

Roles of mitophagy in cellular physiology and development

Jörn Dengjel^{1,2} · Hagai Abeliovich^{3,4}

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Abstract The autophagic degradation of mitochondria, or mitophagy, has been shown to occur in eukaryotic cells under various physiological conditions. Broadly, these fall into two categories: quality-control related mitophagy and developmentally induced mitophagy. Quality-control related mitophagy, which is the lysosomal/vacuolar degradation of malfunctioning or superfluous mitochondria, is an important housekeeping function in respiring eukaryotic cells. It plays an essential role in physiological homeostasis and its deregulation has been linked to the progression of late-onset diseases. On the other hand, developmental processes such as reticulocyte maturation have also been shown to involve mitophagy. Importantly, there are clear differences between these processes. Unlike our knowledge of the more general degradation of soluble cytosolic content during starvation-induced macro autophagy, the mechanisms involved in the selective autophagic degradation of mitochondria have only recently begun to receive significant attention. Here, we review the current literature on these topics and proceed to provide specific examples from yeast and mammalian systems. Finally, we cover experimental

approaches, with a focus on proteomic methods dedicated to the study of mitophagy in different systems.

Keywords Mitophagy · Autophagy · Physiology · Development · Membrane trafficking

Mitochondria, genetic disease, and aging

Mitochondria perform a variety of essential physiological functions in all eukaryotic cells. The production of ATP by oxidative phosphorylation is clearly the best recognized of these roles. However, no less essential are the roles played by mitochondria in fatty acid oxidation and the biosynthesis of isoprenoids, Heme, amino acids, iron-sulfur clusters and nucleotides, among others. Since the 1950s it has been recognized that oxidative metabolism harbors the potential for damage. In fact, it is estimated that anything between 0.1 to 4 % of the total O₂ consumption ends up as reactive oxygen species (ROS) under normal respiratory metabolism (Balaban et al. 2005). The localized production of ROS causes damage, which is manifested in mutagenesis of mitochondrial DNA, as well as protein and lipid oxidation reactions that have direct functional consequences. However, it can also indirectly lead to disruption of mitochondrial compartmentalization, further leading to leakage of cytochrome c and other cytotoxic factors. In addition, mitochondria with defective chemiosmotic coupling “short circuit” the ATP generating process, leading to an energy drain.

Gradual accumulation of mitochondrial DNA mutations has been invoked as an explanation for aging phenomena. In addition, many maternally-inherited forms of late onset diseases, such as type II diabetes, deafness, mitochondrial encephalomyopathy and optic neuropathy, to mention a few, have been linked to maternally-inherited mutations in the

✉ Hagai Abeliovich
ahagai@agri.huji.ac.il

¹ Department of Dermatology, Medical Center, Freiburg Institute for Advanced Studies (FRIAS), ZBSA Center for Biological Systems Analysis, University of Freiburg, Habsburgerstr. 49, 79104 Freiburg, Germany

² Department of Biology, University of Fribourg, Chemin du Musée 10, 1700 Fribourg, Switzerland

³ Department of Biochemistry and Food Science, Hebrew University of Jerusalem, Rehovot 76100, Israel

⁴ FRIAS Senior Fellow and Marie Curie Fellow of the European Union Freiburg Institute for Advanced Studies (FRIAS), University of Freiburg, Albertstr. 19, 79104 Freiburg, Germany

mitochondrial genome (Wallace 1999, 2005). In these heritable late-onset mtDNA diseases, mitochondria harboring deleterious mutations initially only occur as a fraction of the total mtDNA pool. With age, the mutant mtDNA pool expands through unknown mechanisms and begins to dominate cellular physiology, often in a tissue-specific manner (Wallace 1999, 2005). Within the framework of this theory, a role has been proposed for a quality control mechanism that identifies and deletes malfunctioning mitochondria, thus preventing clonal takeover of the system by the defective mitochondrial cohorts (Harman 1956; Wallace 1999, 2005; Ahlqvist et al. 2015).

A disease that is related to mitochondrial dysfunction but is not necessarily linked to inherited mitochondrial DNA mutations, is Parkinson's disease (PD). PD is a disorder in which neurons accumulate an amyloid form of α -synuclein, a small, synaptic vesicle-associated protein (Wang and Hay 2015). This accumulation correlates with degeneration and death of dopaminergic neurons in the substantia nigra region of the brain, ultimately leading to major symptoms of the disease, such as resting tremor and rigidity of limbs. Agents that compromise mitochondrial function, such as rotenone and paraquat, induce a PD-like condition in rats (Greenamyre et al. 1999). Familial forms of PD are known to be caused by a set of mutations that map to specific genes, including those encoding the proteins α -synuclein, LRRK2, DJ-1, ATP13A2, PINK1 and Parkin (Kumaran and Cookson 2015). As discussed below, two of these proteins, PINK1 and Parkin, are directly involved in mitochondrial quality control and in mitophagy.

Mitophagy as a form of selective autophagy

Autophagy is a general term referring to mechanisms that transport cytoplasmic material directly into the lumen of the lysosome or vacuole in eukaryotic cells. Three general types of autophagy are usually pointed out: macroautophagy, microautophagy and chaperone-mediated autophagy. Of these, macroautophagy is by far the best characterized. During macroautophagy, cellular membranes engulf cytoplasmic material to generate a double-membrane bound vesicle termed an autophagosome. Autophagosomes then fuse with vesicles derived from the endosomal system, thus forming amphisomes (Gordon and Seglen 1988; Sanchez-Wandelmer and Reggiori 2013) before finally fusing with lysosomes to generate autolysosomes in mammalian cells, or directly with the vacuole in yeast and plant cells. As a result of these fusion events the inner membrane of the autophagosome is exposed to lysosomal lipases that degrade it. This in turn exposes the cytosolic content sequestered within the inner membrane to additional lysosomal/vacuolar hydrolases leading to its degradation. Microautophagy is a conceptually similar mechanism in which the vacuolar/lysosomal membrane invaginates

directly and pinches off an intraluminal vesicle that is then degraded by hydrolases. The main conceptual difference between microautophagy and macroautophagy is the absence of the intermediate, the autophagosome, in microautophagy. Chaperone-mediated autophagy is a completely different concept, in which individual unfolded protein molecules are thought to translocate across the lysosomal membrane through a channel created by the LAMP2A protein and driven by the ATP hydrolysis activity of cytosolic and lysosomal Hsc70 family members (Schneider and Cuervo 2014). A fourth mechanism that can be considered is multivesicular body (MVB) trafficking but this process is usually considered in the context of endocytosis and lysosomal targeting of secretory pathway components (Saksena et al. 2007; Scott et al. 2014).

