Free Radical Research, March 2011; 45(3): 253–265

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REVIEW ARTICLE

Autophagy, reactive oxygen species and the fate of mammalian cells

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(*Received date: 9 July 2010; In revised form date: 16 September 2010*)

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Abstract
The paper reviews the rapidly expanding pool of information on cellular and molecular mechanisms of autophagy, including autophagy types, macroautophagy induction, formation of autophagosomes and cross-talk between autophagy and apoptosis. Special attention is given to generation of reactive oxygen species (ROS) in various cellular compartments of cells under stress conditions inducing autophagy. The roles of hydrogen peroxide and superoxide in autophagy are analysed based on the recent experimental work. The relation between ROS and life span prolongation is briefly discussed, with the final conclusion that the paradox of dual role of ROS in life and death may be solved to a considerable extent due to research on autophagy.

Keywords: *Autophagy, ATG genes,* ATG *proteins, reactive oxygen species, autophagy-apoptosis cross-talk, ageing.*

Abbreviations: *AKT, protein kinase B, serine-threonine kinase; Alfy, autophagy-linked FYVE protein; AMBRA1, activating molecule in beclin-1-regulated autophagy-1; AIF, Apoptosis-inducing factor; AMPK, AMP-activated kinase; ATF4, Activating transcription factor 4; ATF6, Activating transcription factor 6; ATG, Autophagy related gene; Bax, Bcl2-associated X protein; Bcl-2, B-cell lymphoma 2; BH3, Bcl-2 Homology 3; BI-1, Bax Inhibitor 1; BIF-1, BAX-interacting factor-1; BNIP3, Bcl-2/E1B 19 kDa interacting protein, a pro-apoptotic member of the Bcl-2 family; CHOP, C/EBP homologous protein; DOR, diabetes- and obesity-regulated; DRAM, damage-regulated autophagy modulator; eIF2α, eukaryotic Initiation Factor-2α; ER, Endoplasmic reticulum; ERO1, ER oxidase1; FOXO forkhead transcription factor, target of PI3K/ PKB signalling; GATE16, Golgi-associated ATPase enhancer of 16 kDa; GFP, green-fluorescent protein; GSH/GSSG, glutathione reduced/oxidized; HMGB1, high-mobility group box 1; IGF1, Insulin-like growth factor 1; IRE1, Inositol requiring serine-threonine kinase and endoribonuclease; JNK, c-Jun N-terminal kinase; LAMP, lysosome-associated membrane protein; LC3, microtubule-associated protein light chain 3; LKB1, STK11 serine/threonine kinase 11; mTOR, mammalian target of rapamycin; NAF-1, nutrient-deprivation autophagy factor-1; MAPK, Mitogen activated protein kinase; MEK, a dual threonine and tyrosine kinase that phosphorylates and activates mitogen-activated protein kinase (MAPK); NBR1, neighbour of BRCA1 gene 1 protein 1; NF-κB, Nuclear Factor κB; PDIs, protein disulphide isomerases; PE, phosphatidylethanolamine; PERK, (PKR)-like ER kinase, RNA dependent protein kinase; PI3K I, class 1 phosphatidylinositol-3-kinase; PI3K III, class III phosphatidylinositol-3-kinase; PINK1, PTEN-induced kinase 1, a mitochondrial serine/threonine-protein kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RAF, serine/threonine-protein kinase; RAGE, receptor for advanced glycation end-products; RAS, small GTP-ase, signal transducer from cell surface receptors; ROS, reactive oxygen species; Rubicon, RUN domain and cysteine-rich domain containing; SOD1, copper-zinc superoxide dismutase (Cu, Zn-SOD); SOD2, manganese superoxide dismutase (Mn-SOD); Smac/DIABLO, second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI; UPR, unfolded protein response; UVRAG, ultraviolet (UV) radiation resistance associated gene; VPS34, vacuolar protein sorting factor protein 34 (also known as PI3K III); Xbp1, X-box binding protein 1.*

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10715762.2010.525233

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Introduction

Autophagy (self-eating in Greek) is an ancient, evolutionarily conserved cellular defence process present in eukaryotes. It provides adaptation to various stress conditions such as nutrient deprivation, oxidant-inflicted damage, endoplasmic reticulum stress due to misfolded proteins or necessity to destroy damaged organelles. It involves delivery of proteins and other macromolecules as well as whole organelles to lysosomes for degradation (see [1–6] for recent reviews). So far, 409 proteins have been identified in the autophagy proteome in a network of 751 interactions under basal conditions [7].

The peculiar feature of autophagy is that—under stress conditions—it may lead to survival or death, depending on the severity of damage, duration of the degradation process (whether it reaches a point of no return or not) and the expression profiles of autophagy-related genes (cellular context). Both these end-effects—cell survival and death—may be an advantage: macromolecule degradation provides nutrients that enable survival, whereas elimination of severely damaged cells may be safer for the organism as a whole. Autophagy is sometimes called a type II programmed cell death but this, in fact, may be unfounded, as discussed by Kroemer and Levine [8]. These authors challenged the notion of autophagic cell death. Logically, autophagy inhibition or knockout of *ATG* genes should protect against lethality; there is, however, no convincing evidence to prove such a cytoprotective effect. Further, it is difficult to discern between death *with* autophagy and death *caused* by autophagy [8]. Accordingly, some autophagy investigators consider it to be a form of programmed cell survival [9]. A particularly spectacular support for the protective role of autophagy has been provided by the discovery that lack of some autophagy genes counteracts the calorie restriction effect on life prolongation; other related reports followed showing that autophagy prolongs life span [10–12].

The dual character of autophagy is well illustrated by its effects on tumour development (review in [13]). Autophagy inhibition promotes carcinogenesis, most probably because of accumulation of damaged mitochondria. This leads to increased generation of reactive oxygen species (ROS) and, in consequence, to genome damage and increased mutation frequency. In contrast, once cancer cells develop, autophagy may favour their adaptation to a low nutrient environment or hypoxia and, thus, enhance survival.

This review gives the basic information on the main features of autophagy for those who are not familiar with this topic, relatively less well known than apoptosis. Further, ROS generation in various cellular compartments and their cellular functions as well as interactions between autophagy and apoptosis are discussed.

