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Mitochondrial clearance: mechanisms and roles in cellular fitness

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Mitophagy is one of the selective autophagy pathways that catabolizes dysfunctional or superfluous mitochondria. Under mitophagy-inducing conditions, mitochondria are labeled with specific molecular landmarks that recruit the autophagy machinery to the surface of mitochondria, enclosed into autophagosomes, and delivered to lysosomes (vacuoles in yeast) for degradation. As damaged mitochondria are the major sources of reactive oxygen species, mitophagy is critical for mitochondrial quality control and cellular health. Moreover, appropriate control of mitochondrial quantity via mitophagy is vital for the energy supply-demand balance in cells and whole organisms, cell differentiation, and developmental programs. Thus, it seems conceivable that defects in mitophagy could elicit pleiotropic pathologies such as excess inflammation, tissue injury, neurodegeneration, and aging. In this review, we will focus on the molecular basis and physiological relevance of mitophagy, and potential of mitophagy as a therapeutic target to overcome such disorders.

Keywords: adaptor; aging; autophagy; inflammation; mitochondria; neurodegeneration; ubiquitin

Mitochondria are double-membrane subcellular blocks that house essential metabolic processes such as ATP production, phospholipid biosynthesis/transport, amino acid synthesis, and iron–sulfur cluster formation [1–4]. In addition to their roles as polyvalent metabolic factories, mitochondria serve to control calcium signaling,

Abbreviations
ALLO-1, Allophagy-1; ATG, Autophagy-related protein; BCL2L13, B-cell lymphoma 2-like 13; BNIP3, BCL2 and adenovirus E1B 19-kDa-interacting protein 3; BNIP3L, Nip3-like protein X (NIX)/BNIP3-like protein; CCCP, Carbonyl cyanide m-chlorophenylhydrazone; cGAS, Cyclic GMP-AMP synthase; CK2, Casein kinase 2; ERMES, ER-mitochondria encounter structure; FIP200/RB1CC1, FIP200/RB1-inducible coiled-coil protein 1; Fis1, Mitochondrial fission 1 protein; FKBP8/FKBP38, FK506-binding protein 8; FUNDC1, FUN14 domain-containing protein 1; GABARAP, GABA type A receptor-associated protein; GABARAPL1/2, GABA type A receptor-associated protein like 1/2; GFP, Green fluorescent protein; Keap1, Kelch-like ECH-associated protein 1; LC3A/B/C, Microtubule-associated protein 1 light chain 3 alpha/beta/gamma; LIR, LC3-interacting region; LRRK2, Leucine-rich repeat kinase 2; MARCH5/MITOL, Membrane-associated ring-CH-type finger 5; MBP, Maltose-binding protein; MICOS, Mitochondrial contact site and cristae organizing system; Miro, Mitochondrial Rho; MUL1, Mitochondrial E3 ubiquitin protein ligase 1; NBR1, Neighbor of BRCA1 gene 1; NDP52/CALCOCO2, Nuclear dot protein 52 kDa/calcium binding and coiled-coil domain 2; NLRP3, NLR family pyrin domain-containing 3; OPTN, Optineurin; p62/SQSTM1, p62/Sequestosome 1; PARL, Presenilin-associated rhomboid-like protein; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PGAM5, PGAM family member 5, mitochondrial serine/threonine protein phosphatase; PINK1, PTEN-induced kinase 1; PS, Phosphatidylserine; RABGEF1, RAB guanine nucleotide exchange factor 1; ROS, Reactive oxygen species; Src, SRC proto-oncogene, nonreceptor tyrosine kinase; STING, Stimulator of interferon genes; TBC1D9, TBC (Tre-2/Bub2/Cdc16) 1 domain family member 9; TBC1D15, TBC (Tre-2/Bub2/Cdc16) 1 domain family member 15; TBC1D17, TBC (Tre-2/Bub2/Cdc16) 1 domain family member 17; TBK1, TANK-binding kinase 1; TOMM/TOM, Translocase of the outer mitochondrial membrane; TORC1, Target of rapamycin complex 1; UBAN, Ubiquitin-binding domain in ABIN proteins and NEMO; ULK1, Unc-51 like autophagy activating kinase 1; USP, Ubiquitin-specific protease; VDAC, Voltage-dependent anion channel.

