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# ROS, mitochondria and the regulation of autophagy

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**Accumulation of reactive oxygen species (ROS) is an oxidative stress to which cells respond by activating various defense mechanisms or, finally, by dying. At low levels, however, ROS act as signaling molecules in various intracellular processes. Autophagy, a process by which eukaryotic cells degrade and recycle macromolecules and organelles, has an important role in the cellular response to oxidative stress. Here, we review recent reports suggesting a regulatory role for ROS of mitochondrial origin as signaling molecules in autophagy, leading, under different circumstances, to either survival or cell death. We then discuss the relationship between mitochondria and autophagosomes and propose that mitochondria have an essential role in autophagosome biogenesis.**

## Autophagy has a dual role in the cellular response to oxidative stress

High levels of ROS (Box 1) can oxidize cell constituents, such as lipids, proteins and DNA, and thus pose a threat to cell integrity (Box 2). Various defense mechanisms have been developed to protect cells against oxidative stress, such as up-regulation of antioxidants, removal of specific proteins by the ubiquitin–proteasome system [1] and removal of damaged proteins and organelles by autophagy [2]. Autophagy is a major pathway for delivery of proteins and organelles to lysosomes in mammals or to the vacuole in yeast, where they are degraded and their components recycled (Box 3). A role for autophagy in the response to ROS is highlighted by the accumulation of oxidized proteins in aged cells under normal growth conditions [3,4], where autophagic pathways are compromised with age [2,5,6] and in age-related disorders, such as Alzheimer's disease [7] and diabetes mellitus [8], where there is also a decrease in autophagy. Although autophagy is largely considered nonselective, preferential autophagy of damaged or excess organelles, such as peroxisomes [9], endoplasmic reticulum (ER) [10–13] and mitochondria [14], can occur and there is accumulating evidence for selective autophagic processes in response to ROS. Mitophagy, the selective degradation of mitochondria, can be induced by several stimuli (Box 3) and was proposed to decrease the potential oxidative damage from defective mitochondria [14]. In mammalian cells, the pathway of chaperone-mediated autophagy (CMA) selectively degrades cytosolic proteins containing the KFERQ signal motif through direct translocation into the lysosome [15]. Oxidized substrate proteins of this pathway translocate into lysosomes more efficiently than their unaltered counterparts [16,17]. In addition, lysosomes subjected to mild oxidative stress show a higher tendency to bind and internalize substrates by CMA [18]. In plants, which lack the CMA pathway, macroautophagy has been shown recently to act in the degradation of oxidized proteins following severe oxidative stress [19]. Collectively, this suggests that autophagy provides the front line of defense against oxidative stress (Figure 1).

When survival mechanisms fail, death programs are activated in response to oxidative stress. In recent years, it has become accepted that autophagy, in addition to its role in cell survival, can also lead to cell death (referred to as type II cell death) [20–22]. For example, recent reports show that this form of autophagic cell death is activated in the nervous system in response to oxidative stress. In Parkinson's disease, oxidation of dopamine induces oxidative stress, autophagy and cell death [23]. Autophagy therefore has a dual role in the cellular response to oxidative stress, as summarized in Figure 1.

But could the involvement of ROS in the induction of autophagy be limited to oxidative stress? It has now become accepted that low levels of ROS can serve as regulators of cell signaling by reversibly oxidizing essential signaling components [24,25]. In this Opinion article, we discuss recent data suggesting such a regulatory role for ROS in autophagic survival and death processes and postulate that mitochondria provide a redox signal regulating autophagosome formation.

## A signaling role for ROS in autophagic cell death

The main characteristic of ROS and the basis of their destructive nature is their high reactivity. This characteristic also makes ROS excellent signaling molecules when controlled tightly. Indeed, ROS act as signaling molecules in a variety of intracellular processes, leading to proliferation, apoptosis, immunity and defense against microorganisms [24,25]. That ROS might have such a signaling role in autophagy (Figure 2) was first suggested in the pathway leading to neuronal autophagic cell death in response to nerve growth factor (NGF) deprivation [26,27]. NGF-deprived sympathetic neurons accumulated mitochondrial ROS, which causes lipid peroxidation and loss of the mitochondrial inner membrane lipid cardiolipin, resulting in autophagic cell death [26]. More recently, tumor necrosis factor (TNF)-α was shown to induce autophagic cell death through a ROS-dependent mechanism

