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Review

Mitophagy and mitochondrial dynamics in *Saccharomyces cerevisiae*☆

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ABSTRACT

Mitochondria fulfill central cellular functions including energy metabolism, iron-sulfur biogenesis, and regulation of apoptosis and calcium homeostasis. Accumulation of dysfunctional mitochondria is observed in ageing and many human diseases such as cancer and various neurodegenerative disorders. Appropriate quality control of mitochondria is important for cell survival in most eukaryotic cells. One important pathway in this respect is mitophagy, a selective form of autophagy which removes excess and dysfunctional mitochondria. In the past decades a series of essential factors for mitophagy have been identified and characterized. However, little is known about the molecular mechanisms regulating mitophagy. The role of mitochondrial dynamics in mitophagy is controversially discussed. Here we will review recent advances in this context promoting our understanding on the molecular regulation of mitophagy in *Saccharomyces cerevisiae* and on the role of mitochondrial dynamics in mitochondrial quality control. This article is part of a Special Issue entitled: Mitophagy.

1. Introduction

Mitochondria have a central role in cell survival and are involved in many essential cellular processes including generation of ATP by oxidative phosphorylation, the citric acid cycle, haem biosynthesis, formation of iron sulfur clusters, and β-oxidation of fatty acids. They are key regulators of programmed cell death pathways, and have crucial functions in multiple signalling pathways. Mitochondria are one main source of reactive oxygen species (ROS) which are generated as by-products by complex I and III of the electron transport chain [1,2]. Besides acting as important second messengers, ROS can lead to protein modifications, lipid peroxidation and DNA damage when produced in excess. Increased oxidative stress can lead to dissipation of the mitochondrial membrane potential and to cell death by the release of pro-apoptotic proteins [3].

Mitochondria are characterized by a double membrane architecture [4]. Two structurally and functionally distinct membranes, the mitochondrial outer membrane (OM) and the inner membrane (IM), separate the cytosol from the mitochondrial matrix [5,6]. Between both membranes a small aqueous space, the intermembrane space (IMS), exists. The inner membrane structure is highly diverse as it shows characteristic infoldings, termed 'cristae', which are highly variable in appearance [7]. The IM can be subdivided into the inner boundary membrane (IBM) that closely opposes the OM and the cristae membrane (CM) which represents the majority of the inner membrane surface in most cells. Both parts of the IM are connected by a small pore- or slot-like structure, the crista junction (CJ). CJs are proposed to restrict diffusion of molecules between intra-cristae compartments and the peripheral IMS and also within the IM [8]. This is supported by studies showing that the protein composition is different between the IBM and the CM and that it can adapt dynamically dependent on the physiological state of mitochondria [9–11].

The genome of mitochondria (mtDNA) is organized in circular double-stranded molecules, packed in compact particles, termed 'nucleoids' [12,13]. Human mtDNA is maternally inherited and contains 37 genes encoding for 13 subunits of complexes I, III, IV, and V; 2 ribosomal RNAs, and 22 tRNAs [14] while the majority of about 1000 – 2000 mitochondrial proteins is encoded in the nucleus. Thus, most mitochondrial

Abbreviations: AIM, Atg8 family interacting motif; ALP, alkaline phosphatase; Ape1, aminopeptidase I; ATG, autophagy-related; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CJ, crista junction; CM, cristae membrane; CMA, chaperone-mediated autophagy; Cvt, cytoplasm to vacuole targeting; cytALP, cytosol-localized ALP; ER, endoplasmic reticulum; ERMES, ER mitochondria encounter structure; GFP, green fluorescent protein; GSH, reduced glutathione; GTP, guanosine triphosphate; IBM, inner boundary membrane; IM, inner membrane; IMS, intermembrane space; LIR, LC3 interacting region; MAPK, mitogen-activated protein kinase; mtALP, mitochondria-localized ALP; mtDNA, mitochondrial DNA; NAC, N-acetylcysteine; OM, outer membrane; PAS, phagophore assembly site; PE, phosphatidylethanolamine; PINK1, PTEN-induced putative kinase 1; PKA, protein kinase A; ROS, reactive oxygen species; rRNA, ribosomal RNA; *S. cerevisiae*, *Saccharomyces cerevisiae*; SQA, synthetic quantitative array; TMD, transmembrane domain; TOR, target of rapamycin; TORC1/2, target of rapamycin complex 1/2

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proteins are synthesized in the cytosol and are subsequently imported into the organelle by distinct protein translocases ensuring proper targeting to the different mitochondrial subcompartments [15,16].

Mitochondria are highly dynamic as they constantly undergo balanced fusion and fission events [17,18]. The dynamics of mitochondria is important for various cellular functions such as mtDNA inheritance and intracellular distribution [19,20]. The opposing action of fission and fusion needs to be tightly regulated in order to adapt mitochondrial morphology to altered physiological needs. In *Saccharomyces cerevisiae*, at least three large GTPases and their interaction partners ensure these processes. The dynamin-related large GTPase Mgm1 is responsible for inner membrane fusion. The large GTPase Fzo1 mediates docking and fusion of the mitochondrial outer membranes. Mitochondrial fission is executed by the dynamin-related GTPase Dnm1 which binds to the outer membrane via Fis1 and Mdv1 [21]. Dnm1 assembles into higher oligomers at the mitochondrial surface promoting the formation of rings and spirals around the organelle. These oligomers divide the organelle in a GTP-dependent manner presumably in a similar way as classical dynamins act during endocytosis [21,22]. For most of these core components required for fusion and fission of mitochondria orthologs have been identified ensuring mitochondrial dynamics in mammals [23].