Macroautophagy is known to be induced in all eukaryotic cells under nitrogen and amino acid starvation conditions. In some ways, this is considered a nonspecific process. For example, a mutant form of yeast vacuolar alkaline phosphatase, Pho8 Δ 60, is mistargeted to the cytoplasm due to the absence of its N-terminal 60 amino acids. As such, it is a perfect innocent bystander in the sense that it is not a native cytosolic protein and does not carry out specific molecular interactions within the cytosolic milieu. Nonetheless, Pho8 Δ 60 is—presumably unspecifically—carried to the vacuole by starvation-induced macroautophagy with an efficiency of around 10 % over a 12-h starvation period (Noda et al. 1995). This, however, is not the complete story. Many cellular components require a selective receptor in order to be degraded through macroautophagy under starvation conditions (see below) and on the other hand other components appear to be completely spared.

Over the last 20 years, approximately 40 gene products have been identified as contributing to autophagy. Most of these have been discovered through genetic screens in yeast, thanks to the pioneering work of Yoshinori Ohsumi (Takeshige et al. 1992; Tsukada and Ohsumi 1993), Dan Klionsky (Klionsky et al. 1992; Harding et al. 1995) and Michael Thumm (Thumm et al. 1994). Several excellent and comprehensive recent reviews give a detailed account of our current knowledge and provide tables summarizing the core autophagy factors and their functions (Feng et al. 2014; Ktistakis and Tooze 2016). In what follows, we present a concise overview of these functions, focusing on their roles in mitochondrial autophagy. Overall, the vast majority of the factors composing the autophagic machinery fall into several major mechanistic classes that seem to be phylogenetically conserved. As original screens were performed in *Saccharomyces cerevisiae*, we stick to the respective yeast nomenclature:

1) The Atg12 ubiquitin-like modification system

Atg12 is a 186 residue polypeptide that exhibits structural similarity to ubiquitin. Like ubiquitin it has a C-terminal glycine, which can be conjugated to primary

amine groups. Unlike ubiquitin it is only found to modify a second autophagy protein, Atg5, at lysine 149 (Mizushima et al. 1998), although in mammalian cells it was also reported to conjugate with Atg3, which is the E2 enzyme for Atg8 (Radoshevich et al. 2010; see below). Atg12 is conjugated via an enzymatic cascade very similar to that of ubiquitin. It is first activated by adenylation coupled to ATP hydrolysis, followed by formation of a thioester linkage to a cysteine residue on the E1-like enzyme Atg7. It is then transferred to a cysteine residue on the E2-like enzyme Atg10, which mediates the conjugation reaction with Atg5. The Atg5-Atg12 conjugate has been observed to form large, membrane associated lattices in combination with the Atg16 protein (Kuma et al. 2002).

2) The Atg8 ubiquitin-like modification system

Atg8 is a small, 116 residue polypeptide that also displays a similar fold to ubiquitin (Paz et al. 2000; Sugawara et al. 2004; Koopmann et al. 2009; Noda et al. 2011). Unlike other UBLs, the C-terminal residue is an arginine. To uncover the reactive glycine, a redox-sensitive cysteine protease/amidase (Scherz-Shouval et al. 2007), Atg4, selectively removes the arginine residue, exposing glycine 115 as the C-terminal residue. Glycine 115 is then activated by Atg7 (which is a common E1-like enzyme for both UBL systems in autophagy) and transferred to a dedicated E2, Atg3. The activated Atg8 is then transferred, not to an amino group on a protein but on a lipid, phosphatidylethanolamine, in a reaction that is catalyzed by an E3-like activity that is assigned to the Atg12-Atg5-Atg16 lattice (Ichimura et al. 2000; Hanada et al. 2007). Atg8 has been shown to play a role in determining the size of autophagosomes, possibly by facilitating membrane fusion events at the growing sequestering membrane (Abeliovich et al. 2000; Nakatogawa et al. 2007; Xie et al. 2008). Atg8 is conjugated to both the outer autophagosomal membrane, facing the cytosol, and to PE in the inner membrane, facing the cytoplasmic cargo (Kirisako et al. 1999). These two populations of Atg8 have different fates: the Atg8 molecules on the outer membrane are hydrolytically released from PE in an Atg4-catalyzed reaction, while the Atg8 molecules in the inner membrane are degraded via autophagy. In addition to its role in autophagosome expansion, Atg8 also plays a role in the recruitment of specific cargo destined for autophagic trafficking or degradation (see below) and this may reflect a coupling mechanism between cargo availability and the expansion of the sequestering membrane. While yeast express only one species of Atg8-like protein, in mammalian cells there are at least 6 different members of this protein family. They include the MAP1LC3 sub-family, which includes LC3A, LC3B and LC3C and the GABARAP sub-

family that includes GABARAP, GABARAPL1 and GABARAPL2/GATE16 (Shpilka et al. 2011). The relative roles of these proteins in autophagosome biogenesis is a topic of ongoing research (Behrends et al. 2010).

3) The Atg1 protein kinase complex

Amongst the core autophagy factors that were identified by genetic screening in yeast, Atg1 is the only protein kinase. It is an 890 amino acid residue protein with the kinase domain located in the N-terminal 350 residues. Atg1 seems to function within a complex containing proteins such as Atg13, Atg17, Atg31, Atg29 and Atg11. The precise makeup of the complex is thought to change as a function of the physiological state of the cell, viz-a-viz activation of macroautophagy. The phosphorylation state of Atg1 and its function seem to be regulated by the TOR signaling pathway, although roles for PKA and Sch9 have also been proposed (Budovskaya et al. 2004; Yorimitsu et al. 2007; Soultard et al. 2010; Egan et al. 2011).