The three types of autophagy

The uptake and degradation of cytoplasmic organelles and macromolecules in lysosomes can occur as macro- or micro-autophagy. The most clear descriptions are given by Kunz et al., page 9987 in [14] as follows:

> In macroautophagy, nascent autophagosomes engulf parts of the cytoplasm and subsequently fuse with the lysosome. During microautophagy, vesicles bud into the lysosomal lumen by direct invagination of the boundary membrane, resulting in degradation of both cytoplasmic components and lysosomal membrane.

Microautophagy, further divided into type I (soluble cytoplasmic components) and type II (defective organelles) is defined mainly in yeast. Many authors use terms which define specific 'cargo', like mitophagy (mitochondria) or pexophagy (peroxysomes)—a selective type of autophagy involving the sequestration and degradation of the indicated organelle and occurring by a micro- or macro-autophagic process.

The third type of autophagy, the chaperone-mediated autophagy (reviewed in [15]) is a pathway of degradation of proteins that contain a peptide sequence called KFERQ motif. It consists of a Q (glutamine) flanked on either side by four amino acid residues of basic (K—lysine, R—arginine), acidic (D—aspartic acid, E—glutamic acid), bulky hydrophobic (F—phenylalanine, I—isoleucine, L—leucine, V—valine) and a repeated basic or bulky hydrophobic amino acid (K, R, F, I, L, V). The chaperone protein, Hsc70 (constitutively expressed form of heat shock protein of 70 kDa) and its co-chaperones recognize a region in the proteins to be degraded that includes the KFERQ motif. Further, the chaperones unfold the proteins and translocate them across the lysosomal membrane. A receptor in the lysosomal membrane, the lysosome-associated membrane protein (LAMP) type 2A and intra-lysosomal hsc70 (lyhsc70) are required for the substrate protein entry into the lysosomal lumen. The substrate proteins are degraded by lysosomal proteases, whereas the hsc70 chaperone complex is released from the lysosomal membrane and returns to the cytosol to bind another substrate.

Autophagy in mammalian cells

The term autophagy as used here will exclusively mean macroautophagy, a relatively well characterized phenomenon in mammalian cells. Chaperone-mediated autophagy and micro-autophagy are out of scope of this review.

Autophagosome formation

Autophagy is best characterized in yeast, where 31 *ATG* (AuTophaGy related genes) have been identified,

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whereas in mammalian cells identification still is underway [16–18] and certain less well characterized steps are presumed to be analogous to those in yeast. A recent addition to the list is that of four genes (epg-2, -3, -4 and -5); their protein products clear away aggregates of protein and RNA known as P granules in somatic cells during embryogenesis of *Caenorhabditis elegans*; three of them are present in worms, flies and mammals and active in starvation-induced autophagy [18]. The main steps of autophagy are diagrammatically presented in Figure 1, with the main protein players indicated.

First, a phagophore structure is formed, presumably from a phosphatidylinositol 3-phosphate enriched fragment ('omegasome') of membrane from mitochondria [19], endoplasmic reticulum [20–22] or plasma membrane with the involvement of the heavy chain of clathrin [23]; then, cytoplasmic material and organelles are sequestered by such double-membraned structure, creating a pre-autophagosome. The formation of the initial autophagosomal membrane requires a multi-component complex (Figure 2) with various activators and inhibitors, the presence of which depends on the cell status [24–33]. The phagophore membrane chooses its 'cargo' (cytoplasm fragment, mitochondrium or peroxysome) and elongates until the edges fuse forming the double-membraned autophagosome.

Membrane elongation is supported by two ubiquitin-like conjugation systems:

1) the mammalian homologue of yeast ATG8, microtubule-associated protein 1 light chain 3 or LC3 conjugates with PE (phosphatidylethanolamine) with the help of ATG4, ATG7 and ATG3 (yeast ATG8-PE corresponds to LC3-II); and
2) ATG5-ATG12 (formed with the help of ATG7 and ATG10).

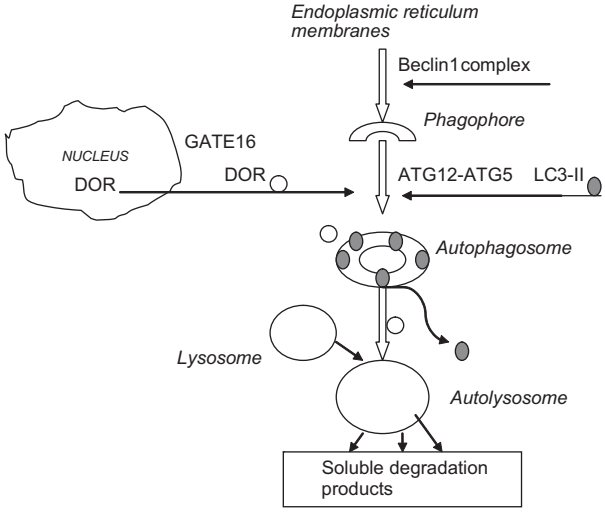

Figure 1. The main steps of autophagy. For detailed description, see text. Beclin-1 interacting proteins are listed in the legend to Figure 2.