Since mitochondria display a key role in maintaining cellular homeostasis it is not surprising that mitochondrial dysfunction is associated with many pathological conditions including neurodegenerative diseases, cancer, diabetes and obesity [24–26]. Human mitochondrial disorders arise from mutations in mitochondrial and/or nuclear DNA [27]. Mitochondrial damage and mtDNA mutations are causally linked to the ageing process in eukaryotic cells [28,29]. Post-mitotic cells such as neurons and muscle cells strongly depend on mitochondrial function and are in particular susceptible to the deleterious consequences of pathogenic mutations as they usually cannot be replaced by neighbouring cells. Mitochondrial dysfunction in neurons can lead to several neurodegenerative disorders, such as Parkinson's and Alzheimer's disease [30]. Some of these neuropathies are associated with mutations in genes affecting mitochondrial dynamics. For example, autosomal dominant optic atrophy type I, a quite common neuropathy affecting retinal ganglion cells of the optic nerve, is caused by mutations in the gene encoding OPA1, the mammalian ortholog of the fusion factor Mgm1 from baker's yeast [31,32].

In order to restrict mitochondrial damage and ensure organelle integrity, eukaryotic cells have evolved distinct quality control mechanisms acting at different levels [33]. First, intra-mitochondrial molecular protein quality control is exerted by highly conserved molecular chaperones and proteases [34]. They monitor the assembly of mitochondrial proteins and selectively remove misfolded, damaged or excess proteins from the organelle. In case mitochondrial damage cannot be controlled at this level, proper mechanistic steps are carried out at the organellar level to limit damage and improve function. Mitochondrial dynamics plays a pivotal role because continuous mixing of mitochondrial contents due to constant fusion and fission contributes to a homogenous inter-organelle complementation of damaged proteins and mtDNA molecules [35]. Mitochondrial fission has been proposed to separate dysfunctional mitochondria from the integral network in mammalian cells [36]. In yeast and mammals, those mitochondria incapable to re-fuse with intact organelles will be removed selectively via an autophagy-related process, termed 'mitophagy' [36–39]. Mitophagy is critical for the cellular regulation of steady-state mitochondrial turnover, and by that determines the amount of mitochondria in response to changing environmental conditions and during development [40–42]. PINK1 and PARKIN have been reported to cause early onset hereditary forms of Parkinson's disease [39,43]. Several studies propose that PINK1-/PARKIN-dependent mitophagy mediates the selective removal of damaged and dysfunctional mitochondria and by that ensures organelle quality control [36,44–47]. Another mechanistic link between mitochondrial dysfunction and autophagy, two processes well known to play pivotal roles in neurodegeneration and ageing, was proposed by a study in *S. cerevisiae* showing that mitochondrial respiratory dysfunction impairs induction of the cellular autophagic response in general [48]. Overall, all this clearly points to the possibility of modulating mitochondrial degradation as a potential therapeutic treatment of human neurodegenerative disorders [49,50]. However, the detailed mechanism and implication of mitophagy in mitochondrial quality control are still not fully understood. When damaged mitochondria cannot be effectively removed by autophagy, apoptosis is induced by the release of pro-apoptotic proteins from the intermembrane space as a third possibility of quality control occurring at the cellular level [51]. How the different levels of quality control are linked and coordinated will be a matter of future research.

2. Mechanisms and regulation of mitophagy

Mitochondria as well as other cytosolic constituents can be degraded by non-selective autophagy [52]. This occurs constitutively at a low basal level but is drastically induced upon starvation and other physiological signals including hormones, growth factors, and certain pathogens. In general three types of autophagy are described [see 53, 54]. (1) Macroautophagy (hereafter referred to as autophagy) defines the degradation pathway of cytoplasmic components involving their engulfment by autophagosomal membranes (Fig. 1). In this process the outer membrane of an autophagosome then fuses with the vacuole/lysosome and releases its remaining content into the acidic lumen where it is degraded by resident vacuolar/lysosomal hydrolases; (2) microautophagy defines a direct uptake of cytoplasmic material via invagination of the vacuolar/lysosomal membrane; and (3) chaperone-mediated autophagy (CMA) is found in higher eukaryotes and mediates the direct translocation of unfolded proteins across the lysosomal membrane requiring cytosolic and lysosomal chaperones [55]. More recently, the importance of selective forms of autophagy became evident. In yeast, several forms are well studied [56]. One example is the Cvt pathway, a biosynthetic process that occurs constitutively under nutrient-rich conditions. It mediates the selective transport of specific precursor enzymes into the vacuole and shares common mechanistic features with macroautophagy [57,58]. At least two vacuolar hydrolases, aminopeptidase I (Ape1) and α-mannosidase I (Ams1), synthesized as inactive proenzymes, assemble and form a large oligomeric complex, the 'Cvt complex', which serves as cargo in this selective type of autophagy.

The term 'mitophagy' was introduced by John Lemasters after first experiments in *S. cerevisiae* suggesting that mitochondrial degradation is a selective process [40,46]. This field has made considerable progress in the meantime improving our molecular understanding of mitophagy in yeast and higher eukaryotes. *S. cerevisiae* is employed as a highly suitable model to study the molecular mechanisms of mitophagy for various reasons including: i) there are numerous genes/proteins orthologous to human genes/proteins which fulfil essentially the same function; ii) it is probably the best characterized model organism with numerous whole-genome, transcriptome, and proteome data sets; iii) and finally, compared with other model systems, genetic manipulation and study of multiple intracellular processes in *S. cerevisiae* is easy, fast, and cheap.