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**Box 1. Reactive oxygen species**

Reactive oxygen species (ROS) are generally small, short-lived and highly reactive molecules, formed by incomplete one-electron reduction of oxygen. ROS include oxygen anions, free radicals, such as superoxide and hydroxyl radical, and peroxides, such as hydrogen peroxide ($H_2O_2$). These species are produced by ionizing radiation of biological molecules, as a byproduct of respiration in mitochondria or are synthesized by specific enzymes of the NADPH oxidase (NOX) and dual oxidase (DUOX) family. NOX and DUOX oxidize NADPH and reduce oxygen across the plasma membrane to generate superoxide, which can then form $H_2O_2$, which crosses the membrane and enters the cell [38,39]. The activity of NOX and DUOX is regulated tightly, producing ROS in various cells and tissues in response to growth factors, cytokines and calcium signals [40,41]. Mitochondria produce low levels of ROS as an inevitable consequence of oxidative metabolism [42]. The mitochondrial respiratory chain, which comprises four enzyme complexes, transfers electrons from NADH- and $FADH_2$-reducing equivalents to molecular oxygen, thus generating the mitochondrial membrane potential that drives the synthesis of ATP by the $F_1F_0$-ATPase and almost all other mitochondrial functions. The complete transfer of electrons to molecular oxygen produces $H_2O$. However, partial one-electron reduction often occurs, primarily at complexes 1 and 3, resulting in the formation of superoxide, which, again, could form $H_2O_2$ [42].

Low levels of ROS are normally reduced by non-enzymatic and enzymatic antioxidizing agents, such as glutathione, thioredoxin, superoxide dismutase (SOD), catalase and peroxidases. Glutathione is the main redox buffer of the cell. It is present, primarily in its reduced form (GSH), in the cytosol and to a lesser extent in the mitochondria and nucleus and is manipulated by glutathione reductase and glutathione peroxidase to maintain a reducing environment and to detoxify ROS [43]. Thioredoxin is similar to glutathione in its ability to undergo rapid oxidation–reduction reactions; however, its lower concentration in the cell (μM as opposed to mM range of glutathione) suggests that it is less significant as a general antioxidant but rather acts in redox control of specific factors, such as transcription factors and kinases [43]. SOD, present both in the cytosol and in mitochondria, converts superoxide into $H_2O_2$, which is further detoxified into $H_2O$ and $O_2$ by catalase or peroxidases, such as the glutathione peroxidase [44].

**Box 2. Oxidative stress**

Oxidative stress results from exposure to high levels of ROS, which are not detoxified by cellular antioxidizing agents [45]. ROS can oxidize cell constituents, such as DNA, proteins and lipids. Oxidation of DNA results in strand breaks and base modifications, which lead to various pathologies, including cancer, neurological diseases and cell death [56]. Oxidized proteins present a threat to cell survival because, apart from losing their functionality, they tend to form toxic aggregates, termed lipofuscin. These compounds, comprising lipids and a heterogeneous mix of proteins, compromise the activity of lysosomes and could eventually lead to cell death [9,10]. Accumulation of ROS within the mitochondria risks the functionality of this organelle owing to mitochondrial DNA mutations, lipid peroxidation and possibly the opening of mitochondrial membrane channels including the mitochondrial permeability transition pore (MPTP) and the inner membrane anion channel (IMAC) [53]. Opening of these channels leads to a collapse of mitochondrial membrane potential and a transient increase in ROS generation by the electron transfer chain, a phenomenon referred to as ROS-induced ROS release (RIRR) [57]. Furthermore, opening of the MPTP increases the permeability of the mitochondria, which might lead to a decrease in the concentrations of ATP and $Ca^{2+}$, to mitochondrial swelling and to release of cytochrome *c*. It has been postulated that, depending on the severity of damage, these phenomena result in autophagy, apoptosis or necrosis ([53].