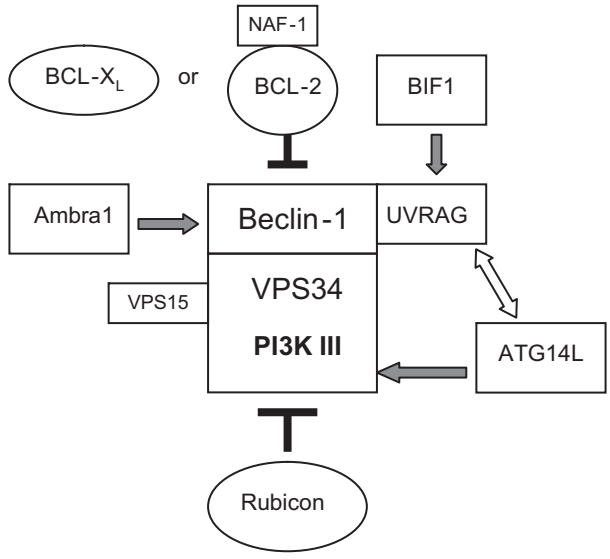

Figure 2. The components of Beclin-1 complex necessary for formation of the initial autophagosomal membrane and its further maturation. It consists of Beclin-1 (Bec1; coiled-coil, myosin-like BCL2-interacting protein; yeast Atg6), membrane-anchored kinase, VPS15 (vacuolar protein sorting factor protein 15, myristoylated membrane-anchored kinase) as well as VPS34 (vacuolar protein sorting factor protein 34 also known as the class III phosphatidylinositol-3-kinase, PI3K III) [17,22,24]. UVRAG (UV irradiation resistance-associated gene) seems to be exchangeable with Atg14L (yeast Atg14-like, also known as Barkor, Beclin 1-associated autophagy-related key regulator) [24,25]. Atg14L and Rubicon (RUN domain and cysteine-rich domain containing) bind to Beclin-1 at different stages of autophagy and reciprocally regulate its course [27,28]. Atg14L enhances VPS34 (PI3K III) lipid kinase activity and up-regulates autophagy at early stages, whereas Rubicon reduces VPS34 activity and down-regulates autophagy at late stages. Other constituents of the complex comprise Bif-1 (endophilin B1), Ambra 1 (activating molecule in Beclin-1-regulated autophagy) and BCL-2 or BCL-X_L. The latter two proteins constitutively bind to the BCL-2 homology-3 (BH3) domain of Beclin1 and repress its function, whereas under stress conditions, in the presence of BH3-only proteins, they dissociate, thus enabling the PI3KIII complex to act [29–32]. A recent addition to the list is NAF-1 (nutrient-deprivation autophagy factor-1); it stabilizes the interaction of BCL-2 with Beclin 1 and is required for BCL-2 to functionally antagonize Beclin 1-mediated autophagy [33].

Activity of these interdependent conjugation systems leads to the conversion of the soluble form of LC3 (LC3-I) to the autophagic vesicle-associated form (LC3-II), a marker of autophagy. In a recent review, Noda et al. [34] describe the role of ATG5-ATG12-containing ATG16L complex in LC3-II formation and closing of the autophagosome vesicle. The ATG16L complex remains localized on the outer surface of the autophagosome and its detachment follows the closure of the vesicle. It has been proposed by these authors, page 984 in [34] that this autophagy step proceeds 'in a topologically similar but reverse order to membrane fusion during the escape of influenza virus from the endosome'. Apart from these ATG proteins, autophagosome maturation has been reported to depend on transglutaminase 2 mediated post-translational modifications

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[35] and a late endosome-/lysosome-associated small GTP binding protein, Rab7 colocalized with LC3 [36–39]. Auto-phagosome formation also is stimulated by the diabetes- and obesity-regulated (DOR) nuclear protein which leaves the nucleus and interacts with GATE16 (Golgi-associated ATPase enhancer of 16 kDa) [40] and LC3 [41].

In the subsequent autophagy step the autophagosome looses LC3 molecules by deconjugation from PE, a step carried out by protease ATG4 [42,43]; then, the autophagosome fuses with lysosome, forming the autolysosome. The fusion step is preceded by detachment of DOR and assisted by microtubules. The role of the cytoskeleton has further been emphasized by the discovery that the ubiquitin-binding deacetylase, histone deacetylase-6 (HDAC6) [44], has the ability to bind ubiquitin as well as to associate with microtubules and the F-actin cytoskeleton. This enzyme is indispensable for the clearance of protein or damaged mitochondria aggregates and fusion of autophagosomes and lysosomes ('quality control autophagy'), this function, however, is not needed in starvation-induced autophagy [44].

Inside the autolysosome, the vacuolar H+ ATPase (V-ATPase, proton pump) maintains an acidic environment necessary for the catalytic activity of the proteases which carry out degradation of the 'cargo' molecules and/or organelles.

Recently, it was found [45] that mammalian autophagy can occur not only through the ATG5- and ATG7-dependent (canonical) pathway but also through an alternative ATG5- and ATG7-independent pathway. So far, it is not clear what are the pre-requisites for activation of the canonical or non-canonical pathway, the latter, however, seems to involve autophagosome formation from late endosomes [45].

Selectivity

Recent evidence indicates that autophagy, previously considered as a non-selective process, mediates selective removal of protein aggregates, organelles and micro-organisms [46–48]. Clearance of 'cargo' constituted by dysfunctional organelles or misfolded protein aggregates proved to be selective due to the presence of tags recognized by receptor proteins, which subsequently interact with proteins from the Atg8 family. Receptor proteins contain WXXL-like sequences (tryptophane-X-X-leucine), which Noda et al. [34] termed the Atg8-family interacting motif (AIM). For example, protein aggregates are tagged by ubiquitin; the tag is recognized by receptors, p62 (polyubiquitin-binding protein p62/SQSTM1 sequestosome 1), Alfy (autophagy-linked FYVE protein) or NBR1 (Neighbour of BRCA1 gene 1 protein 1). These, in turn, are recognized by LC3, the protein that is essential for autophagosomal membrane formation.

Another example of the recently resolved selectivity is that of mitophagy. Dysfunctional mitochondria with decreased membrane potential accumulate increased amounts of PINK1 (PTEN-induced kinase 1, a mitochondrial serine/threonine-protein kinase). This constitutes a signal needed to induce Parkin-catalysed K63- and K27-linked polyubiquitylation of mitochondria [49–54]. Parkin is a PARK2 gene product with a E3 ubiquitin ligase activity [55]. Its action on mitochondria is controlled by Nix, a member of BH3-only Bcl-2 family. Interestingly, another member of this family, BNIP3, also can selectively induce mitophagy, albeit by a different mechanism [56]. Nix can directly interact with LC3 and participate in Parkin-independent mitophagy [54]. The role of p62 is to direct the ubiquitinated mitochondria to autophagosomes.

Methods of autophagy detection

The presence of smooth, ribosome-free double membranes forming vesicles in the cytoplasm can be detected by electron microscopy and is the chronologically first method of macroautophagy (henceforth referred to as autophagy) detection. The LC3-II protein is a popular marker of the completed autophagosomes and usually is detected by western blotting. The use of green-fluorescent protein (GFP)–LC3 construct allows one to estimate autophagosome formation by immunofluorescence. Staining with acidotropic dyes (e.g. monodansylcadaverine, acridine orange, LysoTracker Red) detects lysosomes and autolysosomes, but not the early autophagic vacuoles. A detailed discussion of methods of autophagy detection, inhibition and autophagy flux monitoring, as well as practical recommendations and definitions, can be found in the 'Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes' [57].

