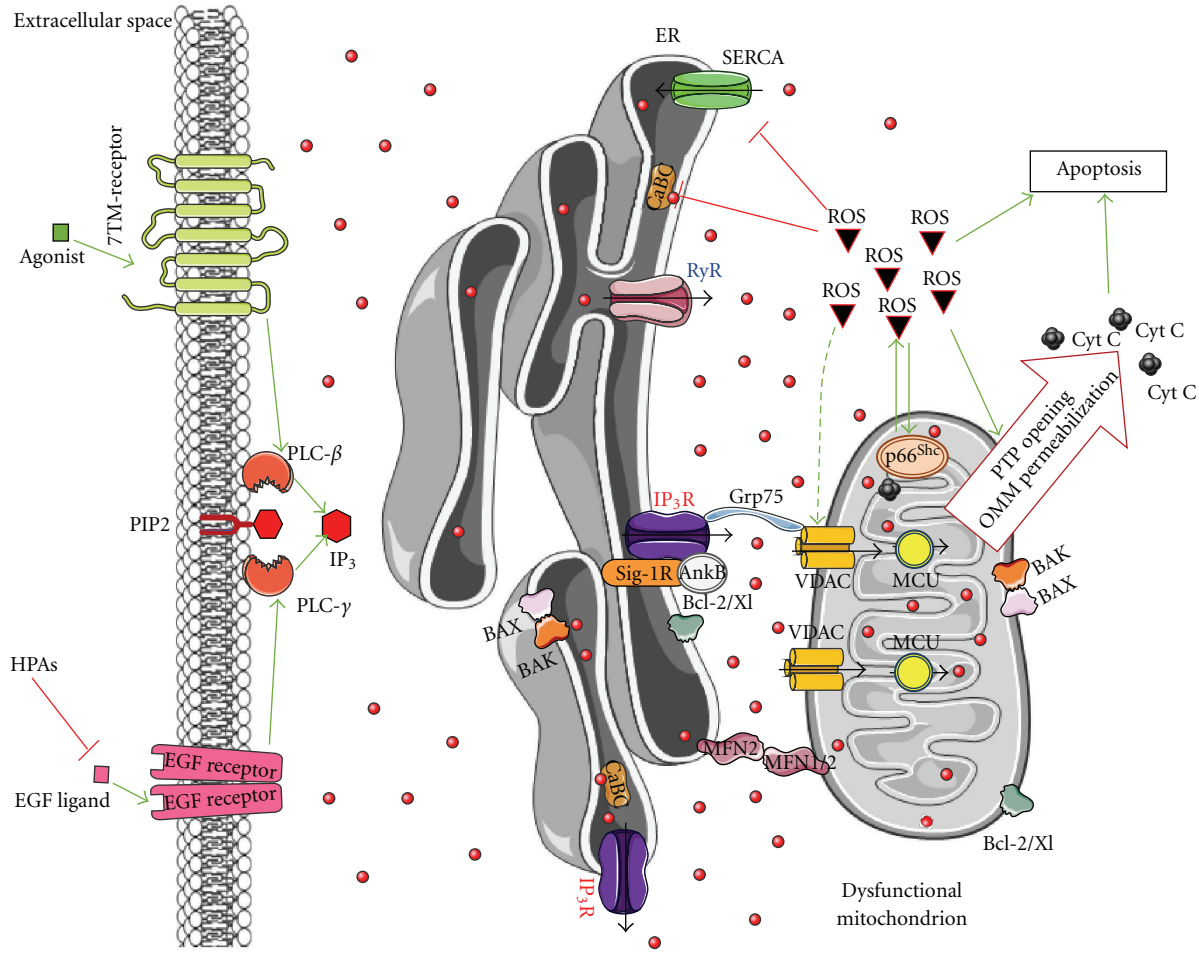

Figure 3: Ca^{2+} signalling and key events involved in aging. Aging cells display decreased function or expression of ER proteins (IP_3Rs, RyRs, SERCAs, Ca^{2+}-binding chaperones (CaBC)), increased cytosolic [Ca^{2+}], suppressed agonist-mediated signaling, and accumulation of damaged mitochondria due to declined autophagic activity. The simultaneous increase in disorganization and dysfunction of the Ca^{2+}-handling proteins and the decline in autophagy will result in the exaggerated production and excessive accumulation of ROS. These events may lead to both ER stress and mitochondrial dysfunction, like PTP opening and OMM permeabilization with the consequent release of apoptogenic factors and cell death. $p66^{Shc}$ and sirtuins take part in this scenario. $P66^{Shc}$ translocates to mitochondria upon oxidative-stress-induced PKCβ phosphorylation and peptidylprolyl isomerization by Pin1, thereby supporting ROS production. Sirtuins are downregulated and unable to exert its antiaging effect. It is important to note that while $p66^{Shc}$ ablation leads to lifespan extension, high levels of $p66^{Shc}$ have been observed in centenarians. While in normal cells, ROS help to detect and remove altered mitochondria through autophagy, thereby maintaining cellular health, the excessive release of ROS in combination with the decline in autophagy observed during aging may underpin the age-related cell-death processes. In this respect, the recently identified inhibitors of EGF-receptor signaling, the high-performance advanced age phenotype proteins (HPA-1 and HPA-2), whose knockdown promotes locomotory health span of *C. elegans*, may point towards an important role of proper agonist-induced Ca^{2+} signaling via the IP_3R axis. The relevance of these ligands or of attenuated agonist-induced signaling in humans needs to be established. However, recent evidence indicates that dysfunction of IP_3Rs during ER stress promotes cell death and underlies a neurodegenerative disease, like Huntington's disease. Given the central role of proper IP_3R function for mitochondrial bioenergetics and ATP production, the decline of IP_3R activity observed during ER stress or attenuated upstream signaling linked to IP_3 may be very relevant for age-related apoptosis but require further investigation. Green arrows: stimulation; red lines: inhibition; black arrows: Ca^{2+} flux; dashed-green arrow: stimulation/damage.

the OMM permeabilization is critically controlled by the elevation of the mitochondrial Ca^{2+} concentration, thereby serving as a coincidence detector with ROS [116]. Next, we will focus on the signaling cascade involving sirtuins, $p66^{Shc}$, and autophagy in the regulation of mitochondrial function. A schematic overview of the interaction between the different molecular key players in aging is provided in Figure 3.