[28]. In this study, a specific ROS, $H_2O_2$, was both sufficient and essential to induce this effect. The involvement of autophagy in this pathway was supported by the finding that siRNA knockdown of two proteins known to regulate autophagy, beclin1 [29] and Atg7 [30], reduced cell death. In another study, autophagy was implicated in the death of lipopolysaccharide (LPS)-activated macrophages. Treatment of these cells with the caspase inhibitor Z-VAD blocked classic apoptosis but nevertheless induced cell death in a caspase-independent pathway, suggested to involve autophagy [31]. ROS, in this study, were both sufficient and essential to induce autophagic cell death. The specific ROS involved in this pathway was not characterized, however, treatment with either superoxide- or nitric oxide-generating agents resulted in autophagic cell death of these macrophages. In the yeast *Saccharomyces cerevisiae*, rapamycin, an inducer of autophagy, led to a cell cycle arrest and loss of viability [32]. This type of death-related autophagy has been shown recently to be mediated by ROS production, leading to lipid oxidation in the mitochondria [33]. Finally, it has been reported recently that a short mitochondrial form of the tumor suppressor protein ARF, termed smARF, induces autophagic cell death when over-expressed, however, this is prevented by siRNA knockdown of beclin1 and Atg5 [34]. Although ROS were not reported as factors in this study, it further suggests a role for mitochondria in autophagy.

The crosstalk between redox and death-related autophagy works in the other direction as well, as shown in a unique example of selective autophagy of the ROS scavenger catalase [35]. Autophagy is considered a nonselective degradation pathway. Nevertheless, caspase inhibition leads to preferential degradation of catalase through autophagy, thereby promoting accumulation of ROS in the mitochondria and, consequently, cell death. In this case, autophagy acts upstream and induces accumulation of ROS. Based on the finding that only catalase and not superoxide dismutase (SOD) is selectively degraded, we postulate that $H_2O_2$, formed by SOD and degraded by catalase (see Box 1 for details), is accumulating specifically, which suggests a signaling role for $H_2O_2$ in this type of cell death. This conclusion is consistent with the involvement of $H_2O_2$ in TNF-α-induced cell death, mentioned earlier [28]. This study [35] raises several questions: is catalase inactivated and for that reason targeted for degradation? Is the active enzyme degraded selectively? Are these cells dying of bulk autophagy or is the involvement of autophagy in this death limited to the degradation of catalase? And, finally, could all processes of autophagic cell death detailed here be mediated by this pathway, in which autophagy induces ROS accumulation and is not only its outcome? The answer to the final question might lie in a feedback loop between ROS accumulation and autophagy because, even in this experimental setup [35], small amounts of ROS are detected long before catalase degradation is evident. It is therefore possible that low levels of ROS induce autophagy, which, in turn, degrades catalase, thereby increasing the level of $H_2O_2$, which further induces autophagy. Notably, autophagy induced by nutrient starvation did not lead to selective catalase degradation [35], although ROS were suggested to be involved in this process, as detailed later [36]. This implies that perhaps degradation of catalase, resulting in a prolonged $H_2O_2$ signal, is responsible for shifting the

**Box 3. Autophagy**

Autophagy is a general term referring to pathways for the degradation of cytosolic constituents by the lysosome/vacuole [58,59]. Autophagy has an essential role in differentiation and development in addition to its role in the cellular response to stress. It is activated during amino acid deprivation and has been associated with neurodegenerative diseases, cancer, pathogen infections and myopathies. There are three main autophagic pathways: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Macroautophagy involves the sequestration of cytosolic portions, including proteins and organelles, through double-membrane structures, termed autophagosomes, to the degradation in lysosomes. In microautophagy, cytosolic constituents are engulfed directly by lysosomes through invaginations of the lysosomal membrane. Both these pathways are conserved from yeast to mammals; however, the latter pathway is far less characterized, especially in mammalian systems. The third autophagic pathway, CMA, is found only in mammalian cells and degrades selectively cytosolic proteins that contain a specific signal motif, KFERQ. This signal motif is recognized by a specific chaperone, which translocates the protein into the lysosome through interaction with the Lamp2a receptor [15]. A fourth pathway, unique to yeast, is the cytosol-to-vacuole targeting (CVT) pathway, used for the delivery of vacuole-resident hydrolases through small vesicles (smaller than autophagosomes).

Macroautophagy, the best-described autophagic pathway (throughout this review the terms macroautophagy and autophagy are used interchangeably), is initiated by the engulfment of cytosolic portions, including proteins and organelles, by a crescent-shaped isolation membrane or phagophore. Fusion of the edges of the phagophore with each other forms a closed double-membrane structure, called autophagosome. Finally, the outer membrane of the autophagosome fuses with a lysosome to become an autolysosome and its content is degraded by lysosomal hydrolases. The origin of the autophagosomal membrane is as yet unknown, however, in yeast, a single pre-autophagosomal structure (PAS), to which many autophagy-essential genes localize, has been characterized as being involved in autophagosome formation [50].