Autophagy inhibitors

Autophagy can be inhibited in many ways, depending on the choice of step to be interfered with. Figure 3 shows the often used inhibitors and their sites of action. Since autophagy pathways are closely intertwined with those of apoptosis and numerous signalling pathways mentioned below, most inhibitors applied both *in vivo* and *in vitro* cannot be considered strictly specific for autophagy. Inhibitors of PI3K III which interfere with the sequestration step also act on PI3K I. Microtubule poisons inhibit fusion of autophagosomes with lysosomes. Inhibitors of lysosomal proteases such as leupeptin, pepstatin A and compounds that elevate the lysosomal and autolysosomal pH (bafilomycin A1, ammonium chloride, weak base amines, e.g. methylamine) counteract protein degradation not necessarily caused by macroautophagy.

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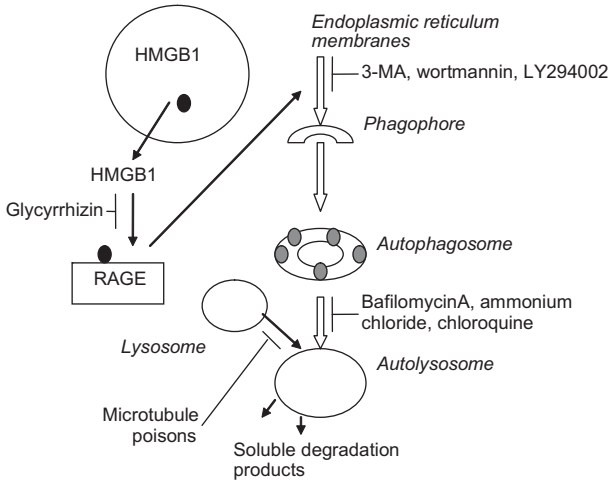

Figure 3. Autophagy steps and sites of action of the most often used inhibitors. HMGB1, high-mobility group box 1 protein; RAGE, Receptor for Advanced Glycation Endproducts; 3-MA, 3-methyladenine; LY294002, 2-morpholin-4-yl-8-phenylchromen-4-one, reversible inhibitor of phosphatidylinositol-3-kinases. For detailed description, see text.

Figure 3 also shows one more way of autophagy inhibition by blocking an early step of phagophore assembly, namely, by prevention of the nuclear export of HMGB1 (high-mobility group box 1) with ethyl pyruvate or quercetin. After cytoplasmic translocation HMGB1 associates with RAGE (Receptor for Advanced Glycation Endproducts) and this complex activates the beclin-1/PI3K III-dependent autophagosome formation. This pathway can be intercepted by glycyrrhizin (a triterpenoid saponin glycoside of glycyrrhizic acid, present in licorice roots) which binds to the A- and B-HMB boxes, thus preventing interaction with RAGE. A description of various autophagy inhibitors, their applications and the respective references can be found in Livesey et al. [9] and Klionsky et al. [57].

Since the inhibitors listed above exert varied biological effects, strictly specific inhibition of autophagy in experiments at the cellular level is best obtained by using RNA interference to silence genes coding for proteins involved in the autophagic processes. Example of the use of mutated ATG family genes to induce a desired effect is given in Fujita et al. [58], where over-expression of an inactive Atg4B mutant inhibited autophagosome maturation. A less direct effect on autophagy can also be exerted by the use of inhibitors of signalling pathways involved in autophagy regulation (mentioned in the following section).

Signalling pathways involved in autophagy induction

The autophagic process is inhibited or activated in response to a variety of intra- and extracellular stimuli. Figure 4 shows in a simplified way some of the main signalling pathways which play crucial roles in almost all cellular functions; they also are involved in activating or inhibiting autophagy. Since the signalling pathways are inter-related, the outcome of their interactions and responses to specific stimuli

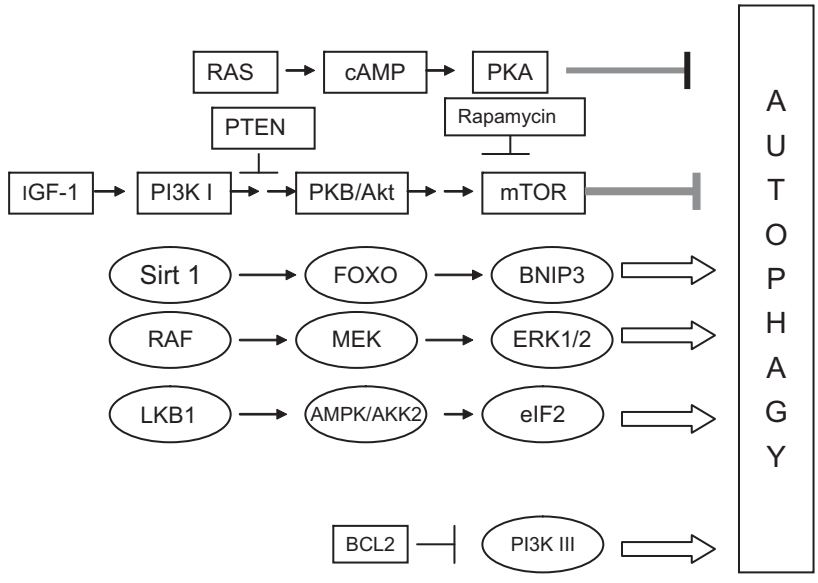

Figure 4. The main signalling pathways involved in activating (arrows) or inhibiting (inverted T) autophagy, presented in a simplified way, with some steps and interactions omitted for clarity. AKT or PKB, protein kinase B, serine-threonine kinase; AMPK, AMP-activated kinase; BNIP3, Bcl-2/E1B 19 kDa interacting protein, a pro-apoptotic member of the Bcl-2 family; cAMP, cyclic AMP; eIF2, eukaryotic initiation factor 2, required in the initiation of translation; ERK, extracellular-signal-regulated kinase; FOXO forkhead transcription factor, target of PI3K/PKB signalling; IGF1, insulin-like growth factor 1; LKB, STK11 serine/threonine kinase 11; MEK, a dual threonine and tyrosine kinase that phosphorylates and activates mitogen-activated protein kinase (MAPK); mTOR, mammalian target of rapamycin; PI3K I, class 1 phosphatidylinositol-3-kinase; PI3K III, class 3 phosphatidylinositol-3-kinase; PKA, protein kinase A; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RAF, serine/threonine-protein kinase; RAS, small GTP-ase, signal transducer from cell surface receptors; SIRT1, sirtuin1, histone deacetylase.