More than 30 autophagy-related (*ATG*) genes have been identified in yeast and other fungi [54,59]. The majority of ATG genes is required for all forms of autophagy as they are necessary for induction, formation of the isolation membrane, assembly of the preautophagosomal structure (PAS), and maturation of autophagosomes (see Fig. 1). Still, some ATG genes are specific for selective forms of autophagy. Consequently, the same core ATG machinery is required for mitophagy as well as for non-selective autophagy [60]. Interestingly, mitophagy can in principle occur via micro- or macroautophagy which apparently depends on the mode of induction [38,47,56,61].

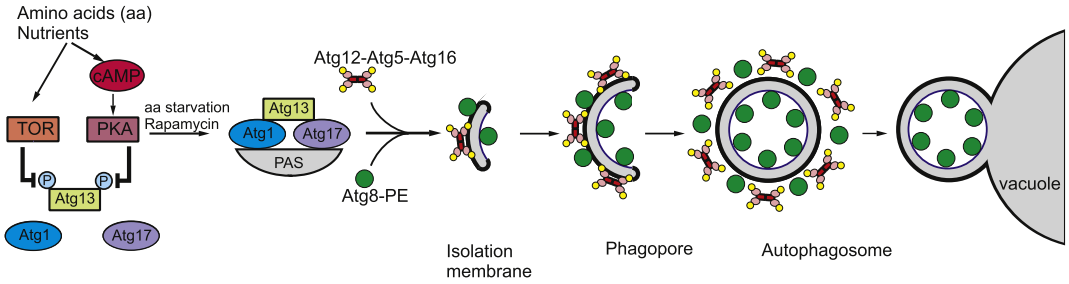

Fig. 1. Autophagy induction in yeast. Under nutrient-rich condition, the TOR kinase phosphorylates Atg13, inhibiting formation of the Atg1–Atg13–Atg17 complex while PKA phosphorylates Atg13 blocking Atg1–Atg13–Atg17 association with the pre-autophagosomal structure (PAS). Under starvation or after rapamycin treatment Atg13 is dephosphorylated and autophagy is initiated, leading to formation of an isolation membrane, growing of the phagophore, and finally resulting in autophagosome maturation (adapted from [119]).

2.1. Induction of autophagy and mitophagy

Inhibition of the target of rapamycin (TOR) kinase is a common way for triggering non-selective autophagy [62,63]. The TOR kinase is a master regulator of nutrient signalling pathways in eukaryotes (Fig. 1). Under nutrient-rich conditions active TOR kinase is a negative regulator of autophagy. It is a serine-threonine protein kinase that is activated under normal growing conditions and thereby promotes cell growth, cell cycle progression and protein synthesis. It is inactivated when nutrients are limiting (e.g. lack of sufficient amino acids due to nitrogen deprivation). This in turn results in induction of autophagy and down-regulation of anabolic pathways [64,65]. The TOR kinase activity is efficiently inhibited by rapamycin inducing autophagy even under nutrient-rich conditions [66]. In yeast, two homologous Tor proteins, Tor1 and Tor2, build up two functionally distinct TOR-complexes termed 'TORC1' and 'TORC2'. Only TORC1 is sensitive to rapamycin and involved in autophagy regulation [67,68]. Starvation conditions cause inactivation of TORC1 and rapid dephosphorylation of Atg13 which results in binding of Atg13 to Atg1 and activation of the Atg1 kinase complexes [69]. This TORC1–Atg1 signalling pathway is highly conserved among eukaryotes and represents an important level of regulation for autophagy induction or inhibition [70]. In addition, also the protein kinase A/Ras/cAMP signalling (PKA) pathway has been linked to regulation of autophagy in yeast (Fig. 1). The Atg1 kinase complex is a substrate of PKA inhibiting the assembly of the Atg1 kinase complex and thus inhibits autophagy [71,72].

Only a few conditions are currently known that induce mitophagy in *S. cerevisiae* [73]. In all known cases mitophagy is induced concomitantly with non-selective autophagy. A specific mitophagy-inducing condition not affecting other forms of autophagy was not described in yeast so far. Mitophagy in baker's yeast was shown to be induced at stationary phase in media containing a non-fermentable carbon sources such as lactate or glycerol [42,60,74]. When cells stop growing cellular energy is conserved and nutrients are generated by removing any surplus of cellular material including mitochondria. Another way to induce mitophagy efficiently is by inhibition of the TOR kinase complex via nitrogen starvation or by rapamycin treatment [40,75]. Mitochondrial degradation was suggested to be further promoted upon nitrogen starvation when the cells are additionally shifted to a medium containing fermentable carbon sources, such as glucose, possibly by making mitochondria more dispensable for energy conversion [74]. It is well supported that mitochondrial dysfunction leads to mitophagy in yeast cells. Priault et al. reported increased degradation of dysfunctional mitochondria in a temperature sensitive mutant, *fmc1*, which exhibited impaired F_1F_O ATP synthase assembly and showed reduced mitochondrial membrane potential [37]. Similarly, deletion of Mdm38, a gene coding for an essential component of the mitochondrial K+/H+ exchange system, caused mitochondria swelling and fragmentation as well as reduction of membrane potential, which subsequently led to increased selective removal of damaged mitochondria by autophagy [38]. However, dissipation of mitochondrial membrane potential by treatment with the uncoupling agent carbonyl cyanide m-chlorophenyl hydrazone (CCCP) did not result in a significant induction of mitophagy in yeast [40,75] although this was repeatedly reported for mammalian cells [39,43,76–78]. Interestingly, mitophagy induced by rapamycin or nitrogen starvation is impaired when yeast cells are treated with N-acetylcysteine (NAC) without affecting general autophagy [79]. Under these conditions the cellular glutathione (GSH) pool was increased raising the possibility that mitochondrial ROS levels and the cellular redox state serve as indicators for mitochondrial damage rather than the mitochondrial membrane potential.