Although considered originally nonspecific, 'bulk' degradation pathways, it is now accepted that preferential autophagy of damaged or excess organelles, such as peroxisomes [9], ER [10–13] and mitochondria [14], occurs both through micro- and macro-autophagy, under certain conditions. The selective degradation of damaged mitochondria through autophagy, termed mitophagy, occurs in response to various stimuli, both in yeast and in mammalian cells. In yeast, nitrogen starvation induces mitophagy in a process mediated by the *Uth1* gene, a regulator of oxidative stress responses that is one of four 'youth' genes that prolong yeast life span [52,60]. Mitophagy was also observed in yeast cells harboring a temperature-sensitive mitochondrial proton ATPase, under anaerobic conditions, at restrictive temperature [55]. Finally, stationary-phase yeast cells were reported to undergo mitophagy in a pathway that requires the *Aup1* gene [54]. In mammals, mitophagy was reported in rat hepatocytes exposed to nutrient starvation or to photodamage. In both cases, this process was associated with a mitochondrial permeability transition (MPT, see Box 2 for further explanation); however, no mitophagy-specific genes have been reported yet.

outcome of autophagy from survival to death. Analyzing whether catalase is degraded selectively in response to different autophagy-inducing stimuli might resolve this hypothesis.

## ROS as signaling molecules in survival-prone autophagy

In addition to the harmful activities of ROS, both in oxidative damage (Figure 1) and as signaling molecules