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much depends on the cell type, its microenvironment, nutrient and oxygen supply, energy status, available growth factors, to list only a few factors causally related to autophagy induction. A detailed review of these pathways and their relation to ageing and life span prolongation can be found in Yen and Klionsky [59].

The pathway involving mTOR (mammalian target of rapamycin) is the central one that blocks the cell's path towards autophagy; hence, mTOR inhibitor, rapamycin, efficiently induces this process. Inhibitors of kinases in pathways activating autophagy (listed in Figure 4) or those of Sirtuin 1, a NAD-dependent deacetylase can affect autophagy, but their specificity is low, as these signalling pathways are involved in numerous cellular functions.

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Generation of ROS in cellular responses leading to autophagy

The main autophagy inducers are erroneous folding, inhibited glycosylation and maturation of newly synthesized proteins, disturbed proteasomal protein degradation, defective mitochondrial respiration, nutrient and/or oxygen deficiency, lacking or disturbed growth factor signalling. Generation of ROS takes place mainly in three cellular compartments, depending on the type of autophagy-inducing stimulus: in the ER, mitochondria or cytosol. It is, however, increasingly clear that at all three locations the ROS generating processes mutually affect each other and also are influenced by exogenous ROS.

Mitochodrial respiration is the main source of ROS but also is a target of ROS produced at other cellular locations, e.g. by cytosolic NADPH oxidases or in the endoplasmic reticulum (ER). As discussed further in the text, interactions between various types of ROS at different cellular locations shape the cell's response to the environmental stress.

Endoplasmic reticulum and unfolded protein response

Unfolded protein response (UPR) is a common way of autophagy induction. It is initiated in the endoplasmic reticulum (ER) where the proper folding and post-translational modifications of the freshly synthesized protein molecules depend on energy supply, proper calcium ion concentration, redox status and last but not least, the chaperone control system for checking the folding of polypeptide chains and the specific protein disulphide isomerases (PDIs) (see [60–67] for reviews). As remarked by Malhotra and Kaufman [60], maintenance of proper conditions for protein-folding reactions in the ER can be easily disturbed by environmental insults. When these conditions are not fulfilled, misfolded proteins accumulate as aggregates and UPR is initiated. In particular, alterations in redox status, exogenous or endogenous ROS can directly and/or indirectly affect ER functions and protein folding.

There are three ER stress transducers, transmembrane proteins that respond to elevated levels of unfolded and misfolded proteins in the lumen of ER:

1) RNA-dependent protein kinase PERK (PKR-like ER kinase; PKR is a double-stranded RNA-activated protein kinase), which phosphorylates eukaryotic translation factor 2 on the alpha subunit (eIF2α) to attenuate mRNA translation initiation; exceptionally, eIF2· phosphorylation increases translation of Atf4 mRNA which encodes a transcription factor required to activate the UPR-induced genes;
2) ATF6 (activating transcription factor 6) is cleaved in the Golgi complex and transferred to the nucleus where it acts as a transcription factor; and
3) IRE1 (Inositol REquiring serine-threonine kinase and endoribonuclease) cleaves a 26 base intron in Xbp1 (X-box binding protein 1) mRNA which subsequently generates a transcription factor.

Additionally, ER-stress causes a release of Ca^{2+} from the ER, thus increasing the cytosolic free Ca^{2+}. All these events activate various pathways that lead to apoptosis and autophagy. The balance between these two processes depends, among others, on the expression/activation of the pro-apoptotic transcription factor CHOP (C/EBP homologous protein) [68] and BAX inhibitor-1 (BI-1) [69]. The latter blocks BAX mediated apoptosis by enhancing the anti-apoptotic function of the anti-apoptotic BCL2 family members (cf [65] for detailed discussion).

PDIs are the enzymes that act as thiol oxidases and/or isomerases in the ER environment, in the relatively oxidizing conditions (GSH/GSSG ratios 2–3:1) producing intra- and inter-molecular disulphide bonds in nascent proteins. Reduced PDIs are then oxidized by the thiol oxidoreductase ERO1 (endoplasmic reticulum oxidase-1), a glycosylated flavoenzyme associated with the lumenal face of the ER membrane, which in turn is re-oxidized by FAD-mediated electron transfer to oxygen [70]. In this process, hydrogen peroxide is generated, whereas ERO-1-dependent oxidative activity is balanced by cytosolic glutathione pool [71]. It is estimated that disulphide bond formation and breakage of mispaired bonds generate ~ 25% of cellular ROS and consume GSH [60].

ROS generation by the cytosolic NADPH oxidases

NADPH oxidases (NOX1-5) reduce molecular oxygen to superoxide, which is further converted to various ROS; other members of this oxidase family, dual oxidases DUOX1 and DUOX2, produce hydrogen peroxide. Their function is controlled by a complicated regulatory system (review in Takeya and

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Sumimoto [72]) and also is modulated by interactions with other ROS producing cellular systems. Interestingly, PDIs were reported to interact with NADPH oxidase complexes and to regulate their activity, at least in vascular smooth muscle and endothelial cells [73]. Since these oxidases belong to the key cellular sources of ROS, this presents an interesting possibility of interaction between UPR and NADPH oxidases. In particular, the hydrogen peroxide or superoxide-producing NADPH oxidase 4 (NOX4) responds by activity increase to such UPR triggering agents as tunicamycin, protein glycosylation inhibitor or thapsigargin, inhibitor of calcium ion pumps which discharges intracellular Ca^{2+} stores [67].