6.2.1. Ca^{2+} Signaling in Aging. Altered intracellular Ca^{2+} signaling is a hallmark of neurodegeneration, like in Alzheimer's and Huntington's disease [117–120]. Different models have been proposed for familial Alzheimer's-disease-linked presenilin mutations, including the function of presenilins as Ca^{2+}-leak channels [121], an increase in the expression level of IP_3Rs [122], or the direct activation of IP_3Rs or

RyRs [123–125]. In any case, it is clear that exaggerated Ca^{2+} signaling is an upstream event in the pathophysiology of Alzheimer's disease and contributes to the ROS-mediated cell toxicity [126]. However, the changes in Ca^{2+} signaling that occur in neurodegenerative diseases may be dependent on the type of disease. For instance, a mouse model for Huntington's disease revealed dysfunctional IP_3R Ca^{2+}-release channel activity in the cerebrum and striatum, which was caused by a prominent decline in the association of Grp78, a positive regulator of the IP_3R1-channel formation, with the IP_3R1 [86].

Other age-related diseases also display altered Ca^{2+} signaling. Cardiac hypertrophy, for example, is characterized by enhanced IP_3 signaling, leading to spontaneous Ca^{2+}-release events that underlie arrhythmias [127]. Also chronic heart failure can be a consequence of excessive phosphorylation of RyR, leading to an increased Ca^{2+} leak [128] (Figure 2).

However, the role and mechanism of ER Ca^{2+} signaling in aging is less clear [129], although most studies suggest altered Ca^{2+} signaling during aging (Figure 2). In most cell types, ER Ca^{2+} dyshomeostasis was caused by a decreased ER Ca^{2+} content and a decreased Ca^{2+} release from the ER, while the cytosolic $[Ca^{2+}]$ was increased. These effects were the result of a decline in SERCA and/or IP_3R and/or RyR activity, caused by changes in mRNA or protein levels, phosphorylation events, or oxidative damage to SERCA [7]. In addition, intraluminal Ca^{2+}-buffering protein levels often decline during age, in part also through oxidative damage [130] (Figure 2). Also VDAC undergoes posttranslational modifications in aged cells, possibly through oxidative break-up of tryptophan residues, thereby increasing the susceptibility to apoptosis [131]. This is in line with evidence showing that superoxide can lead to mitochondrial permeabilization in a VDAC-dependent manner [132]. In yeast, this phenomenon can be protected by Cu/Zn-superoxide dismutase, a protein known for its protective role against aging [133].