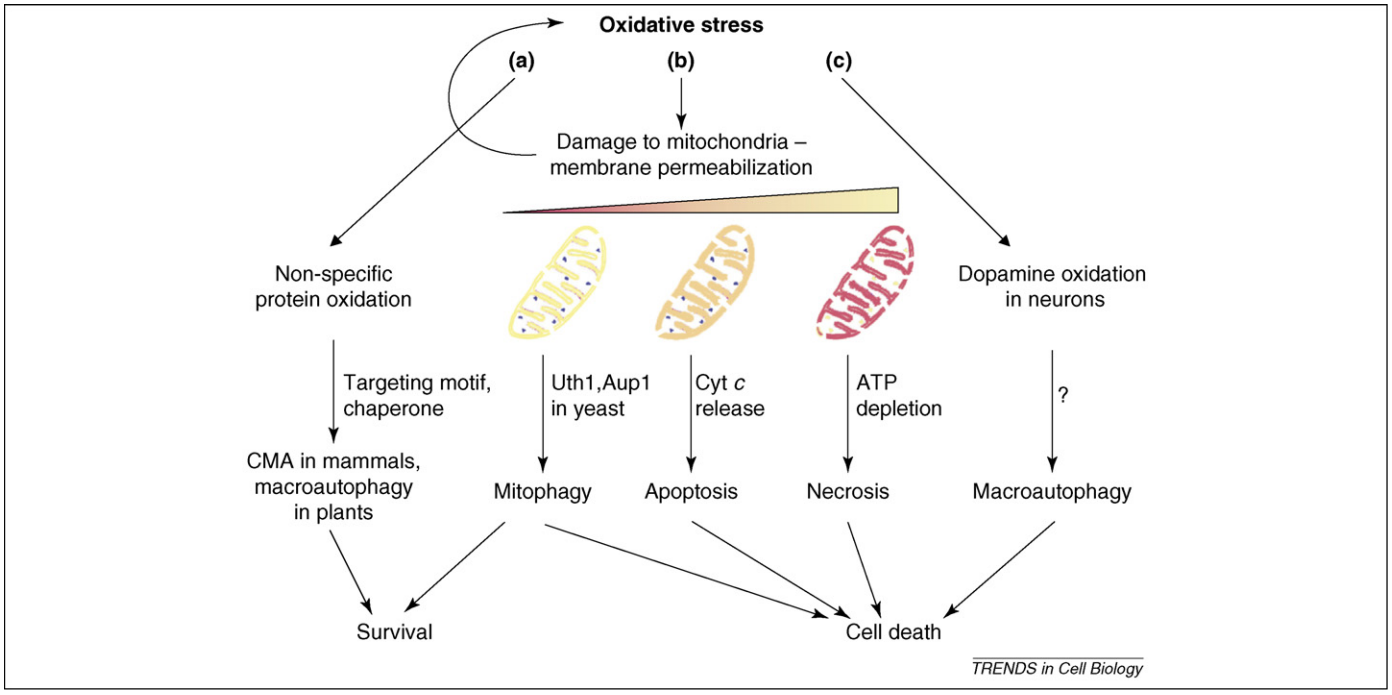


**Figure 1**. Different pathways of autophagy are activated in response to oxidative stress. Massive oxidative stress leads to autophagy through three different pathways. **(a)** Non-specific protein oxidation caused by oxidative stress activates CMA in mammals [18] and macroautophagy in plants [19] as a survival pathway. **(b)** Mitochondrial permeability transition induced by oxidative stress triggers one of several processes, depending on the severity of the oxidative damage: mild damage (pale yellow) induces mitophagy, as either survival or death pathway, through Uth1 and Aup1 in yeast and yet unknown factors in mammals [14,52–55]; increased damage (orange) triggers apoptosis in mammals following permeabilization and release of cytochrome *c*; and severe damage (red), both in yeast and mammals, results in necrosis owing to ATP depletion. Massive accumulation of ROS in the mitochondria can trigger the release of additional ROS from the mitochondria, which further increases the oxidative stress in the cell [50]. **(c)** In the nervous system, oxidative stress induces dopamine oxidation that, in turn, leads to autophagic cell death [23]. Where known, factors associated with the different processes are indicated.

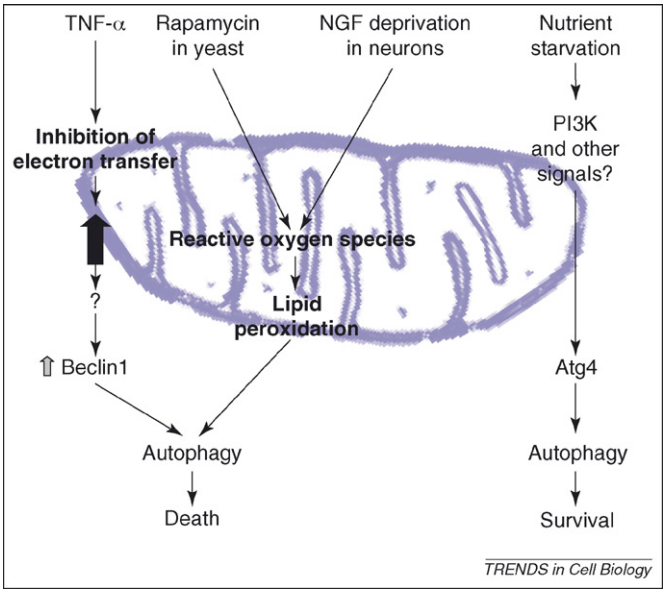


**Figure 2**. ROS formation in the mitochondria is a fundamental regulatory event in autophagy. Different pathways that require ROS formation in the mitochondria for induction of autophagy, resulting either in survival or death, are described: TNF-α induces accumulation of $H_2O_2$ [28], possibly through inhibition of mitochondrial electron transfer [46]. This signal induces the expression of Beclin 1 by an as yet unknown mechanism, leading to autophagic cell death [28]. ROS accumulation induced by rapamycin in yeast [33] or NGF deprivation in neurons [26,27] leads to mitochondrial lipid peroxidation, which signals for autophagic cell death; this mechanism, at least in yeast, is not mediated by a known mitophagy-associated factor [33]. Accumulation of $H_2O_2$ induced by nutrient starvation, mediated partially through class III phosphoinositide 3-kinase (PI3K), activates autophagy as a survival pathway through inhibition of the Atg4 protease [36].