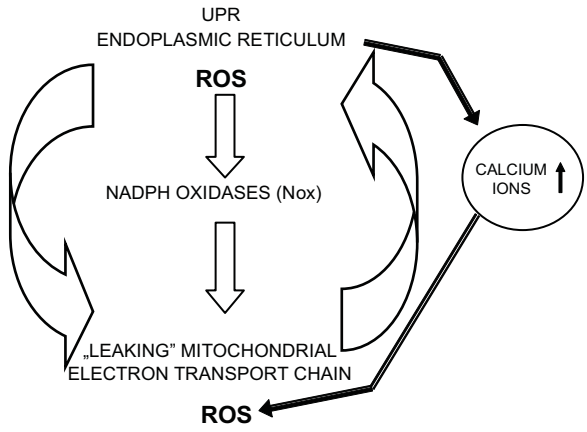

Figure 5. Interactions between cellular ROS sources and feedback loops due to which an amplification of the initial ROS production is achieved. For detailed description, see text.

Mitochondria as the ROS source in stress responses

During mitochondrial respiration, ~ 2–3% of oxygen is incompletely reduced [74]; thus, generation of superoxide anion radicals takes place by complexes I and II into the mitochondrial matrix, whereas complex III produces superoxide on both sides of the inner mitochondrial membrane. Under hypoxic conditions or high calcium concentration in the cytosol, superoxide production is enhanced. Superoxide's breakdown is carried out by the mitochondrial manganese superoxide dismutase (MnSOD, SOD2) and the mitochondrial membrane is permeable to the product, hydrogen peroxide. Superoxide may also be transferred to the cytosol through the voltage-dependent anion channels [75] to become the substrate for the cytosolic Cu, Zn-SOD (SOD1). Hydrogen peroxide may then meet iron or copper ions and engage into Fenton type reaction to produce the highly reactive hydroxyl radical.

Figure 5 shows the interactions between the above-mentioned cellular ROS sources and the feedback loops due to which an amplification of the initial ROS production is achieved. When malfunctioning, 'leaking' mitochondria produce increased amounts of ROS, this affects the redox conditions in the ER, leading to disturbed protein folding, UPR stress, ROS production by PDIs and ERO-1 and calcium ion release into the cytosol. Calcium becomes concentrated in the inner matrix of the mitochondria, leading to disruption of the respiratory electron-transport chain and further production of ROS. As mentioned above, PDIs also increase NOX-mediated ROS production. Further, NOX-produced cytosolic ROS cause mitochondrial dysfunction and, thus, trigger ROS production [76]. Examples of interactions between the ROS-producing cellular systems are reviewed by Daiber [77].

Senescent lysosomes as an ageing-related ROS source

It should be mentioned that in senescent cells there is still another source of ROS. In such cells, lysosomes contain lipofuscin, a pigmented material derived from cross-linked undegraded proteins, accumulating with age. Many metalloproteins are directed for degradation into lysosomes (among them ferritin and metalloproteins from malfunctioning mitochondria degraded in the course of mitophagy). Hence, lipofuscin contains iron and copper ions able to catalyse Fenton reaction and, thus, is a source of hydroxyl radicals. The necessary substrate, hydrogen peroxide, can diffuse into lysosomes from the cytoplasm. This age-related functional change is the cause of decreased degradation capacity of lysosomes and, hence, diminished efficiency of autophagy, as well as enhanced oxidative stress in ageing cells [78]. Also, this is the cause of disturbed mitochondrial function, as explained by the mitochondrial–lysosomal axis theory of ageing based on analysis of the relations between lipofuscin accumulation, decreased autophagy, increased ROS production and mitochondrial damage in senescent cells [79].

Cross-talk between autophagy and apoptosis

It seems significant for cell survival or death under various stress conditions but also somewhat confusing that ROS are instrumental in inducing both apoptosis (death receptor independent or intrinsic) and autophagy. The latter can be envisaged as a cellular quality control for proteins and organelles, whereas apoptosis is a supracellular quality control system that eliminates faulty cells from the organism. The balance between the life-sustaining adaptation strategy represented by autophagy and the apoptotic death chosen as a lesser evil for the organism is maintained by a complicated control system. The cellular surveillance machineries for both processes are partly overlapping, whereas in some cases autophagy and apoptosis are mutually exclusive (reviewed in Maiuri et al. [62]). This feature is proposed to be exploited in cancer therapy, since in cells with defective apoptosis

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autophagy may contribute to cell death [80]. As an example of the molecular mechanism, where apoptotic machinery counteracts autophagy can serve the effect of caspase cleavage of Beclin-1. It prevents Beclin-1-induced autophagy and, furthermore, apoptosis is enhanced by the C-terminal fragment of Beclin 1 that results from this cleavage and promotes the release of pro-apoptotic factors from mitochondria [81].

As discussed above, BCL-2 and BCL-X_L exert an anti-apoptotic and anti-autophagic effect, whereas the pro-apoptotic BH3-only proteins (e.g. BNIP3) also activate the key autophagic Beclin-1 complex. ROS-generating cellular systems provide signals both for autophagy and apoptosis. An example of such dual role is provided by the mitochondria with uncoupled electron transport chain. They produce ROS that amplify the pro-autophagic and pro-apoptotic signalling and also liberate pro-apoptotic proteins such as cytochrome c, Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI), Omi/HtrA2 protease, contributing to caspase activation or apoptosis-inducing factor (AIF) and endonuclease G, both acting in the nucleus as caspase-independent factors (review in Saelens et al. [82]).

Both apoptosis and autophagy are activated by signal transduction pathways involving IGF-1-AKT-mTOR or LKB1-AMPK that are under control of p53 [83,84]. Certain pro-autophagic proteins are encoded by p53 target genes, among them DRAM (damage-regulated autophagy modulator), a lysosomal protein [85]. These proteins also have different functions in apoptosis induction, as discussed in Crighton et al. [85]. Whereas nuclear p53 acts as pro-autophagic transcription factor, it inhibits autophagy when present in the cytoplasm [86,87].