Some cell types, however, display Ca^{2+} dyshomeostasis in a different way (Figure 2). Studies in aged rat hearts, for example, showed increased IP_3R levels [134]. Also aged neuronal cells displayed reduced sensitivity towards caffeine, which may be caused by a decline in the steady-state ER Ca^{2+} levels [135–137]. The latter may be due to a decreased SERCA Ca^{2+}-pump activity, a limited supply of ATP or an increased Ca^{2+} leak from the ER. Other studies pointed to a prolonged Ca^{2+}-induced Ca^{2+} release, resulting in an inhibition of synaptic strength and long-term potentiation [138, 139].

Interestingly, IP_3R characteristics also appear to be altered in aged brain tissues [140], as IP_3R density and IP_3 binding to the IP_3R were decreased in aged rat cerebellum. The same observation of decreased IP_3 binding was made in aged mice cerebellum [141]. However, the cellular IP_3 content increased with age [142]. These findings suggest a role for the phosphoinositide/Ca^{2+} signaling in the impaired neuronal responsiveness during aging. In this respect, more recent work revealed that stimulation of IP_3Rs in old astrocytes increased protection against ROS and subsequently neuroprotection [143].

Moreover, in aged MII-stage eggs, it was found that the IP_3R1 was proteolytically cleaved by caspase-3, resulting in a leaky 95-kDa C-terminal IP_3R1 fragment containing the channel pore [144, 145]. In contrast, when the C-terminal channel domain was recombinantly expressed in the mouse oocytes, the sperm-factor-induced Ca^{2+} oscillations were abolished and the eggs displayed an apoptotic and fragmented phenotype. Previously, we had shown that caspase-3-dependent cleavage of the IP_3R augmented the late phase of apoptosis by providing a prolonged ER Ca^{2+} leak [146]. However, in healthy cells, the Ca^{2+} leak through a recombinantly expressed C-terminal channel domain was very small. Hence, the caspase-3-dependent cleavage of the IP_3R may participate in cellular Ca^{2+} overload via a second-hit mechanism. In the case of aged oocytes, accumulated ROS may be the second hit. Currently, it is not clear whether IP_3R cleavage contributes to the aging process by overloading the mitochondria with Ca^{2+} and sensitizing them towards ROS accumulation. In addition, ROS may also directly regulate IP_3R activity, since it is known that oxidizing agents like thimerosal sensitize IP_3Rs by stimulating intramolecular interactions between the suppressor and ligand-binding domain [147]. Taken together, IP_3R/Ca^{2+} signaling appears to be affected in aged cells. Abnormal Ca^{2+} signals may then affect many processes (ROS production/protection, autophagy, apoptosis, synaptic transmission, etc.) that are altered during aging (summarized in Figure 5). Nevertheless, the overall changes in ER Ca^{2+} handling observed during aging seem relatively small compared to the changes found in Alzheimer's disease [129].

Recently, an elegant study on *Caenorhabditis elegans* re-enforced the paradigm that the activation of IP_3R pathways may be considered in therapeutic applications for treating age-related decline in skeletal muscle function (sarcopenia) [148]. Indeed, using an RNAi screen, the authors identified two critical factors that delayed the age-associated decline in locomotory health span of *C. elegans* in a high-performance advanced age phenotype (HPA-1 and HPA-2). The concept underpinning this study was that locomotory decline in humans contributes to frailty and loss of independence. Although the exact mechanism is not yet known, it is clear that HPA-1 and HPA-2 attenuate epidermal-growth-factor-(EGF-) dependent signaling via the EGF receptor [148]. When HPA-1 and HPA-2 are disrupted, EGF signaling via the EGF receptor will increase. The activation of the EGF-signaling pathway normally leads to cell proliferation, survival, integrity, and differentiation. Importantly, phospholipase C-γ (PLC-γ) and IP_3Rs were demonstrated to act downstream of EGF-receptor signaling, thereby contributing to prolonged health span in these animals. This is the very first report considering the role of EGF signaling in aging. Therefore, the exact mechanism of how these signaling pathways affect human aging remains to be further clarified, but restoring the attenuated IP_3R-mediated Ca^{2+} signaling and reestablishing normal mitochondrial function may be an attractive hypothesis in combination with chemical induction of autophagy (Figure 4). Nevertheless, a decline in G-protein-coupled receptor-dependent signaling has been observed in the skeletal muscle and intestine of aged