in death-related autophagy (Figure 2), recent findings show that ROS also regulate starvation-induced autophagy, which is clearly a survival pathway [36]. Nutrient starvation was reported to lead, partially through class III phosphoinositide 3-kinase, to accumulation of $H_2O_2$ in the mitochondria, which was essential for the induction of autophagy. The oxidative signal in this experimental setup appeared minutes after induction of starvation and did not cause cell death. Furthermore, Atg4, an essential protease in the autophagic pathway, has been identified as a direct target for oxidation by $H_2O_2$. This protease cleaves the c-terminus of the Atg8 family of ubiquitin-like proteins, as a prerequisite for their conjugation to phosphatidyl ethanolamine (PtdEtn) on the autophagosomal membrane [37]. This ubiquitin-like conjugation, mediated by Atg7 and Atg3 as E1 and E2 modifiers (respectively) is essential for autophagosome maturation [30]. Conjugated Atg8 acts as another substrate for Atg4, cleaving and removing it from the mature autophagosome for recycling [37]. The finding that this protease is redox-regulated signifies a novel signal transduction pathway in which ROS function as signaling molecules to trigger autophagy as a survival mechanism (Figure 2). Once again, $H_2O_2$ appears to be the signaling ROS in this pathway. $H_2O_2$ is an attractive candidate for signaling because it is relatively stable and long lived compared with other ROS and its neutral ionic state enables it to exit the mitochondria easily. Indeed, $H_2O_2$ was implicated in various signal transduction pathways as a modifier of thiol-containing proteins [24,25]. But is Atg4 the only target of $H_2O_2$ in this pathway? And, more importantly, considering that the basic autophagic machinery appears to be conserved regardless of the specific inducer and outcome, is the inhibition of Atg4 unique to starvation-induced autophagy or will it turn out to be a general characteristic of autophagy? These questions remain to be answered.

## Mitochondria as a source of ROS to regulate autophagy

Where does the redox signal originate? Several sources of ROS exist in cells, the most prominent being NADPH oxidase (NOX), dual oxidase (DUOX) [38–41] and the mitochondria [42] (Box 1). Mitochondrial ROS are normally detoxified by superoxide dismutase (SOD), NADH and glutathione, and also by catalase in the cytosol [43–45]. Disruption, however, of the delicate balance between ROS production and elimination might lead to accumulation of ROS in the mitochondria. Several studies implicate mitochondrial ROS in the induction of autophagy (Figure 2): (i) rapamycin and NGF deprivation both induce mitochondrial lipid oxidation [26,33]; (ii) TNF-induced ROS production [28] was reported previously to result from the inhibition of mitochondrial electron transfer [46]; and (iii) nutrient starvation induces significant co-localization of ROS-positive structures with mitochondria [36]. Therefore, we propose a new role for mitochondria as the source for redox regulation of autophagy (Figure 2).

## A role for mitochondria in autophagosome biogenesis

Is the role of mitochondria in the regulation of autophagy limited to ROS production? Atg9 is one of only two integral-membrane autophagy-related proteins identified to date, which was reported, in yeast, to cycle between peripheral sites in the cell and the pre-autophagosomal structure (PAS; see Box 3 for further explanation). These two characteristics make it a favorable candidate for membrane recruitment for autophagosome biogenesis [47]. Interestingly, some of the peripheral Atg9-positive structures were reported to localize near mitochondria [47]. Two mammalian Atg9 orthologues have been described [48,49], one of which (Atg9L2) harbors a putative mitochondrial localization signal; however, its endogenous localization was not yet characterized and ectopic expression of Atg9L2 did not reveal mitochondrial localization. The other orthologue (Atg9L1) was reported to localize to the trans-Golgi network (TGN) and was suggested to cycle between the TGN and endosomes [49]. It is intriguing to speculate that the different Atg9s supply membranes from different sources for autophagosome biogenesis; however, the involvement of mitochondria in this process remains to be elucidated.

The PAS, localized at the vicinity of the vacuole in yeast, was identified as a structure to which many autophagic factors co-localize before autophagosome formation [50]. No such single structure is observed in mammalian cells; however, it is conceivable that, similar to the existence of multiple degradation sites (lysosomes), there are also multiple organizing sites. Indeed, multiple phagophores (see Box 3 for further explanation) are dispersed in the cytosol [51]. In addition, Atg5 and Atg16, two markers of autophagosome biogenesis [51], co-localize near mitochondria [36]. We propose that mitochondria act as one of the major sources of signal for the induction of autophagy and