The function of the kinase activity of Atg1 is somewhat controversial. Atg1 is known to autophosphorylate (Matsuura et al. 1997; Abeliovich et al. 2003) and an apparent phosphorylation activity of Atg1 has also been documented towards Atg2, Atg13, Atg6, Atg9, (Papinski and Kraft 2014; Papinski et al. 2014; Kamber et al. 2015) as well as additional proteins (Lin and Hurley 2016). Apart from autophosphorylation, the importance of these phosphosites for the induction of autophagy is not entirely clear. Using analog-sensitive kinase domain mutants, it was shown that the kinase activity is required for selective autophagy and not for starvation-induced autophagy (Abeliovich et al. 2003), although that result was later contested (Kabeya et al. 2005). More recently, it was shown using an orthogonal substrate-kinase pair that selective autophagy receptors and scaffold proteins activate Atg1 kinase activity and that rapamycin treatment overrides the need for these receptors in activating kinase activity (Kamber et al. 2015). It has not been verified whether the functional role of Atg1 kinase activity is as a self-regulating structural switch (Abeliovich et al. 2003) or a part of a signaling cascade that regulates downstream proteins such as Atg6 and Atg9 (Kamada et al. 2000; Kabeya et al. 2005; Papinski and Kraft 2014), or both. Atg1 is found in a complex with a second protein, Atg13 and it was shown that, upon starvation or inhibition of TOR kinase, the Atg1-Atg13 heterodimer associates with an Atg17-Atg29-Atg31 heterotrimer to form a dimer of pentamers (Köfinger et al. 2015) that can function to tether Atg9 vesicles (Suzuki et al. 2015; Rao et al. 2016) (see below). This interpretation would be consistent with a structural role for Atg1. In addition, *in vitro* phosphorylation of Atg13 by Atg1, which mimics the native phosphorylation pattern observed on Atg13 under nutrient replete conditions, inhibits the Atg9 vesicle tethering

activity of the pentameric form (Rao et al. 2016). Atg1 can also directly bind to phosphoinositide-containing liposomes in a curvature-dependent fashion, although the biological significance of this activity is currently unclear (Ragusa et al. 2012; Rao et al. 2016).

In mammalian cells, two Atg1 orthologs have been identified: ULK1 and ULK2 (Unc-51 Like). These proteins are expressed ubiquitously in all mammalian tissues and interact with mammalian Atg13 (Chan et al. 2009; Hosokawa et al. 2009a) as well as with a putative Atg17-like protein, RB1CC1/FIP200 (Hara et al. 2008; Alers et al. 2011). The mammalian ULK complexes lack Atg29 and Atg31 homologs but contain an additional protein, Atg101, which is missing from the yeast complex (Hosokawa et al. 2009b; Mercer et al. 2009). As in yeast, however, mammalian Atg13 is a phosphoprotein and its phosphorylation is under mTOR regulation. Knockdown of ULK1 but not ULK2, leads to an autophagy defect in HEK293 cells. However *ulk1*^{-/-} knockout mice have a very limited phenotype that is much less severe than that of classical autophagy mutants such as *atg5*^{-/-} or *atg7*^{-/-} knockouts. The *ulk1*^{-/-} mice survive birth and do not display defects in classic starvation-induced autophagy but appear to be defective in mitochondrial clearance in erythrocyte maturation and display increased mitochondrial biomass in fibroblasts (Cheong et al. 2011). While *ulk2*^{-/-} mice show no defects, the phenotype of the double *ulk1*^{-/-} *ulk2*^{-/-} MEFs supports a redundant role of these two kinases in supporting autophagy (Cheong et al. 2011).

4) A type III phosphatidylinositol 3-kinase complex

Eukaryotic cells contain three distinct types of phosphatidylinositol 3-kinase (PI3K) activities with different biochemical attributes: types I, II and III. Each of these has additional sub classifications and variants as well, further complicating the picture. Type I PI3Ks play an indirect role in regulating autophagy in higher eukaryotes, by regulating mTOR activity in response to extracellular signals. However, type III PI3K is known to directly function in autophagic trafficking and this role is conserved in all eukaryotes. Type III PI3Ks are the only enzymes that phosphorylate PI to generate PI3P. PI3P is a lipid that is characteristic of the endolysosomal system and plays vital roles in vacuolar/lysosomal biogenesis as well as in endocytosis and related processes (Simonsen et al. 2001). In autophagy, type III PI3Ks are found as large protein complexes that are tethered to membranes. These complexes always include the catalytic subunit, Vps34, as well as a membrane-anchored (myristylated) protein kinase, Vps15/p150 and a third subunit, Vps30/Atg6/Beclin 1 (Stack et al. 1995). In addition to these invariant subunits, the holoenzyme also includes at least one additional component that seems to be function-specific. In yeast, there are two type III PI3K holoenzymes. In one complex, the

additional subunit is Vps38. This complex plays a vital role in vacuole biogenesis and targeting processes, as well as in endocytosis. In the second complex, Vps38 is replaced by a different component called Atg14. This second complex (sometimes confusingly referred to as complex I) is essential for autophagic trafficking. In mammalian cells, there appear to be three type III PI3K complexes; two of them are analogous to the two yeast complexes. In one, a protein with analogous function to yeast Atg14, Atg14L/barkor, pairs with Beclin 1 (the mammalian Atg6/Vps30 homolog) to generate a complex that is essential for autophagy. In a second complex, Atg14L/barkor is replaced with UVRAG, a protein with some resemblance to yeast Atg38 and this second complex is important for lysosomal trafficking/endocytosis. A third mammalian Vps34 complex contains UVRAG as well as Rubicon, a protein with no known yeast homolog. This third complex seems to negatively regulate both autophagy as well as lysosomal trafficking (Tabata et al. 2010). It was shown that PI3K activity is essential for generation of specialized PI3P patches on ER membranes (Axe et al. 2008), which then recruit PI3P binding proteins such as WIPI1 and deform into proto-autophagosomal structures known as omegasomes (Proikas-Cezanne et al. 2004; Polson et al. 2010).

5) Atg9 and ancillary proteins

Surprisingly, only one of the core autophagic factors that are absolutely required for all forms of macro autophagy is an integral membrane protein. Yeast Atg9 is a large 997 AA residue protein with 6 predicted transmembrane domains. In yeast cells, Atg9 localizes to small cytoplasmic vesicles. Upon induction of autophagy by nitrogen starvation or TOR inhibition, additional vesicles are formed de novo by budding from the trans-Golgi network and each round of autophagosome biogenesis requires the coalescence of approximately three such Atg9 vesicles. De novo formation of these Atg9 vesicles requires Atg23 and Atg27 (Mari et al. 2010; Yamamoto et al. 2012). These Atg9-containing vesicles are then tethered in a step dependent on the Atg1 pentameric complex (see above) to nucleate autophagosomes.