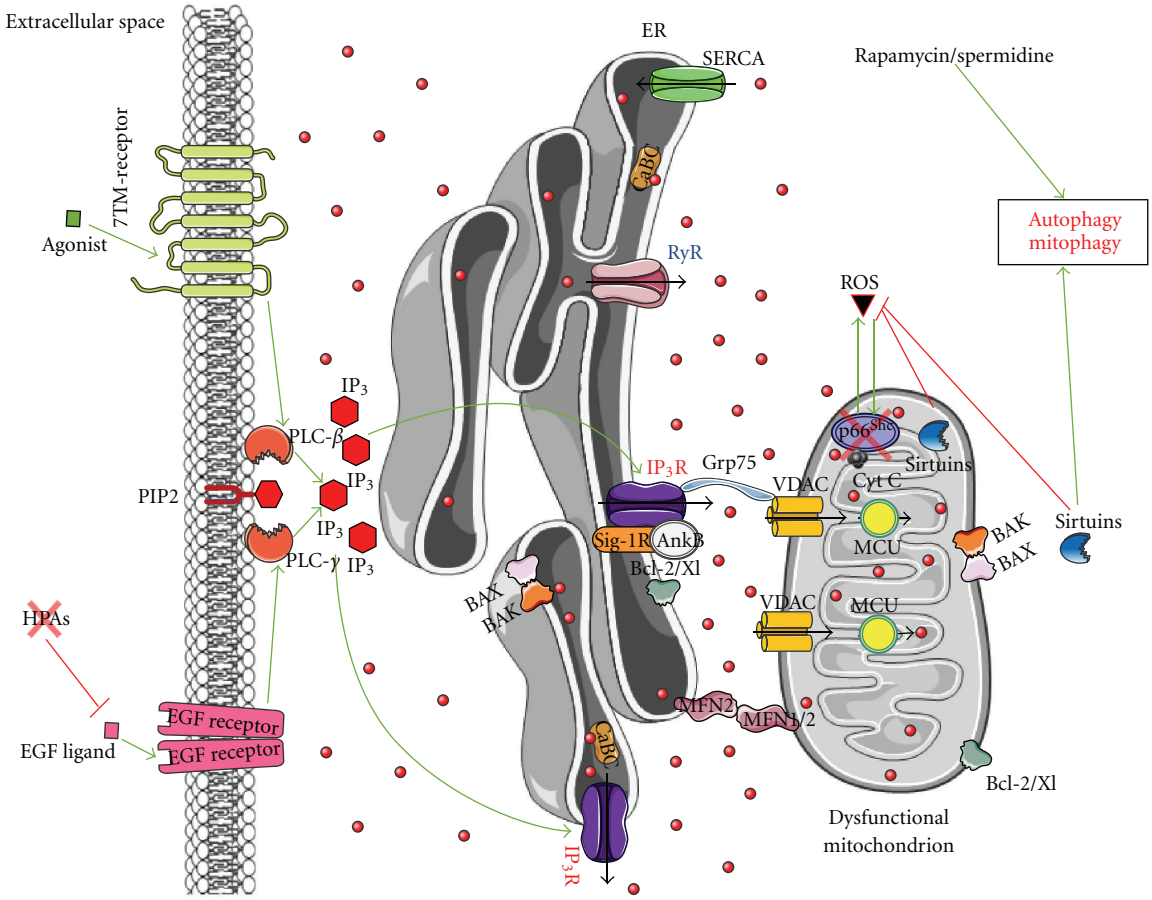

FIGURE 4: A speculative antiaging strategy based on restoring IP_3R-mediated Ca^{2+} signaling and chemical induction of autophagy. Provided the concept that aging cells are characterized by suppressed IP_3 signaling or attenuated IP_3R, Ca^{2+}-release activity is relevant in humans, and elevating IP_3 levels may compensate for the decline in the IP_3/IP_3R-signaling axis. This may contribute to a decline in the $p66^{Shc}$-mediated ROS production, an activation of sirtuin-dependent mitochondrial biogenesis, and the lowering of ROS production. The final step of this compensatory response consists in the autophagic removal of the damaged mitochondria. Hence, chemical induction of autophagy (e.g., by rapamycin or spermidine) is likely critical for successful and healthy aging in human beings. It is important to note that this concept is based on a recent report on *C. elegans*, in which ablations of inhibitors of EGF signaling enhance IP_3R signaling and promote healthy lifespan extension. Green arrows: stimulation; red lines: inhibition; black arrows: Ca^{2+} flux.

rats [149]. The underlying mechanism involved a prominent decrease in the levels of $G_{q/11}$ and G_i protein levels.