The degradation of mitochondria was shown to occur selectively, requiring the interaction between Atg32 and Atg11 [56,61]. Similar to other selective types of autophagy in yeast, the degradation of mitochondria was shown to depend on adaptor-like protein Atg11 which can bind to different cargos including mitochondria, peroxisomes and Cvt-complexes (Fig. 2A) [74]. Furthermore, mitophagy appears to occur at a specialized phagophore assembly site (PAS) which is spatially separated from the site of Cvt vesicle or non-selective autophagosome formation [56]. An ortholog of Atg11 has not been found in higher eukaryotes so far. Still, also in mammalian cells selectivity is determined by an interaction of cargo-specific proteins with components of the autophagosome [80]. Cargo recognition occurs either directly by binding to receptors that are localized to the cargo or by adaptors that bind cargo-specific proteins (Fig. 2B). For mitochondria, the first case is represented by NIX, a mitochondrial outer membrane protein directly binding to the LC3/GABARAP protein family [41,81,82]. The NIX pathway is primarily thought to be required for the elimination of excess mitochondria during maturation of red blood cells. Recognition of mitochondria via the PINK1/PARKIN pathway is more complex and requires the following steps: (1) PINK1 accumulation in the outer membrane as a result of the reduction of the membrane potential across the mitochondrial inner membrane; (2) PINK1-dependent recruitment of PARKIN, an E3 ubiquitin ligase, to the outer membrane of damaged mitochondria; (3) ubiquitinylation of several outer membrane proteins; (4) recruitment of adaptor proteins such as p62; and (5) binding of mitochondrial-bound adaptor proteins to the LC3/GABARAP protein family [39,78,83–88]. The putative main purpose of the latter pathway is the elimination of dysfunctional mitochondria [for review see 89,90].

2.2. Assays for monitoring mitophagy in yeast

Engulfment of mitochondria by autophagosomes is classically detected by electron microscopy. However, this method is quite time-intensive, yet it allows for a detailed morphological analysis of distinct steps during mitophagy [47,56,91]. Other approaches involve to determine degradation of mitochondrial proteins by western blot analysis or to study the appearance of fluorescent mitochondrial proteins in the vacuole by fluorescence microscopy [40,92]. For example, the mitochondrial outer membrane protein Om45 fused with a green fluorescent protein (GFP) or mitochondrially targeted red fluorescent protein