Once an autophagosome forms, Atg9 is located on the outer membrane of the autophagosome and is recycled to the Golgi. The location within cells at which recycling occurs is controversial, with some reports indicating direct recycling from the autophagosome while others indicate recycling of Atg9 from the vacuole after autophagosome–vacuole fusion (Mari et al. 2010; Yamamoto et al. 2012).

In mammals, mATG9 is also essential for autophagy, as the respective knockout mice do not survive the neonatal starvation period, similar to ATG5^{-/-} mice and the *atg9*^{-/-} MEFs show a defect in autophagy (Saitoh et al.

2009). Under basal conditions, mammalian Atg9 shows a bimodal distribution, localizing to both the trans-Golgi network (TGN) and late endosomes (Young et al. 2006; Orsi et al. 2012). Upon the induction of autophagy by nutrient deprivation or mTOR inhibition, the distribution of mAtg9 shifts and under these conditions the protein is found mostly colocalized with peripheral endosomes. This redistribution of mAtg9 localization appears to depend on ULK1, mVps34 and WIPI (Young et al. 2006; Orsi et al. 2012). The model that has been proposed to explain these results is that mAtg9 shuttles between endosomes and the TGN and that induction of autophagy correlates with an inhibition of the endosome-to-TGN route, leading to accumulation of the protein on endosomes. One model for Atg9 function, in both yeast as well as mammalian cells, is that it plays a role in the delivery of lipid and membranes to feed the expansion of the sequestering membrane, although formal proof of this concept has been elusive thus far.

6) Selective autophagy receptors

In recent years, it has been acknowledged that many selective forms of autophagy exist and some of these can be uncoupled from starvation conditions. The classical form of selective autophagy is the yeast CVT pathway. In the CVT pathway, a cytosolic precursor of the vacuolar protease aminopeptidase I (Ape1) is engulfed by autophagic membranes and delivered to the vacuole in a process that depends on many of the same factors as starvation-induced macroautophagy. The Cvt pathway, however, occurs in normally growing yeast cells and does not require nitrogen starvation for its induction. Rather, it requires a receptor (or rather adaptor) protein called Atg19 (Shintani et al. 2002). Atg19 binds to dodecameric Ape1 precursor complexes on the one hand and to Atg8 and Atg11 on the other hand. By linking the cargo, Ape1 with the autophagic machinery Atg19 allows selective delivery of Ape1 to the vacuolar lumen. A second protein α -mannosidase (Ams1) is also known to be delivered to the vacuole using the Cvt pathway. Consistent with our notions of nonselective macroautophagy, knockout of ATG19 abrogates Cvt trafficking of Ape1 but still allows an inefficient transport of precursor Ape1 to the vacuole upon nitrogen starvation, with an efficiency roughly similar to that of Pho8 Δ 60 (Shintani et al. 2002).

The Cvt pathway is a very instructive paradigm for selective autophagy as it embodies the defining characteristics of such processes: all forms of selective autophagy employ adaptors and receptors, which link the designated cargo with the autophagic machinery and usually with

Atg8 or an Atg8-like protein.

There has been a veritable explosion of selective autophagy processes described recently. A partial list includes aggrephagy (Øverbye et al. 2007; Wong et al. 2012) (autophagy of aggregated proteins), ribophagy (Kraft and Peter 2008), ER-phagy (Khaminets et al. 2015; Mochida et al. 2015), nucleophagy (Mijaljica and Devenish 2013; Mochida et al. 2015), lipophagy (Singh et al. 2009), ferritinophagy (Mancias et al. 2014), lysophagy (Hung et al. 2013) (lysosome autophagy), xenophagy (degradation of intracellular pathogens) (Singh et al. 2006; Chauhan et al. 2015), to name a few (for a full review, see Stolz et al. 2014). By definition, each such process designates a receptor or adaptor protein that links the selective autophagic cargo to an Atg8-like protein. The selective autophagy receptors interacting with Atg8-like proteins through dedicated, evolutionarily conserved motifs termed LIR (LC3 interacting region, W/F/YxxL/I/V) or AIM (Atg8 interaction motif) motifs selective macroautophagy, are no exception: As described below in more detail, all forms of mitophagy involve such receptor and adaptor proteins. However, the identity of the receptor/adaptor and its regulation seem to vary between organisms, as well as between different forms of mitophagy in the same organism.

Mitophagy as a quality control mechanism in mammalian cells

Mitochondria exhibit a wide spectrum of quality control mechanisms, which include proteasome-mediated degradation of outer-membrane proteins (OMMAD) as well as endogenous proteases. However autophagy is the only known cellular mechanism that can actually mediate the degradation of entire organelles. Selective mitochondrial autophagy was first described by the Lemasters laboratory (who also coined the term “mitophagy”; Rodriguez-Enriquez et al. 2004, 2006, 2009). Later, it was confirmed that, in specific types of mammalian cells, depolarized mitochondria are selectively degraded by mitophagy (Narendra et al. 2008; Twig et al. 2008). The overall scheme suggested by these studies implies that (1) depolarized mitochondria are unable to undergo further fusion events; (2) 85 % of fission events lead to formation of one depolarized and one hyperpolarized compartment, suggesting an active process segregating intra-mitochondrial material; (3) the post-fission state is the “resting state” in the mitochondrial life-cycle and fusion is rapidly followed by fission; and finally (4) depolarized mitochondria, formed by selective fission, are selectively autophagocytosed.