6.2.2. Sirtuins. Sirtuins are a conserved family of proteins that are linked to longevity and stress tolerance in *Saccharomyces cerevisiae* [150]. Sirtuins have been identified as antiaging genes, since increasing their activity prolonged lifespan not only in yeast, but also in *C. elegans* and *Drosophila melanogaster* and is thought to act similarly in mammals [151–153]. In this respect, age is often associated with reduced sirtuin levels. In aged mouse embryonic fibroblasts, progressive loss of the sirtuin-1 protein, but not mRNA, was observed [154]. However, other studies show that this is at least tissue specific; sirtuin-1 activity was reduced in rat hearts, but not in adipose tissue [155], and reduced sirtuin-1 expression was found only in distinctive parts of the mouse brain [156]. Sirtuins, which retard aging as a function of their gene dosage, display unique biochemical activities, that is, NAD-dependent protein deacetylase [157, 158]. The subsequent deacetylation of sirtuin substrates alters their activity (activation or inhibition). In mammals, sirtuin-1 deacetylates a variety of key transcription factors and cofactors, like p53 [159], FOXO proteins [160, 161], peroxisome proliferation activating receptor (PPAR)-γ coactivator-1α (PGC-1α) [162], and nuclear factor-κB [163]. The effects of sirtuin-1 on these factors elicit stress tolerance and metabolic changes reminiscent of caloric restriction, while caloric restriction upregulates sirtuin-1 levels, and mice lacking sirtuin-1 did not display phenotypic responses upon caloric restriction [160, 164–166]. Since sirtuins are regulated by NAD^+, their activity will be influenced by the NAD^+/NADH ratio and thus by the metabolic state of the cell [167]. Hence, sirtuins may be influenced not only by caloric restriction but also by physical activity, both associated with longevity and increased insulin sensitivity [168, 169].

Importantly, sirtuin-1 also regulates mitochondrial biology [150, 167], another key aspect in aging, since the number