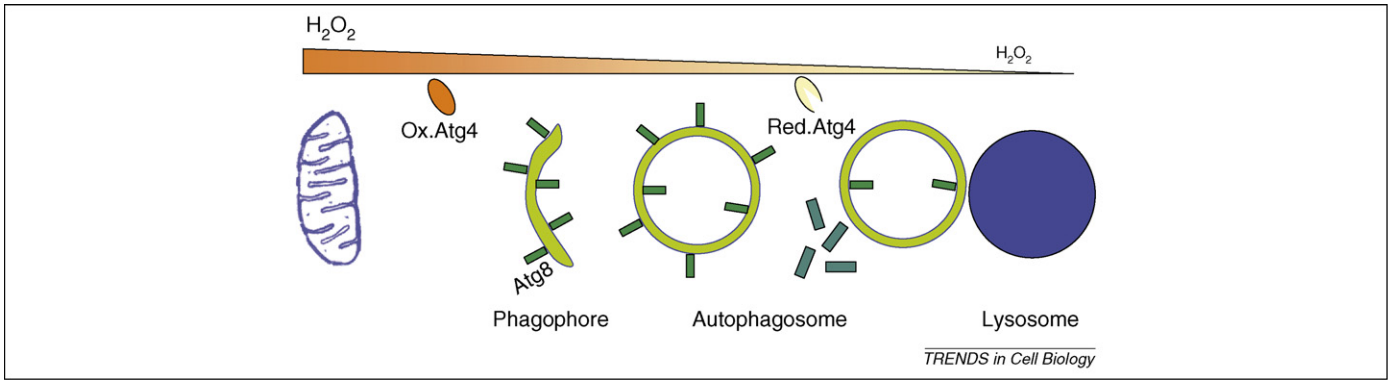


**Figure 3**. A model depicting the involvement of mitochondria in autophagosome biogenesis. Our model suggests that ROS released from the mitochondria create an oxidative gradient, which favors autophagosome formation at the vicinity of mitochondria and lysosomal degradation further away from the mitochondria, where reducing conditions prevail. In the oxidative environment, the protease Atg4 is inactive (depicted as a red oval), enabling formation of the autophagosome. Further away from the mitochondria, in a reducing environment, the protease (depicted as a yellow oval) cleaves and delipidates Atg8 (depicted as green rectangles), thereby preventing formation of new autophagosomes but enabling the recycling of this protein before degradation of the autophagosome in the lysosome.

possibly supply part of the membranes required for autophagosome formation. As depicted in Figure 3, we suggest that ROS formed in the mitochondria transfer to the cytosol, creating an oxidative gradient and signaling for autophagosome formation by oxidative modification of target molecules, such as Atg4, and probably other yet unidentified factors. Once Atg4 is oxidized, it becomes inactive and its substrate, Atg8, can be conjugated to autophagosomes. Because ROS are short-lived molecules, we hypothesize that oxidation occurs only in the vicinity of mitochondria. Further away from the mitochondria, Atg4 will be active and therefore cleave Atg8 from the autophagosomal membrane for recycling. Currently, accurate methods for the detection of oxidative gradients, which would enable us to test this hypothesis, are not available. Notably, mitochondrial ROS could signal for autophagy through mitochondrial targets as well, as suggested by studies showing that lipid peroxidation occurs in response to treatment with rapamycin or NGF deprivation [26,33], however, these pathways remain to be elucidated.

## Concluding remarks

Autophagy was first discovered as a nonselective pathway for the degradation of cell constituents, activated in response to starvation. It is now clear that selective autophagy of specific organelles and proteins occurs in response to diverse stimuli, varying from survival-promoting removal of pathogens, through housekeeping degradation of damaged organelles and proteins, to programmed cell-death. Nevertheless, the basic machinery of autophagosome formation and degradation, as it is known today, is conserved in all of these forms of autophagy. Is ROS part of this core machinery or, in other words, do all pathways of autophagy require ROS formation? What cellular factors, other than lipids and the Atg4 protease, are targeted by ROS in autophagy? Future experiments aimed to determine the exact pathways of autophagy-related ROS signaling might answer these questions. ROS is a collective definition for a large family of molecules with distinct features. Several of the studies discussed here suggest that $H_2O_2$, a rather moderate ROS, is an effector of autophagy. Is it indeed the only ROS involved in the regulation of autophagy? Interference with specific ROS regulators might resolve this issue.

The puzzling duality of autophagy in survival versus death is unavoidable, when discussing the regulation of this pathway. Could the outcome of life-or-death in autophagy be determined, among other signals, by the level of ROS? To answer this question, methods for the accurate quantification of ROS levels in living cells must be developed.

One of the greatest enigmas in the autophagic field today is the membrane source for newly formed autophagosomes. Here, we propose that mitochondria have a major role as the source of ROS required for autophagy. Could this organelle also be a source for the membrane required for autophagosome formation? Hopefully, new advances in live-cell imaging technology, together with biochemical methods, will help to elucidate these questions.

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