Recently, the molecular mechanisms underlying mitochondrial engulfment in mammalian cells have been characterized. However, some details are still under debate. The mitochondrial membrane-anchored protein kinase PINK1 is normally imported into mitochondria. In energized mitochondria, it is processed by presenilin associated rhomboid-like protease (PARL) followed by its degradation, which is suggested to happen either inside mitochondria, or in an ubiquitin- and proteasome-dependent manner following retrotranslocation to the cytosol (Jin et al. 2010; Meissner et al. 2011; Yamano and Youle 2013). In depolarized mitochondria, PINK1 is stabilized on the mitochondrial outer membrane (Narendra et al. 2008; Jin et al. 2010; Matsuda et al. 2010; Narendra et al. 2010) and phosphorylates ubiquitin (Ub) (Shiba-Fukushima et al. 2012; Kane et al. 2014; Kazlauskaitė et al. 2014; Koyano et al. 2014) in addition to phosphorylating Parkin, which is a ubiquitin-conjugating E3 enzyme (Narendra et al. 2008; Jin et al. 2010; Matsuda et al. 2010; Narendra et al. 2010); (Kondapalli et al. 2012; Shiba-Fukushima et al. 2012; Koyano et al. 2014). The combination of phospho-Parkin and phospho-Ub functions as a feed-forward loop amplifying the mitophagic signal (Ordureau et al. 2014). Two phosphoubiquitin-specific autophagy receptors, optineurin and NDP52, have been shown to be recruited to PINK1-presenting mitochondria using mass spectrometry-based proteomics (Heo et al. 2015; Lazarou et al. 2015). Interestingly, both of these proteins have been previously implicated in xenophagy (Thurston et al. 2009; Wild et al. 2011). Optineurin binding leads to TBK1 recruitment, which in turn phosphorylates optineurin, thus further enhancing its ubiquitin binding capacity (Heo et al. 2015; Richter et al. 2016). In addition, TBK1 has been shown to phosphorylate additional autophagy receptors, such as NDP52, TAX1BP1 and SQSTM1 (Heo et al. 2015; Richter et al. 2016). Additional, alternative Parkin receptors have also been suggested in the past: mitofusin 2 was suggested to be phosphorylated by Pink1, with phospho-mitofusin 2 functioning as a Parkin receptor (Chen and Dorn 2013). For a general summary of this sequence of molecular events, see Fig. 1b. As Parkin is activated by phosphoubiquitin, it ubiquitinates numerous mitochondrial proteins and among them are mitofusin 1 and 2 (Narendra et al. 2010; Tanaka et al. 2010). This explains the inability of depolarized mitochondria to fuse with polarized mitochondria. While these results provide a mechanistic explanation for much of the data, several important questions remain. For example, given that 85 % of fission events lead to segregation between depolarized and hyperpolarized mitochondria (Twig et al. 2008), one must hypothesize the existence of some active segregation mechanism between functional and defective

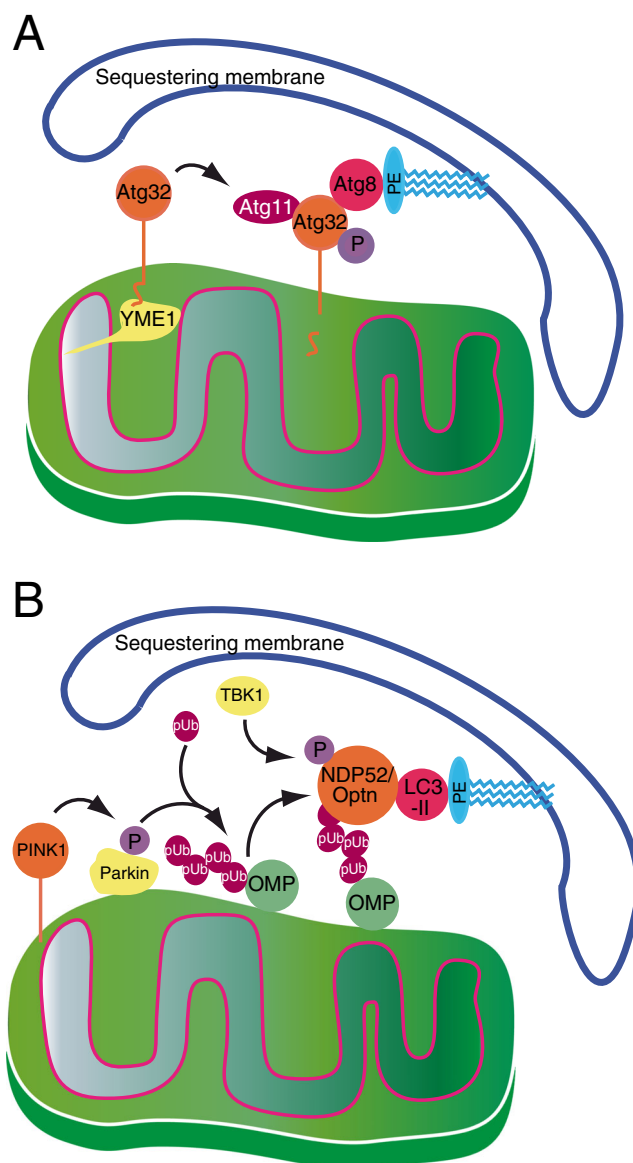


Fig. 1 Comparison of sequestering membrane recruitment to mitochondria in **a** yeast and **b** mammalian cells. In yeast (**a**), activation of Atg32 requires a proteolytic clipping step, mediated by the AAA protease Yme1, as well as phosphorylation. Activated Atg32 is then able to interact with lipidated Atg8 as well as with the Atg11 scaffold protein. **b** In mammalian cells, loss of mitochondrial membrane potential stalls PINK1 import and stabilizes it on the outer membrane, by preventing constitutive PARL-dependent proteolysis of imported PINK1 (not depicted). The stabilized PINK1 phosphorylates both Parkin, as well as ubiquitin. This simultaneously recruits Parkin to mitochondria and activates it to phospho-ubiquitylate mitochondrial outer membrane proteins, which in turn are recognized by the redundant mitophagy receptors NDP52 and Optineurin, thus linking LC3-II (the mammalian ortholog of lipidated Atg8) with outer mitochondrial proteins. Finally, Optineurin activation is regulated by TBK1 through phosphorylation on serine 177

mitochondrial components that somehow senses the Pink1 load of the outer mitochondrial membrane. The nature of such a mechanism is currently ground for speculation and is an area of active research.

Mitophagy during development in multicellular organisms

Erythrocyte maturation in mammals involves the complete removal of organelles, including mitochondria. It was shown that mitophagy contributes to the degradation of mitochondria in differentiating reticulocytes. Thus, knocking out canonical autophagy proteins such as Atg5 abolishes some but not all mitochondrial degradation during erythrocyte maturation. It was shown that a specific mitochondrial outer membrane protein, NIX (also known as BNIP3L) functions as a receptor that interacts with the autophagic machinery to mediate this process (Sandoval et al. 2008; Novak et al. 2010). Thus, in NIX knockout animals, a significant proportion of red blood cells (30–50 %) show persistence of mitochondria in differentiated erythrocytes but no persistence of other organelles such as peroxisomes, secretory pathway members, or ribosomes. Consistent with the assignment of NIX as a mitophagy receptor, it contains a LIR domain and mutations within this domain impair its function (Novak et al. 2010).