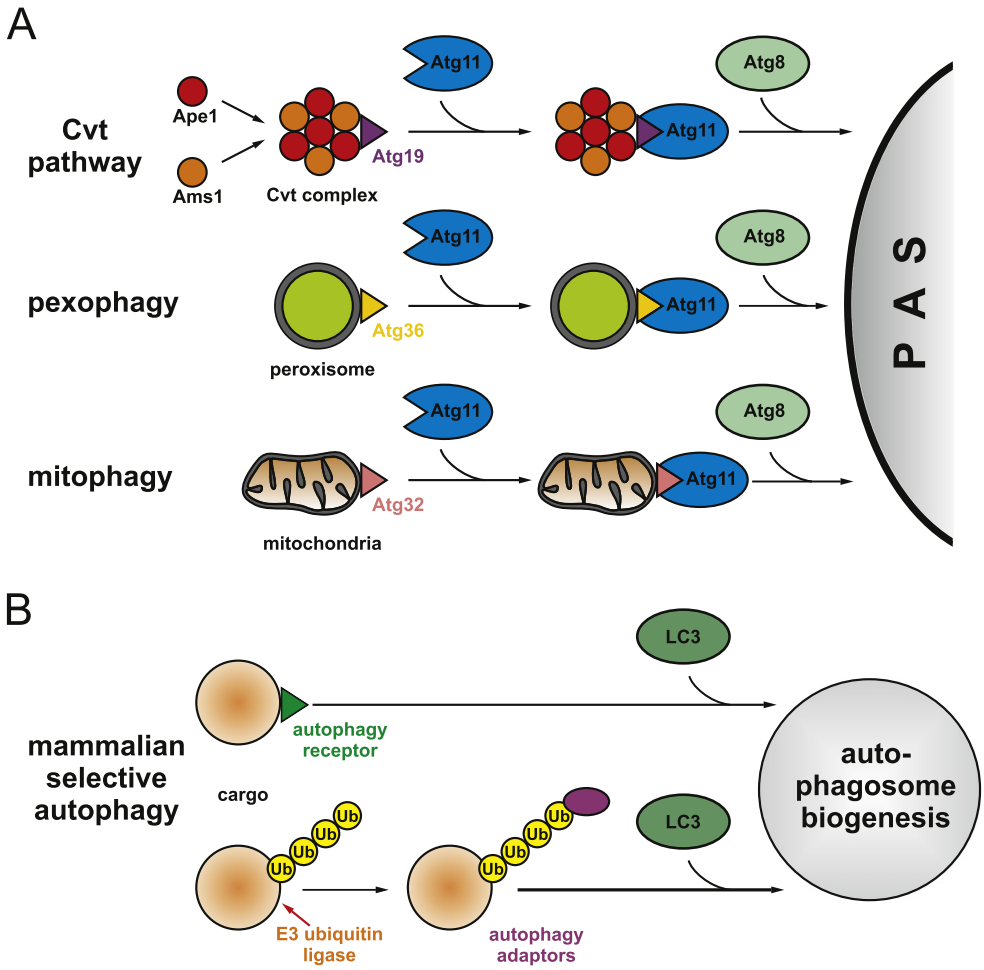

Fig. 2. Selective types of autophagy in yeast and mammals. A. Selective types of autophagy in baker's yeast. Atg11 is an adaptor-like protein enabling different kinds of selective autophagy in yeast. It binds specifically to distinct receptor molecules on the surface of the respective cargo mediating its recruitment to the PAS (adapted from [56]). B. Mitophagy pathways in mammals. Specific autophagy receptors such as NIX either directly bind to the cargo (top) or the cargo becomes recruited after ubiquitinylation (bottom) and binding of autophagy adaptors such as p62 (purple) which both will mediate the interaction with the cargo and with the core autophagy machinery via binding to LC3/GABARAP protein family members.

can be applied for these analyses [74,75,92]. When mitochondria are delivered to the vacuole, the Om45-part of the chimera is rapidly degraded whereas the GFP domain is proteolytically stable and can be determined by western blot analysis or fluorescent microscopy.

To achieve a quantitative analysis of mitophagy levels in yeast, Mendl et al. adapted an alkaline phosphatase-based (ALP) assay [75]. This enzymatic method was originally described for the dissection of non-selective autophagy and has been successfully modified for mitophagy before [93,94]. Here, an inactive proenzyme of alkaline phosphatase is initially targeted to the mitochondrial matrix (mtALP). Upon mitophagy induction, this proenzyme is delivered along with mitochondria to the vacuole where it becomes activated by proteolytic processing by the vacuolar proteinase A. The specific activity of ALP is subsequently quantified by a colorimetric assay reflecting the level of mitophagy induction. For this assay, the endogenous alkaline phosphatase gene, *PHO8*, has to be deleted to reduce background activity. With this principle also non-selective autophagy induction can be quantified by using cytALP, the alkaline phosphatase proenzyme targeted to the cytosol. The mtALP or cytALP proenzymes become only activated when transported into the vacuole upon induction of autophagy. A major advantage of this assay is that it is quantitative and enables the detection of minor changes in mitophagy or autophagy activation. It even allows determining an increase in both processes which is difficult to conclude easily by image-based or immunoblotting-based assays.

2.3. Identification and role of Atg32 in mitophagy

Two genome-wide screens for yeast mutants defective in mitophagy used a library of strains, each lacking a non-essential gene. These studies led to the identification of Atg32 as the first mitophagy-specific protein which is not required for non-selective or other types of selective autophagy [56,61]. Atg32 harbours a single transmembrane domain and is localized to the mitochondrial outer membrane with its N-terminus facing towards the cytosol (Fig. 3). Two serine residues, Ser-114 and Ser-119, in the cytosolic domain are phosphorylated when mitophagy is induced [95]. Atg32 was reported to directly interact with Atg11 in vivo. This interaction strongly increases upon nitrogen starvation. Phosphorylation of Atg32 promoted the interaction with Atg11, but how Atg32 phosphorylation is regulated is unclear (Fig. 3). A recent study, however, revealed a potential role of casein kinase 2 (CK2) in phosphorylation of Atg32 and induction of mitophagy [96].

Atg32 interacts with a central component of the autophagosomal machinery, Atg8, via the conserved consensus sequence W/Y XX I/L/V located near the cytosolic N-terminus (Fig. 3) [61]. The 'Atg8 family interacting motif' (AIM) in yeast or 'LC3 interacting region' (LIR) in mammals is ubiquitously found in different autophagy-receptor proteins ([97]). The interaction of Atg32 with Atg8 was reported to depend on phosphorylation of Atg32 in close proximity to the AIM motif [98]. It was proposed that a ternary Atg32–Atg11–Atg8 initiator complex is