This striking symmetry in apoptosis and autophagy regulation complies with the observations of switching from one process to the other. As indicated by Nishida et al. [88], mitochondria function as an important switch between autophagy and apoptosis. Under conditions of mild stress, autophagy is induced and it functions as a cell survival mechanism. With increasing stress, autophagic activity may be insufficient to maintain cellular homeostasis. Accumulation of misfolded proteins and/or defective mitochondria add to the stress conditions. Malfunctioning mitochondria start to release cytochrome *c*, thus initiating apoptotic cell death. Under extremely unfavourable conditions, mitochondrial membrane depolarization and ATP depletion follow, causing necrosis. So, mitochondria are

Table I. Summary of experimental data [68] proving that superoxide is the main ROS active in autophagy induction.

Treatment	HeLa cells	Intracellular superoxide[a] (48 h)	Intracellular hydrogen peroxide[b] (48 h)	Autophagy(72 h)
Over-expression of SOD2 down-regulates starvation-induced autophagy				
AA starvation	WT vs SOD2	In WT cells significantly higher than in SOD2, $p < 0.01$	Equal levels	WT significantly higher than SOD2, $p < 0.01$
GP starvation	WT vs SOD2	In WT cells significantly higher than in SOD2, $p < 0.01$	In SOD2 cells significantly higher than in WT, $p < 0.01$	In WT cells significantly higher than in SOD2, $p < 0.01$
siRNA knockdown of sod-2 *upregulates autophagy*				
AA starvation	WT control siRNA vs *sod-2* siRNA	In *sod-2* siRNA treated significantly higher than in the control, $p < 0.05$	In *sod-2* siRNA treated significantly lower than in the control, $p < 0.05$	In *sod-2* siRNA treated significantly higher than in the control, $p < 0.05$
GP starvation	WT control siRNA vs *sod-2* siRNA	In *sod-2* siRNA treated significantly higher than in the control, $p < 0.05$	No significant difference	In *sod-2* siRNA treated significantly higher than in the control, $p < 0.05$
Over-expression of SOD2 down-regulates exogenous hydrogen peroxide-induced autophagy				
1 mM hydrogen peroxide 24 h	WT vs SOD2	In WT cells significantly higher than in SOD2, $p < 0.002$	In SOD2 cells significantly higher than in WT, $p < 0.001$	In WT cells significantly higher than in SOD2, $p < 0.05$

AA, amino acids and serum; WT, wild type HeLa cells; SOD2, HeLa cells over-expressing manganese superoxide dismutase (mitochondrial) SOD2; GP, [f] glucose, L-glutamine, pyruvate and serum.

[a]estimated from ethidium fluorescence positive cells.

[b]estimated from dichlorofluorescein fluorescence positive cells.

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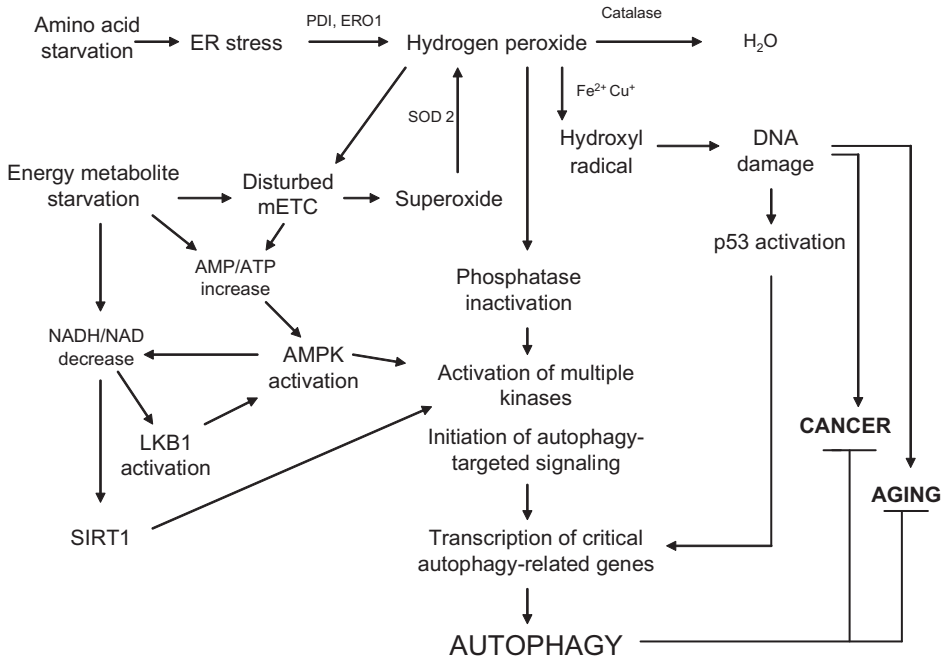

Figure 6. Diagram summarizing the main events in starved cells that lead to autophagy. Main sites of action of ROS are shown. DNA damage denotes both mitochondrial and nuclear DNA. AMPK, AMP-activated kinase; mETC, mitochondrial electron transport chain; ER, endoplasmic reticulum.

an essential part of the cellular system that carries out a concerted response to various stress factors and maintains homeostasis or convicts the cell to death [89].

The role of ROS in autophagy induction

In most early papers on autophagy the term ROS is used without specifying what is the active species. As indicated by Chen et al. [90], usually catalase and N-acetylcysteine are used as antioxidants in experiments *in vitro*—which act specifically against hydrogen peroxide. Also, exogenous hydrogen peroxide often is used for autophagy induction without measuring its actual intracellular concentration. Chen at al. [90], in a very thorough study, were the first to measure in parallel the cellular content of hydrogen peroxide and superoxide and have shown that the level of the latter species is correlated with the extent of autophagy induction. Table I summarizes some of the experimental results which support this conclusion (control data of these experiments are omitted for clarity). This does not mean that hydrogen peroxide is not important in autophagy—as discussed in the preceding section, its generation, e.g. due to ER stress affects the function of mitochondria. Loss of membrane potential is the cause of superoxide production and the level of SOD2 is one of the factors that decide on the ultimate fate of the cell. A startling finding is that exogenous hydrogen peroxide is converted to intracellular superoxide [90] (cf Table I). Nevertheless, the extensive interactions between the ROS producing cell compartments and the feedback loops (Figure 5) explain the presence of all types of ROS in cells under oxidative stress conditions. Consistently, antioxidants such as catalase or N-acetylcysteine counteract the effect of hydrogen peroxide on mitochondria and decrease the autophagic response [90].