In addition to the direct role of mitophagy in erythrocyte maturation, mitophagy has been implicated in developmental transitions in muscle tissue. It was recently shown that, upon differentiation of mouse myoblasts, mitophagy is induced and is necessary for differentiation (Sin et al. 2016). Furthermore, essential metabolic transitions that occur during differentiation of cardiomyocytes have been shown to be mitophagy-dependent (Gong et al. 2015).

Mitophagy in mammalian cells is not limited to development and mitochondrial depolarization. An additional experimental framework in which mitophagy has been observed is during hypoxic treatment of mammalian cells in culture. Hypoxia-induced mitophagy appears to be a protective response (Zhang et al. 2008; Bellot et al. 2009). Later, it was discovered that FUNDC1, a mitochondrial outer membrane protein, binds LC3 and functions as a mitophagy receptor under these conditions (Liu et al. 2012).

Mitophagy in the yeast *S. cerevisiae*

In *S. cerevisiae*, the presence of mitochondria in nitrogen-starvation induced autophagic bodies was demonstrated by EM (Takeshige et al. 1992), similar to what was shown for mammalian cells three decades earlier (Deter et al. 1967). However, those pictures appear to be anecdotal, as only a few mitochondria are actually engulfed by autophagosomes during nitrogen starvation in *S. cerevisiae*. Standard nitrogen starvation protocols, which are used to assay starvation-induced macroautophagy in yeast (transfer from glucose-based minimal medium to glucose-based minimal medium lacking nitrogen; inhibition of TORC1 in minimal and rich

glucose-based medium), do not yield any discernible biochemical or structural manifestations of mitophagy.

The first reports on mitophagy in yeast utilized stress conditions that were induced by mutation. Priault et al. (2005) utilized *fmc1*Δ mutants. These cells fail to properly assemble the F₁F₀ ATPase at high temperatures. Their study showed that under anaerobic conditions and after 24 h at non-permissive temperature, *fmc1*Δ cells appear to accumulate autophagosomes. Concomitantly, levels of ATPase subunits specifically decline, as well as the levels of mitochondrial porins. The authors suggested that these data point to selective autophagy being induced as a result of depolarization but did not demonstrate, either morphologically nor biochemically, that mitochondrial material was actually being degraded in the vacuole under these conditions.

Nowikowsky et al. later showed that upon depletion of K⁺/H⁺ antiporter activity, mitophagy occurs as an early event (Nowikowsky et al. 2007). They reported that the degradation of mitochondria under these conditions occurs via a microautophagic mechanism, in which vacuoles directly engulf mitochondria, thus generating mitochondria-containing autophagic bodies without forming cytosolic autophagosomes. They also demonstrated that Dnm1, a dynamin-like protein that is required for mitochondrial fission, is required for this type of mitophagy. In both of these early studies, the introduction of a genetic lesion causes a physiological defect in mitochondrial function that leads to mitochondrial degradation in the vacuole, via mitophagy.

Initial reports of mitophagy occurring en masse in wild-type *S. cerevisiae* (Kissova et al. 2004) showed that yeast cells grown in rich lactate medium and then transferred to minimal nitrogen starvation on glucose, display mitophagy within 12 h (as measured by transfer of a mitochondrially targeted GFP to the vacuolar lumen). However, as the procedure involves a direct response to starvation coupled with a change in carbon source, it is unlikely to reflect a physiological quality control event.

The observation of mitophagy in *S. cerevisiae* without additional experimental manipulation was first described by Tal et al. (2007) in wild-type yeast cells incubated into stationary phase (beyond 3 days of culture) under respiratory conditions (Tal et al. 2007). This phenomenon may indeed represent a quality control process, as suggested by several observations: (1) standard nitrogen-starvation protocols do not lead to mitophagy; (2) macroautophagy occurs already on day 1 of a 3-day stationary phase mitophagy protocol (Journé et al. 2009) and thus mitophagy is temporally distinct from starvation-induced macroautophagy; and (3) the genetic requirements for starvation-induced mitophagy (coupled to a simultaneous change in carbon source and nitrogen availability) and stationary phase mitophagy do not fully overlap (Kanki et al. 2010).

This stationary phase mitophagy protocol was adapted to carry out large-scale screenings of the yeast knockout collection identifying strains that lost the ability to transfer mitochondrial GFP to the vacuole under respective conditions. These screens identified several interesting genes (Kanki et al. 2009; Okamoto et al. 2009; Kanki et al. 2010). The general message of these studies indicates that mitophagy is a type of selective autophagy. As in all other instances of selective autophagy, mitophagy requires most of the common autophagy machinery (for review, see Feng et al. 2014) and additionally a specific mitophagy receptor. This receptor was identified as Atg32, a type II integral membrane protein of the outer mitochondrial membrane (see Fig. 1a) (Kanki et al. 2009, 2010; Okamoto et al. 2009). Atg32 functions as a mitophagy receptor by interacting with components of the autophagy machinery, e.g., Atg11, which are specific to selective autophagic pathways such as the Cvt pathway and pexophagy, the selective degradation of peroxisomes. Atg32 seems to be expressed uniformly on yeast mitochondria. However, its overall levels increase under mitophagy-inducing conditions in a redox-dependent fashion (Okamoto et al. 2009). Moreover, overexpression of Atg32 leads to an increase in the amplitude of the mitophagic response. More recent work demonstrated that Atg32 undergoes posttranslational modifications that may regulate its activity. It is phosphorylated on serine 114 in its N-terminal cytosolic domain (Aoki et al. 2011) and it is proteolytically cleaved within its intermembrane space C-terminal domain by the mitochondrial AAA protease Yme1 (Wang et al. 2013). The levels of Atg32 respond to the availability of glutathione (Okamoto et al. 2009) and glutathione levels can be manipulated by defects in phospholipid metabolism (Sakakibara et al. 2015), although it is unclear to what degree changes in phospholipid metabolism accompany the onset of mitophagy in wild-type cells. While no direct orthologue of Atg32 is known in mammalian cells, it is clearly functionally analogous to mammalian mitophagy receptors such as optineurin and NDP52 (see Fig. 1).