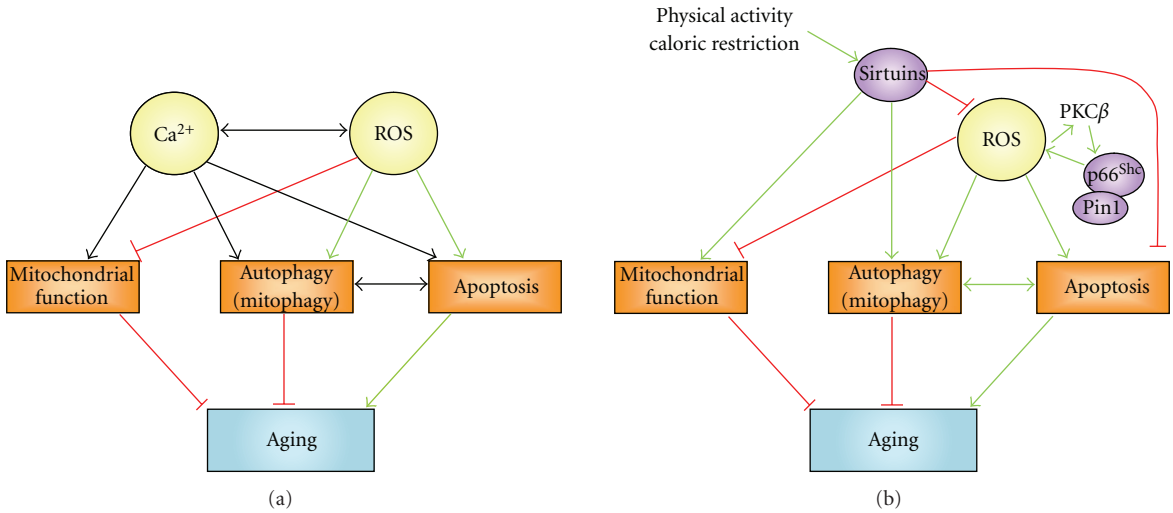

Figure 5: Network of interactions between sirtuins, $p66^{Shc}$, Ca^{2+}, and ROS, which affect mitochondrial function, autophagy, and apoptosis, thereby controlling aging-dependent processes. (a) Ca^{2+} signals may increase or prevent aging. Ca^{2+} signals are characterized by different spatiotemporal characteristics and subsequently different outcomes on mitochondrial function, autophagy, and apoptosis. For example, a constitutive Ca^{2+} transfer from ER to mitochondria would stimulate mitochondrial function and inhibit autophagy and apoptosis, while a mitochondrial Ca^{2+} overload would be proapoptotic. The interplay between mitochondrial Ca^{2+} elevations and ROS production is a critical determinant in the apoptotic outcome at the level of the mitochondria, which function as co-incidence detectors. Therefore, high mitochondrial Ca^{2+} concentrations and ROS act as a double-hit mechanism, triggering mitochondrial-dependent apoptosis. (b) Sirtuins are mainly antiaging genes via the promotion of mitochondrial function and autophagy and inhibition of apoptosis. They also act inhibitingly on ROS. Sirtuin function may be enhanced by restricting caloric intake or increasing physical activity, thereby extending lifespan. Increased ROS activate the Pin1- $p66^{Shc}$ complex, which, in turn, promotes the production of ROS and subsequently mitochondrial damage. Therefore, $p66^{Shc}$ may help to target damaged mitochondria and activate cellular processes that deal with dysfunctional mitochondria and oxidative stress. The outcome, however, can be dual: aging may be enhanced via a complete removal of the cell through apoptosis, while the selective removal of the damaged mitochondria through mitophagy, leaving the cell with predominantly healthy mitochondria, may slow down the aging process. Green arrows: stimulation; red lines: inhibition; black arrows: stimulation or inhibition.

of functional mitochondria is known to decline during aging. This has been proposed to underlie aging in diseases like type-2 diabetes [170, 171]. In contrast, increasing mitochondrial activity will increase the metabolic rate, enhance glucose metabolism, and improve insulin sensitivity. Even without an increase in the metabolic rate, caloric restriction might be beneficial by inducing mitochondrial biogenesis via sirtuin-1 [165, 172, 173]. Activation of sirtuin-1 has been shown to be involved in mitochondrial biogenesis and improved mitochondrial function by deacetylation of PGC-1α, thereby lowering ROS production [162].

Sirtuin-1 also suppressed stress-induced apoptosis, while the lack of sirtuin-1 inhibited autophagy *in vivo* [174]. In addition, the extension of lifespan upon caloric restriction was proposed to be dependent on the induction of autophagy by sirtuin-1 [175]. The underlying mechanism probably involves the deacetylation of certain autophagy proteins, such as Atg5, Atg7, and Atg8 [174, 175]. A schematic overview of the role of sirtuins in aging is depicted in Figure 5.

6.2.3. $p66^{Shc}$. Recent research revealed the role of $p66^{Shc}$, the 66 kDa isoform of the Shc (Src homolog and collagen homolog) family [176]. Although $p66^{Shc}$ forms stable complexes with Grb2, an adaptor protein for the Ras-exchange factor SOS, it has little effect on Ras-mediated signaling [177].

Nevertheless, $p66^{Shc}$ is activated by oxidative stress via phosphorylation on Ser36, and this mechanism is indispensable for $p66^{Shc}$'s lifespan regulation [178, 179]. Mice in which $p66^{Shc}$ has been deleted displayed a prolonged lifespan with a decreased mitochondrial metabolism and ROS production, while lacking pathophysiological characteristics or effects on body size. MEF cells from $p66^{Shc-/-}$ animals displayed resistance towards oxidative-stress-induced apoptosis in a p53-dependent manner [176].

ROS arise from the mitochondrial electron-transfer chain or from exogenous sources, like UV and ionizing radiations. $p66^{Shc}$ is involved in mitochondrial ROS production. In basal conditions, about one fifth of $p66^{Shc}$ is localized to the intermembrane space of the mitochondria, while oxidative stress dramatically increases the mitochondria-associated $p66^{Shc}$ due to its mitochondrial translocation from the cytosol [180]. In the mitochondria, $p66^{Shc}$ interacts with CytC, promoting the shuttling of electrons from CytC to molecular oxygen [181]. The latter may underlie the increased ROS production upon $p66^{Shc}$ overexpression and the decreased ROS production in $p66^{Shc}$ knockout cells. In addition, $p66^{Shc}$ knockout cells displayed decreased oxidative capacity, thereby redirecting metabolic energy conversion from oxidative toward glycolytic pathways. Therefore, $p66^{Shc}$ may provide a molecular switch to oxidative-stress-induced apoptosis by controlling mitochondrial ROS production.

It should be noted, however, that studies in yeast correlated higher respiration rates combined with decreased oxidative stress and increased lifespan [182]. This suggests that the respiration rate *per se* is not the important factor for ROS production, but more likely the electron transmit time and the availability of oxygen [183].