Interestingly, amino acid starvation induces both superoxide and hydrogen peroxide generation, whereas glucose starvation induces only superoxide generation [90]. This, in fact, could be predicted from what is known on the mechanisms of the cellular response to these kinds of stress. Deprivation of amino acids for protein synthesis leads to ER stress and hydrogen peroxide generation by PDIs, as discussed in the preceding sections. Lack of energy metabolites, e.g. glucose, affects the function of mitochondria (see Benard et al. [91] for review). There follows the increase in AMP/ATP ratio which activates AMPK [92], whereas NADH/NAD ratio decreases leading to activation of LKB1 and AMPK [93] as well as SIRT1 [94]. These interactions are depicted in Figure 6.

The abundance of iron ions may modify the cell's response, as hydroxyl radicals produced in the Fenton-type reaction are highly reactive. If generated in the nucleus, they can damage DNA and the resulting p53 activation—depending on the extent of damage and the cellular context—may lead to apoptosis or autophagy. As mentioned previously, ageing cells accumulate iron ions and produce ROS. This leads to disturbed mitochondrial respiration and damage of mitochondrial and nuclear DNA accelerating age-related unfavourable changes in cellular functions [59,79].

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One can ask why are ROS so important in autophagy induction when at least some of the cellular signalling pathways leading to autophagy (Figure 4) can be launched by mechanisms apparently independent of ROS (e.g. inhibition of the mTOR pathway). In experiments carried out *in vitro*, such as mentioned in Table I, autophagy induction is achieved in the absence of glucose or amino acids and serum, which is the source of growth factors. One role of ROS in the cellular response to starvation may be to inactivate redox-sensitive, cysteine-based enzymes. Such enzymatic target of ROS is the cysteine protease ATG4. Its inactivation is necessary to ensure conjugation of ATG8/LC3 to autophagosome membrane and the target for oxidation is a cysteine residue placed near the catalytic site of ATG4 [95]; the same mechanism operates with other cysteine proteases and dehydrogenases. Also protein phosphatases contain a nucleophilic catalytic cysteine highly susceptible to oxidation [96–99]. Inactivation of phosphatases causes a shift in the equilibrium between the phosphorylated and dephosphorylated forms of various kinases. In fact, activation of receptor kinases by EGF or IGF-1 is accompanied by superoxide generation and its dismutation into hydrogen peroxide by SOD1 [99]. Hydrogen peroxide then inactivates the protein tyrosine phosphatases in the vicinity. This mechanism supports the ligand-mediated activation of receptor kinases, but may act independently, e.g. in X-irradiated cells known to respond by activation of growth factor receptors (reviewed in Szumiel [100]). Additionally, transcription factors such as FOXO, Nrf2, HIF-1 and NFκB are redox-controlled; thus, ROS effects on cellular functions may be considerable (reviewed in D'Autréaux and Toledano [101]).

This ROS-driven activation mechanism may be especially important in the case of receptor kinases, normally activated by ligands present in the serum. Although at first glance ROS seem to be a by-product of the cellular processes taking place under stress conditions, their importance is accentuated by the pronounced autophagy inhibition by antioxidants [90]. So, it is plausible to assume that they are indispensable for launching the signalling pathways involved in autophagy induction.

Concluding remarks

The fate of a cell subjected to various kinds of stress is either survival or death. A surviving but functionally altered cell may bring more complications for the multi-cellular organism than that killed and eliminated, as the alteration (genetic or epigenetic) may bring pathological consequences, including early ageing or malignancy. Autophagy may improve the chances of survival under starvation conditions by eliminating dysfunctional organelles, thus preventing pathological processes and favouring life span prolongation. On the other hand, its pathways can bring about cell death. These paradoxical aspects of autophagy have been discussed in the text. Another paradox is the role of ROS in the life of mammalian cells: inducing pro-survival and anti-cancer autophagy in parallel to mutational and causally related ageing effects and apoptotic death (Figure 6).

There are still many gaps in our understanding of autophagy mechanisms and role in the life and death of the mammalian cell. Its importance for ageing is documented but not fully understood [102]. Nevertheless, the knowledge of autophagy has opened new perspectives on ageing and carcinogenesis, as well as new therapeutic possibilities in oncology [9–13, 80,103].

Among the contemporary theories of ageing, the most convincing seems the mitochondrial free radical theory of ageing: increasing production of ROS by mitochondria in ageing individuals causes mitochondrial DNA damage and this is the primary cause of ageing and death and the major determinant of life span. In contrast to predictions, high levels of oxidative damage in mitochondrial DNA do not always decrease longevity and low levels do not guarantee prolonged lifespan [104,105]. On the other hand, calorie restriction is an unfailing way to achieve lifespan prolongation and it is known to stimulate mitochondria biogenesis [106].

It seems that the apparent contradictions can be explained as follows. Destruction of faulty mitochondria which produce potentially destructive ROS is of primary importance in prevention of mitochondrial and nuclear DNA damage; mitophagy carries out this function [107], on the same principle preventing ageing and carcinogenesis. As ROS generation is indispensable for autophagy, the argument that low ROS production does not prevent ageing cannot be taken against the ROS theory of ageing. To achieve the anti-ageing effect, autophagy must be induced and, here, ROS generation at adequate rates is a necessary but not sufficient pre-requirement. Interestingly, SIRT1 promotes both autophagy and mitochondrial biogenesis by deacetylating PGC-1α (peroxisome proliferator-activated receptor-γ coactivator 1α) (review in Lanz and Nair [106]). Similarly, pro-autophagic stimuli cause AMPK activation and the kinase activates PGC-1α, thus promoting biogenesis of mitochondria. This can be taken as an indication that to play a role in life span prolongation, the pro-autophagic stimuli should also include stimulation of mitochondrial biogenesis.

In conclusion, the paradox of dual role of ROS in life and death may be solved to a considerable extent due to research on autophagy. Undoubtedly, there remains much to be discovered and this is an exciting perspective.

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Declaration of interest

The author is supported by the statutory grant of the Ministry of Science and Higher Education to the Institute of Nuclear Chemistry and Technology.

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This paper was first published online on Early Online on 20 October 2010.

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