The interrelationship between redox stress and mitophagy

Within the mitochondrial theory of aging, mitophagy is hypothesized to play a vital role in alleviating the accumulation of defective mitochondrial components. Nonfunctional mitochondria should either hamper cells by generating harmful substances such as reactive oxygen species (ROS), or by being a metabolic burden. Paradoxically, no increase in the levels of ROS under conditions where mitophagy was blocked was found (Kanki et al. 2009). However, Twig et al. (2008) described an increase in oxidized proteins in COS7 cells expressing FIS1 RNAi or a dominant negative DRP1 mutant leading to impaired mitophagy. This result has been

corroborated in yeast by Journo et al. (2009), who found that *aup1Δ* cells, which exhibit decreased mitophagy (Tal et al. 2007), accumulate more oxidized proteins than wild-type cells under conditions of stationary phase mitophagy. Interestingly, Deffieu et al. (2009) showed that the antioxidant N-acetyl-L-cysteine (NAC) can inhibit mitophagy in yeast but not general nitrogen starvation-induced macroautophagy. The effect on mitophagy was due to an increase in glutathione levels and not due to changes in ROS levels. In agreement, glutathione synthetase mutants show a significantly higher level of mitophagy that is not inhibited by NAC. These results suggest that accumulation of oxidized molecules, i.e., proteins, may trigger mitophagy. However, it should also be mentioned that NAC was shown to inhibit ATG32 expression (Okamoto et al. 2009).

Targeting, segregation and engulfment of mitochondria

Mitophagy in *S. cerevisiae* does not lead to the degradation of all mitochondria. This by itself is not surprising, as mitochondria are essential for cell survival. In addition, however, the data of Twig et al. (2008) as well as Narendra et al. (2010) suggest that depolarized mitochondria are generated specifically during fission events and that these depolarized units are specifically targeted for mitophagy. Again, a non-random segregation process is supported by the fact that 85 % of mitochondrial fission events were shown to result in one significantly depolarized and one significantly hyperpolarized daughter mitochondrion. Such segregation, coupled with fission, may ultimately ‘distill’ defective mitochondria out of the dynamic network. Combined with the observation that depolarization of mitochondria leads to ubiquitination of mitofusins (Ziviani and Whitworth, 2010; Ziviani et al. 2010), one may hypothesize that depolarized mitochondria are marked as defective by a specific molecular signature. In mammalian cells, this signal appears to be phospho-ubiquitin (Fig. 1). A respective signal in yeast cells is still missing. However, it is likely to involve post-translational modifications of Atg32, as discussed above.

Several observations suggest that degraded mitochondrial components are not a random representation of mitochondrial biomass and that these components are sorted prior mitophagy. The observation that asymmetric fission results in one hyperpolarized and one depolarized mitochondrion (Twig et al. 2008), together with the fact that mitochondrial dynamics and content mixing and not mitochondrial size, determine the rate of mitophagy suggest a distillation-type mechanism for separating intra-mitochondrial components (Abeliovich 2011; Abeliovich et al. 2013). Interestingly, a direct interaction between Atg11, an autophagy factor functioning in cargo selectivity and Dnm1, a dynamin like ATPase

required for mitochondrial fission has been reported (Mao et al. 2013). Also, mitochondrial fission is known to occur at ER-mitochondrial contact sites (Friedman et al. 2011), which were shown to be important for the induction of mitophagy in yeast (Böckler and Westermann 2014).

Mitophagy as a potential quality control agent in mitigating mtDNA heteroplasmy

As mentioned above, many late-onset mtDNA-linked disorders occur due to the age-dependent clonal takeover of the mtDNA cohort by mutations that are either maternally inherited as heteroplasmic oocyte mtDNA or by somatic mutations that arise due to the error-prone nature of mtDNA replication (Wallace 2005, 2010). The mechanism of clonal takeover is still being debated (Wallace and Chalkia 2013). One popular hypothesis posits that mitophagy can identify mitochondrial fragments that contain such mutant DNA molecules and “purify” the network (Busch et al. 2014). This is consistent with the ‘late-onset’ etiology of these diseases, since autophagy and mitophagy are known to decrease with age in metazoans (Vittorini et al. 1999; Cuervo 2008; Lipinski et al. 2010; Cui et al. 2012). The manner in which recognition of mutant mtDNA could occur is an active area of research. Open reading frames encoded by mtDNA encode, as a rule, polytopic integral membrane proteins of the inner mitochondrial membrane, which function in the electron transport chain. One recent discovery has been that, in contrast with mitochondrial matrix protein, these proteins intermix much more slowly within the mitochondrial network (Wilkens et al. 2013; Busch et al. 2014). In addition, the rate of diffusion of these membrane proteins is similar to that of mitochondrial nucleoids. Thus, it is envisaged that the nucleoids form relatively static “domains” that are enriched in the products of that specific nucleoid. Thus, a mutation in a mitochondrial ORF would generate a defective mitochondrial domain that could be isolated via repeated fission and fusion to generate a defective, ‘edible’ mitochondrion. However, some facts are difficult to reconcile with this hypothesis. For example, some pathological mtDNA mutations, such as the MELAS mutation (Goto et al. 1990) are located in non-protein coding regions such as tRNA molecules, which should be freely diffusible in the matrix. Since tRNA molecules are not hypothesized to be diffusion-constricted, they would not provide the linkage between phenotype and genotype that is essential for purification. Despite this, segregation effects of the MELAS mutation have been reported (Hämäläinen et al. 2013). During iPSC differentiation of MELAS patient-derived iPSCs to neuronal lineages, it was observed that expression of the defective mitochondrial tRNA led to selective degradation of mitochondrial electron transport complex I via mitophagy. This process appears to involve intra-mitochondrial protein segregation

(Hämäläinen et al. 2013; Pickrell and Youle 2013). Additionally, it appears that the mutant DNA also undergoes a segregation effect, as differentiation involved a shift to homoplasmy but did not involve any reduction in mtDNA copy number (Hämäläinen et al. 2013).