In normal cells, oxidative stress leads to compromised mitochondrial Ca^{2+} homeostasis, which is an early event of mitochondrial damage [107, 176]. This is observed as a decreased mitochondrial Ca^{2+} signal upon agonist stimulation in cells challenged with H_2O_2 despite a normal cytosolic Ca^{2+} signal. Importantly, cells lacking $p66^{Shc}$ seemed to be protected against oxidative challenge, since their mitochondrial Ca^{2+} signaling upon agonist stimulation was not impaired in the presence of H_2O_2 [176]. Similar results were found in MEF cells lacking Pin-1, a peptidylprolyl isomerase catalyzing *cis*/*trans* isomerization of phosphorylated Ser-Pro bonds, where the reduction of agonist-induced Ca^{2+} signals in mitochondria upon oxidative stress was significantly smaller. These findings suggest a phosphorylation-dependent conformational change in Pin-1 targets, like $p66^{Shc}$.

Recent work provided important mechanistic insights into the role of $p66^{Shc}$ in the early mitochondrial response to oxidative stress [178, 179]. ROS are known to activate a variety of kinases, including protein kinase C (PKC) β. The activation of PKCβ will cause the phosphorylation of $p66^{Shc}$ on Ser36, although other kinases may also participate in this process. Indeed, the mitochondrial fraction of $p66^{Shc}$ during oxidative challenge was severely reduced after treatment with PKCβ inhibitors. As a result, Ser36-phosphorylated $p66^{Shc}$ will interact with Pin-1. The catalytic activity of Pin-1 may result in *cis*/*trans* isomerization of Ser36-Pro37, thereby triggering the exposure of a mitochondrial targeting sequence or an interaction with mtHsp70, a mitochondrial heat-shock protein. This process may underlie selective targeting of $p66^{Shc}$ to mitochondria undergoing oxidative challenge. The mitochondrial targeting of $p66^{Shc}$ involves its protein-phosphatase-(PP-) 2A-mediated dephosphorylation and dissociation from mtHsp70, although the mechanism of their contribution is not fully elucidated. In the intermembrane space, $p66^{Shc}$ will interact with reduced CytC and enhance intramitochondrial H_2O_2 production. The latter and its more damaging reaction products, the hydroxyl radicals, have been shown to trigger the opening of the PTP [184]. This will perturb mitochondrial structure and function, resulting in mitochondrial permeabilization, CytC release, and apoptosis induction, and subsequently lead to a coordinated cell-death response and the removal of the cell containing damaged mitochondria. However, in addition to apoptosis, autophagy may be involved in removing the subpopulation of compromised mitochondria suffering from oxidative challenge. Interestingly, this autophagy-mediated removal of damaged mitochondria can be triggered through PTP opening [185]. This will result in the removal of the organelles that are damaged by the oxidative stress (a process termed mitophagy), while maintaining the healthy mitochondria. According to these findings, it is interesting to note that aging has been associated with declined autophagy activity [186], while autophagy activity is a requisite for lifespan extension in *C. elegans* [187]. In this way, $p66^{Shc}$ may be important for mitochondrial quality control through the autophagy-mediated removal of damaged mitochondria. However, during aging, the number of mitochondria suffering from oxidative stress may increase, while their cleanup by the autophagic system may become limiting, leading to the accumulation of unprocessed oxidation-damaged mitochondria. Importantly, in mouse models for aging, the levels of $p66^{Shc}$ seemed to decline, while its phosphorylation at Ser36 was enhanced [188]. This correlated with higher free-radical production and accumulation of damage caused by ROS.

Strikingly, fibroblasts obtained from centenarians displayed elevated levels of $p66^{Shc}$ [189], indicating that basal mitochondrial $p66^{Shc}$ plays an important role in normal cell-damage management of stress and in damage repair. Indeed, the selective removal of damaged mitochondria may contribute to lifespan extension. In addition, it is interesting to note that increased physical activity has been associated with lifespan extension and lower mortality, although this is associated with increased mitochondrial ROS production due to an increased metabolic rate. Therefore, it is conceivable that exercise may promote adaptation to ROS by upregulating ROS scavengers, causing a natural resistance against ROS or against cellular damage in general [167]. Hence, it may be worth investigating whether $p66^{Shc}$ levels are affected by exercise and whether this may contribute to increased cleanup of damaged mitochondria or resistance against ROS. A schematic overview of the role of $p66^{Shc}$ in aging is depicted in Figure 5.