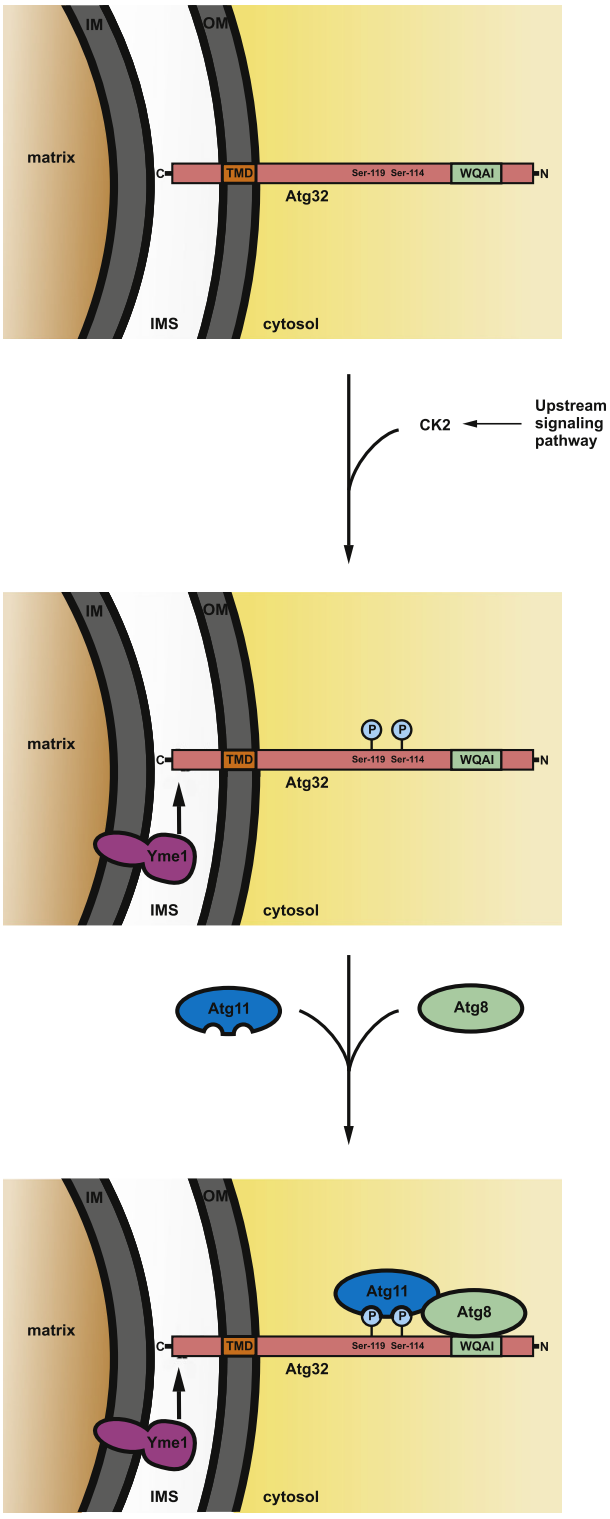

Fig. 3. The mitochondrial receptor for mitophagy Atg32 is regulated by phoshorylation. The mitochondrial outer membrane protein Atg32 is phosphorylated at its cytosolic N-terminus upon induction of mitophagy promoting the interaction with the adaptor-like protein Atg11. Atg32 further interacts with Atg8 via a conserved AIM motif. Yme1-mediated processing of the C-terminal domain in the intermembrane space regulates these interactions. The Atg32–Atg11–Atg8 ternary complex is generated upon mitophagy induction leading to the recruitment of mitochondria to the PAS.

formed upon mitophagy induction mediating the recruitment of mitochondria to the core autophagy machinery (Fig. 3) [99].

The role of the C-terminal, intra-mitochondrial domain of Atg32 was unclear until recently. Wang et al. observed that Atg32 is proteolytically processed by the mitochondrial inner membrane i-AAA protease Yme1 at its C-terminus when mitophagy is induced [100]. This appears to promote the interaction of Atg32 with Atg11 (Fig. 3).

The cytosolic domain of Atg32 is sufficient to promote pexophagy when targeted to the surface of peroxisomes indicating that Atg32 can act as an autophagic degron (polypeptide degradation signal) [99]. A mammalian ortholog of Atg32 is not known so far. Yeast strains lacking the mitophagy receptor Atg32 were reported to exhibit severe mitochondrial defects, small colonies, increased mtDNA damage and ROS levels due to impaired mitochondrial degradation [101].

2.4. Other modulators of mitophagy in yeast

Besides Atg32, a few other factors affecting mitophagy in yeast have been studied over the last years. The mitochondrial outer membrane protein Uth1 was initially proposed as a key player for the selective autophagy of mitochondria as cells lacking *UTH1* were impaired in the degradation of mitochondrial proteins and displayed increased resistance to rapamycin treatment or nitrogen starvation [40,47]. In contrast, another study found Uth1 to be fully dispensable for mitophagy [102]. The mitochondrial protein phosphatase Aup1 was shown to be required for mitophagy indicating its role in removing a surplus of mitochondria [42]. Aup1 further appears to regulate mitophagy by controlling the retrograde signalling pathway (RTG) via the transcription factor Rtg3 [103]. Two mitogen-activated protein kinases (MAPKs), Slt2 and Hog1, have also been linked to the regulation of mitophagy [104]. Slt2 was proposed to act via yeast cell wall integrity signalling pathway without affecting general autophagy or the Cvt pathway. Hog1 plays a key role in osmoregulation and it was interesting to see that it is also specifically required for mitophagy. Atg32 phosphorylation was reported to involve Hog1 and its upstream kinase Pbs2. However, Hog1 apparently does not directly affect Atg32 function but rather acts via the upstream kinase signalling cascade [95].