However, efforts to demonstrate mutant mtDNA purification by mitophagy in whole animals have met with little success. Pickrell and Youle (2013) and Pickrell et al. (2015) found that when Parkin KO mice were crossed with mice carrying mutant mitochondrial DNA polymerase, a lesion that leads to accumulation of mutant mitochondria and early aging phenotypes, the mice displayed L-DOPA reversible motor defects, without effects on non-dopaminergic neurons. Importantly, the double mutant mice did not show any effects that would imply a Parkin-dependent ‘purification’ of mutant mtDNA (Pickrell et al. 2015). In contrast, previous results from work in cell lines showed precisely such a purifying effect of Parkin expression on heteroplasmy (Suen et al. 2010). The lack of a Parkin-dependent purification activity in whole mice may simply reflect the fact that Parkin-dependent mitophagy is not the only form of mitophagy. In agreement with this latter interpretation, Diot et al. (2015) described conditions under which heteroplasmic mtDNA populations in patient-derived primary fibroblasts underwent purification in a mitophagy-dependent manner. Since they could not detect Parkin expression in their cells, they suggested that this purification process is Parkin-independent. In summary, it is possible that mitophagy responds to protein damage and that it attenuates the penetrance of mtDNA mutants in heteroplasmy, without addressing the core genetic defect. However, it should be borne in mind that some studies in cell lines have suggested a role for mitophagy in directly decreasing mutant mtDNA load. Thus, much work still remains to be done in order to clarify whether mitophagy plays an age-dependent role in preventing clonal takeover by mutant mtDNA in heteroplasmies.

Analyses of mitochondrial function and turnover by mass spectrometry-based proteomics approaches

As mentioned above, mass spectrometry-based proteomics has been successfully used to study the regulation of proteins involved in mitophagy, highlighting the power of these approaches (Heo et al. 2015; Richter et al. 2016). Indeed, mass spectrometry-based proteomics has played a vital role in characterizing mitochondrial biology. As mitochondrial proteins are encoded by nuclear and mitochondrial genes, gene expression analyses cannot answer the question which proteins localize to mitochondria and what their function might be. Thus, already more than a decade ago, scientists aimed at generating a comprehensive compendium of mitochondrial proteins. Initially, qualitative experiments were performed: purified

mitochondria were analyzed by shot-gun proteomics approaches to generate a comprehensive organellar inventory (Mootha et al. 2003; Sickmann et al. 2003) and to map post-translational modifications such as phosphorylation of organellar proteins (Reinders et al. 2007). An inherent problem in these early studies was the presence of co-purifying contaminants that could be misidentified as genuine mitochondrial proteins. This is a common problem in biochemical fractionation/purification experiments, as one cannot enrich organelles to purity.

More recently, quantitative proteomic approaches were developed, which minimize these problems. Label-free approaches that incorporate gradient fractionation profiles (Foster et al. 2006) and approaches that monitor degrees of enrichment (Pagliarini et al. 2008), both allow a better discrimination between truly organellar and contaminating proteins. Indeed, this led to the identification of novel mitochondrial proteins as well as the identification of genes mutated in mitochondrial diseases (PMID; Pagliarini et al. 2008). With the introduction of relative quantification approaches such as stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al. 2002), more sophisticated experiments could be designed. Amongst others, mitochondrial sub-compartments, like the inter-membrane space proteome (Vögtle et al. 2012), mitochondrial protein interactomes (Petrungaro et al. 2015) and mitochondrial protein import (Wrobel et al. 2015) have been characterized in detail.

One of the newest methodological developments to study mitochondrial function by mass spectrometry is termed proximity proteomics (Rees et al. 2015). Different approaches have been designed that all rely on the covalent modification of proteins in close proximity of proteins-of-interest, e.g., by biotin, which allows subsequent biochemical enrichment and determination of “protein neighborhoods”. An engineered ascorbate peroxidase (APEX) was initially used to biotinylate mitochondrial matrix proteins generating new insights into inner mitochondrial transmembrane protein topology (Rhee et al. 2013; Lam et al. 2015). Importantly, APEX labeling can also be used for electron microscopy experiments allowing a combinatorial approach based on imaging and mass spectrometry to study the spatial distribution of proteins.

With specific reference to mitophagy, an unbiased mass spectrometry-based proteomic screen has supported the idea of intra-mitochondrial protein segregation prior mitophagic degradation. Mitochondrial matrix proteins have been identified with widely diverging rates of mitophagy (Abeliovich et al. 2013). Strikingly, mitochondrial matrix proteins that are inefficiently degraded by mitophagy clearly segregate within the matrix from those undergoing efficient mitophagy, whereas inefficiently degraded proteins appear to concentrate in specific foci inside the mitochondrial matrix, those proteins undergoing efficient mitophagy appear to be evenly distributed (Abeliovich et al. 2013). One interpretation of these results

is indeed that a distillation-type process is coupled to mitochondrial dynamics as well as to mitophagic engulfment (Dengjel and Abeliovich 2014).

Proteomic studies using mice also provide indirect support of the concept of intra-mitochondrial selectivity during mitophagy (Kim et al. 2012). Thus, the half-lives of proteins from heart and skeletal muscle mitochondria appear to vary from hours to months, a discrepancy that is irreconcilable with non-selective mitophagy.

Concluding remarks

Our mechanistic understanding of mitophagy has been boosted by recent studies in mammalian cells. On the other hand, the discovery of stationary phase mitophagy in respiring yeast cells allowed high-throughput genomic screens and supported the identification of critical factors required for this process. Studies in both systems suggest that distinct signaling mechanisms identify mitochondria that are destined for degradation by mitophagy. These signaling mechanisms, however, must also be coupled to intra-mitochondrial mechanisms sorting defective, perhaps oxidized, factors. It appears plausible that these signals activate and use the mitochondrial fusion and fission machinery to “distill” faulty components out of the general mitochondrial milieu. Thus, one can postulate not one, but at least two signaling events: The original overall physiological burden of accumulated and unsorted mitochondrial damage may induce a “sorting” process, while the segregation of defective mitochondrial compartments could induce a specific “eat me” signal mediated by phospho-ubiquitin in mammals and by Atg32 modifications in yeast. Different classes of genes, which fulfill different tasks during the various stages described, would be predicted to contribute to the overall process. Taken together, much more work, both scientific and intellectual, is needed to test these hypotheses and to identify the underlying molecular machinery, in the mitochondrial compartments as well as in the cytosol and on the membranes of the vesicular compartment.

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