6.2.4. Autophagy. It has become increasingly clear that autophagy plays a central role in the aging process, in which it is involved in the removal of damaged organelles or of protein aggregates by engulfment in autophagosomes followed by lysosomal degradation. First of all, autophagy was demonstrated to decrease with increasing life time [186]. Caloric restriction slowed down the age-related impairment of autophagy in skeletal muscle of rats [190]. In addition, chemical induction of autophagy by spermidine or by rapamycin prolonged lifespan [191, 192]. In contrast, animals with compromised capacity to perform autophagy were short living and displayed neurodegenerative phenotypes, probably due to the accumulation of deleterious accumulation of protein aggregates [193–195]. Moreover, it is clear that damaged mitochondria ought to be removed, while harboring the healthy mitochondria, which are needed for cell survival. In any case, the accumulation of damaged mitochondria and their impaired removal is a hallmark of aging and will contribute to decreased cell viability. Therefore, mitochondrial quality control is essential for proper cell survival.

The "selective" recognition of damaged mitochondria by autophagosomes without affecting healthy mitochondria remains very poorly understood. However, the first components essential for "selective" mitophagy have been identified in yeast: Uth1, an OMM protein, and Aup1, a mitochondrial phosphatase [196–198]. Additional components of

organelle-specific autophagy have been revealed in a systematic screen, including Atg11, Atg20, Atg24, Atg32, and Atg33 [199, 200]. Atg32 is proposed as the receptor for mitophagy via the local recruitment of Atg8, an essential component of the autophagosome formation. NIX/BNIP3L [201, 202], BNIP3 [203], PARKIN [204], and PINK-1 [205–210] were proposed to be involved in mitochondrial degradation in mammalian cells. PARKIN is selectively recruited by dysfunctional mitochondria, thereby mediating the engulfment of these mitochondria by the autophagosomes [204]. A recent study provided clear insights into the underlying mechanism, which required the accumulation of the kinase PINK-1 on damaged mitochondria. In healthy mitochondria, PINK-1 is maintained at a low level by voltage-dependent proteolysis [210]. In mitochondria with sustained damage, PINK-1 levels rapidly accumulated. The latter was required and sufficient to recruit PARKIN to the mitochondria providing a mechanism for the selective removal of damaged mitochondria by autophagy. Importantly, mutations in PINK-1 or PARKIN associated with Parkinson's disease abolished the recruitment of PARKIN by PINK-1 to the mitochondria, allowing the accumulation of damaged mitochondria. Another recent study revealed the mitochondrial protein NIX as the selective mitophagy receptor for the removal of damaged mitochondria by binding and recruiting LC3/GABARAP proteins [211]. The latter are ubiquitin-like modifiers required for the elongation of autophagosomal membranes.

Besides these mitophagy receptors, mitochondrial proteases and chaperones were needed to prevent the accumulation of misfolded and aggregated proteins within the mitochondria [167].

Finally, various studies point towards a role of ROS upstream of autophagy [212]. Accumulation of ROS directly affects different key players essential for the induction of autophagy, including the activation of the protein kinases AMPK and JNK, the inhibition of other kinases (Akt and TOR), and the inhibition of LC3 delipidation. These processes will stimulate autophagy, thereby alleviating the oxidative stress by removing the ROS-generating mitochondria.

7. Conclusions

Upstream Ca^{2+} and ROS signaling tightly control cellular homeostasis by regulating fundamental cell-death and cell-survival processes like apoptosis and autophagy. It is clear that many proteins that mediate apoptosis and autophagy directly affect Ca^{2+} signaling through interaction with the ER and mitochondrial Ca^{2+}-release and/or Ca^{2+}-uptake mechanisms. Furthermore, these Ca^{2+}-signaling proteins contribute to the functional and physical linking between ER and mitochondria. Importantly, the interplay between ER and mitochondrial Ca^{2+} signaling and ROS signaling mediates the detection, the efficient targeting, and removal of mitochondria with sustained damage. This is the key for cellular homeostasis as well as for homeostasis at the level of the whole organism. In this respect, the efficient and selective removal of damaged mitochondria by autophagy is a crucial element in the maintenance of cellular health, whereby the poisonous accumulation of ROS from dysfunctional mitochondria and eventual cell death via apoptosis are avoided. Recent studies point towards a central role for impaired autophagy and inadequate removal of damaged mitochondria during aging. At the level of the organism, apoptosis will be the ultimate resort to remove seriously damaged cells. This will particularly affect the lifespan of nondividing cells, like neurons, thereby affecting the lifespan of the whole organism.

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