As already described, Atg32 was initially identified in two genome-wide screens [61,105]. Both groups employed yeast libraries containing ~5000 non-essential gene deletion strains (see Table 1). Mitophagy was induced by growing cells until stationary phase was reached in a medium with a non-fermentable carbon source. Monitoring of mitophagy was performed by analysing the accumulation of a mitochondria-targeted fluorescent probe in the vacuole. These studies did not reveal Uth1 and Aup1 as potential mitophagy regulators, however, some genes with previously unknown function as well as some non-autophagy associated genes were identified to be required for mitophagy. In sum, Kanki et al. identified 32 mitophagy-defective strains while the screen by Okamoto et al. revealed 36 strains excluding already known *ATG* mutants. However, only 8 mutant strains overlap when comparing both screens. They were primarily deleted for genes linked to normal vacuolar function, vacuolar protein sorting, or membrane fusion processes. The little overlap may result from different experimental conditions applied [106]. Okamoto et al. cultivated yeast cells for five days in medium supplemented with glycerol whereas Kanki et al. used different supplements or growth conditions. The latter study was done in two steps: in the beginning, strains were cultured for 3 days in medium supplemented with lactate. Subsequently, identified mitophagy-defective strains were subjected to nitrogen starvation for mitophagy induction. Furthermore, different fluorescent probes were applied in these two studies.

In each study 23 mitophagy-specific mutants were found that are defective in mitophagy but that do not affect other autophagy-related pathways. These genes included factors involved in various cellular processes including proteins with known mitochondrial location. Only *Δatg32 and Δegd1* were found to be specific for mitophagy in both screens indicating their essential roles in regulating the core mitophagy mechanism under different induction conditions (Table 1). The nascent polypeptide-associated complex (NAC) is composed of two known subunits, Egd1 and Egd2 and was shown to be associated with cytoplasmic ribosomes. It is involved in cotranslational protein folding and

Table 1
Comparison of genomic screens for yeast mutants defective in mitophagy.

	Kanki et al. (2009)		Okamoto et al. (2009)
Number of analyzed single gene deletion mutants	4667		5150
Induction of mitophagy	1.) Stationary phase (3 days, lactate) ↓ 2.) Nitrogen starvation (+ shift from lactate to glucose)		Stationary phase (5 days, glycerol)
Monitoring of mitophagy	Visual screen (Om45-GFP)		Visual screen (mito-GFP)
Mitophagy-defective strains (excluding ATG related genes)	32		36
Overlap		8	
Mitophagy-specific mutants (without affecting autophagy and/or Cvt pathway)	23		23
Overlap		2 (Δ*atg32*, Δ*egd1*)	

posttranslational protein targeting to mitochondria raising the possibility that Egd1 regulates mitophagy via affecting targeting of one or more mitophagy modulators to mitochondria [107].

Recently, a systematic screen for mitophagy modulators in respiratory-deficient yeast cells using a fluorescence-based assay was performed [108]. Among 381 yeast deletion mutants tested, 39 mutant strains showed reduced whereas 54 strains exhibited increased mitophagy after nitrogen starvation compared to controls. The applied assay methodology allowed for the first time a systematic analysis of respiratory-deficient mutant strains.

Another factor specifically required for rapamycin-induced mitophagy is Whi2 [75]. Whi2 was originally described as a stress response factor [109]. It was found to affect mitophagy more or less accidently as the reduced mitophagic activity in the strain deleted for the fission factor Fis1 was demonstrated to be caused by a suppressor mutation in the *WHI2* locus instead [75]. Importantly, Whi2 is specific for mitophagy as the *Δwhi2* strains showed a significantly decreased mitophagic activity while non-selective autophagic activity or the Cvt pathway was not affected grossly indicating that Whi2 is not a major player in other types of autophagy [75]. Whi2 was suggested to regulate degradation of mitochondria by modulating the activation of the general stress response and the PKA/Ras/cAMP signalling pathway [110]. However, the detailed mechanism of Whi2 function in mitophagy still needs to be elucidated.

2.5. Morphology/dynamics of mitochondria during mitophagy

Mitochondrial dynamics and morphology have been shown to be linked to the mitochondrial bioenergetics state. The dynamin-like GTPases Mgm1 exists in two isoforms located in the IM and IMS and is essential for mitochondrial fusion [111–113]. Both the small isoform, s-Mgm1, and the large isoform, l-Mgm1, are required for respiratory growth, maintenance of mitochondrial DNA and normal mitochondrial morphology. l-Mgm1 is processed by matrix processing peptidase which removes the mitochondrial targeting sequence while s-Mgm1 is produced via limited intramembrane proteolysis by the rhomboid protease Pcp1 [113,114]. Later it was shown that generation of s-Mgm1 is dependent on a functional protein import machinery and the ATP level in the mitochondrial matrix [45]. Thus, the mitochondrial bioenergetic state controls the ratio of the two Mgm1 isoforms in a manner that reduced ATP levels cause an increase in the ratio of l-Mgm1 to s-Mgm1 resulting in mitochondrial fragmentation [30,45]. Based on these initial findings we have proposed that such a mechanism helps to distinguish functional from dysfunctional mitochondria and that such a spatial separation of damaged mitochondria might help to ensure efficient removal of such organelles [30,45]. Also constantly occurring fusion and fission events are important to maintain a functional pool of mitochondria [40,41]. In mammalian systems, inhibition of mitochondrial fission leads to impaired mitophagy and accumulation of dysfunctional mitochondria [36]. However, conflicting results about the role of mitochondrial dynamics in mitophagy have been reported in particular in baker's yeast when the role of the fission factor Dnm1 was studied. Of all fission factors, including Dnm1, Fis1, Mdv1 and Caf4, only Dnm1 was identified as a positive regulator of mitophagy [105]. Strains lacking the fission factors Fis1, Mdv1 or Caf4 did not show apparent defects in mitophagy. Nowikovsky et al. found that deletion of Dnm1 prevented mitophagy induced by deletion of Mdm38 [38]. These studies are in line with Mao et al. reporting that Dnm1-mediated mitochondrial fission is required for efficient mitophagy induction by nitrogen starvation in yeast [115]. Here, it was also demonstrated that Dnm1 interacts with Atg11 suggesting that Dnm1 is recruited to mitochondria undergoing mitophagy. These studies point to a role of Dnm1 in yeast mitophagy.

However, the genetic screen by Okamoto et al. did neither reveal Dnm1 nor any of the other fission factors [61]. Also Mendl et al. demonstrated that deletion of essential components of the mitochondrial fission machinery such as Dnm1, Fis1, Mdv1 or Caf4 as well as overexpression of a dominant-negative variant of Dnm1 did not result in an alteration of rapamycin-induced mitophagy indicating that mitochondria fragmentation was not required for mitophagy induction under these conditions [75,110]. In *Ustilago maydis*, it was also reported that Dnm1-dependent fission is only required for mitophagy under certain conditions confirming that in lower eukaryotes Dnm1 is not always required [116]. Overall, it remains possible that Dnm1 has a specialized function in mitophagy which might not be linked to its fission activity. Alternatively, it cannot be excluded that certain strains lacking Dnm1 have accumulated suppressor mutations. Furthermore, inhibition of oxidative phosphorylation by various inhibitors does not trigger mitophagy in yeast. Taken together, all these results indicate that in yeast Dnm1-independent pathways for mitophagy exist; at least under certain conditions. It is also clear that, unlike in mammals, Dnm1-mediated fission is not essential for mitophagy as only mild reductions in mitophagy occur when observed at all. It is furthermore well possible that fission is required for enabling basal levels of mitophagy which are technically difficult to detect. Moreover, other additional triggers may be necessary for inducing fission-dependent mitophagy. Considering the relative small size of yeast cells and consequently of the mitochondrial network, it is conceivable that sufficient mitochondrial fission enabling mitophagy is mediated by other mechanisms such as cell division, by the autophagy machinery itself, or by an yet unknown Dnm1-independent pathway [110].

2.6. Regulation of mitophagy in Sacharomyces cerevisiae by (de-)ubiquitination

Recently, we performed a quantitative genome-wide screen for modulators of rapamycin-induced mitophagy in *S. cerevisiae* applying an enzyme-based high-throughput screening technology which we termed Synthetic Quantitative Array (SQA) technology [117]. In total 86 positive and 10 negative modulators of mitophagy were identified. 63 of those were specific for mitophagy. Interestingly, the same two

genes (*Δatg32* and *Δegd1*) as identified previously in two other genome-wide screens [56,61] were also found here supporting the central role of these factors. Our study further revealed that Ubp3 and Bre5, two core components of the Ubp3–Bre5 deubiquitination complex, are inhibitors of rapamycin-induced mitophagy but, conversely, activators of non-selective autophagy as well as of other selective types of autophagy [117]. This includes ribophagy which was shown previously to be promoted by the Ubp3/Bre5 complex [118]. Both proteins were shown to dynamically translocate from the cytosol to mitochondria upon induction of mitophagy. This demonstrates a so far unrecognized role of (de-)ubiquitination in the regulation of mitophagy in yeast, a fact well accepted for mammalian cells. Moreover, it shows for the first time a reciprocal regulation of distinct autophagy pathways and that switching between different forms of selective autophagy is regulated by deubiquitination. Further studies in yeast will elucidate the role of other components of the ubiquitin conjugation/de-conjugation machineries in mitophagy regulation and will unravel the molecular mechanisms and physiological roles underlying the reciprocal way of regulating distinct autophagy pathways.

3. Conclusions

Mitophagy has gained enormous attention over the last decade, mainly as it has been recognized as an important cellular mechanism coping with mitochondrial dysfunction [33,44]. It is a well-orchestrated and tightly regulated process that is partly conserved from yeast to humans [60,110]. Mitophagy gained further interest upon studies demonstrating that mutations in PARKIN and PINK1, two genes required for removal of dysfunctional mitochondria in mammals, cause autosomal recessive early-onset Parkinson's disease [49,50,39,43,78,86,87]. Obtaining a detailed molecular understanding of this process is thus highly relevant for identifying strategies for counteracting ageing and a number of human disorders linked to mitochondrial dysfunction.

Studies in the model organism *S. cerevisiae* have deepened our knowledge in this regard tremendously as they led to the identification of conserved factors required for mitophagy and revealed regulatory principles. Still, one drawback certainly is that there is currently no specific induction method for mitophagy described in yeast. Atg32 was identified as the first specific mitophagy receptor and its regulation by phosphorylation is a good example for the complex regulatory network ensuring mitophagy. Mitochondrial morphology/dynamics is another aspect ensuring proper mitochondrial quality control and certainly contributes to the efficiency of mitophagy at least under certain conditions. Hopefully, with the continued rapid pace of current discoveries in the field we can successfully tackle the following challenges in the future: (1) to decipher the signalling cascades regulating mitophagy positively as well as negatively; (2) to dissect the molecular steps for the selective removal of dysfunctional mitochondria; (3) to determine the regulatory mechanisms ensuring that mitophagy is compensated by sufficient mitochondrial biogenesis and that excess of mitophagy is prevented; (4) to identify ways to modulate mitophagy which have the potential to be used as therapeutic strategies combating diseases linked to mitochondrial dysfunction.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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