inflammatory responses, and apoptosis. Since mitochondria generate ROS (reactive oxygen species) during electron transport through the OXPHOS (oxidative phosphorylation) complexes, they are constantly challenged by oxidative stress [5,6]. Excessively accumulated ROS could randomly react with mitochondrial proteins, lipids, and DNA (mtDNA), ultimately disrupting mitochondrial integrity. The resultant dysfunctional mitochondria could easily release their inner contents such as ROS and mtDNA, which further induce cellular oxidative stress and excess inflammation, respectively, that potentially perturb cell/tissue fitness [7,8]. Accordingly, appropriate removal of damaged mitochondria is essential to maintain biological homeostasis. Moreover, mitochondrial activity and abundance are flexibly adjusted in response to cellular demands. In several tissues such as brain, skeletal muscle, heart, liver, and kidney that consume a massive amount of ATP, mitochondrial biogenesis is strongly promoted and/or mitochondrial respiration is highly activated to meet the cellular needs. When these cells are shifted from energy-consuming conditions to steady-state conditions, mitophagy is often induced to decrease mitochondrial quantity, thereby adapting to cellular metabolic changes. Thus, proper management of mitochondrial quantity is also critical to maintain biological elasticity.

Autophagy, one of the membrane trafficking pathways that delivers cytoplasmic constituents to lysosomes (vacuoles in yeast), plays key roles in quality and quantity control of organelles (Fig. 1, upper part) [9–11]. In this catabolic pathway, double membrane-bound structures called autophagosomes serve as containers for cargo delivery to lysosomes. Autophagosome biogenesis occurs at a relatively low level under non-stressed conditions, whereas it is drastically induced by several stress signals such as amino acid deprivation and nitrogen starvation. Induction of autophagy leads to *de novo* formation of cup-shaped structures called isolation membranes (or phagophores) that expand to become spherical and eventually close to form autophagosomes [10]. During this dynamic process, a portion of the cytoplasm containing proteins and organelles is randomly engulfed by the autophagosome. Finally, autophagosomes fuse with lysosomes for the degradation of autophagosomal content by lysosomal hydrolases. Hence, autophagy

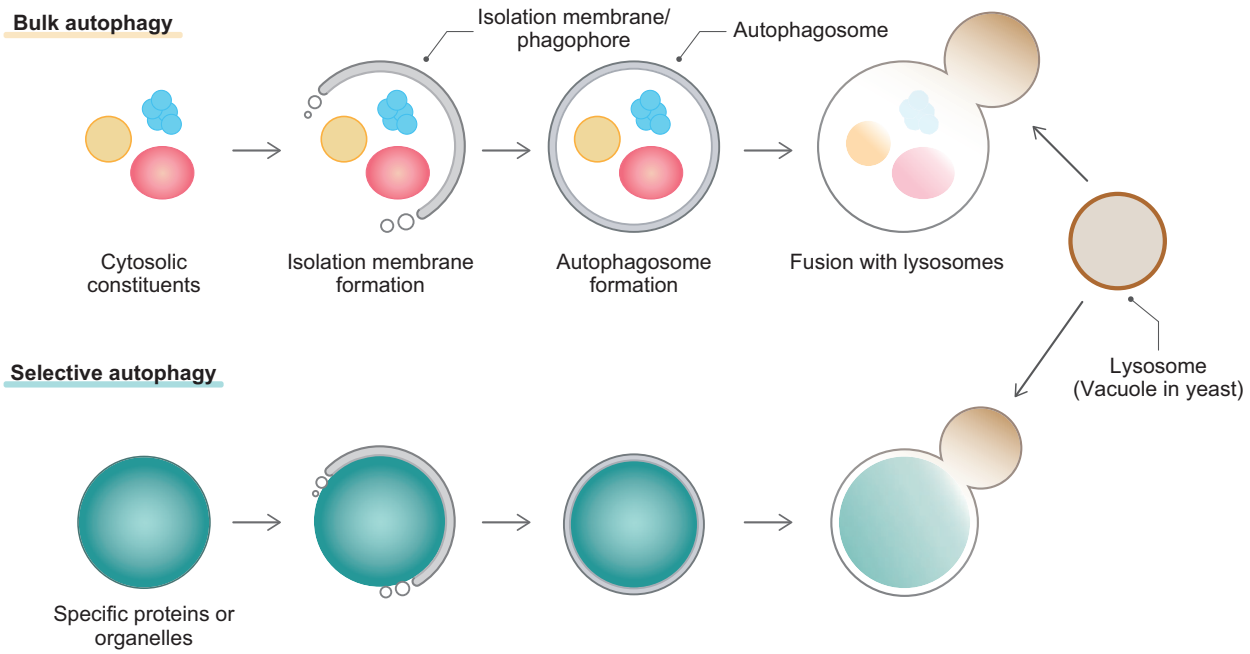

Fig. 1. Two distinct pathways of autophagy. Autophagy can act as either bulk (nonselective) or selective degradation process. In bulk autophagy, cytoplasmic constituents such as proteins, lipids, nucleotides, and organelles are engulfed by the flattened isolation membrane. The isolation membrane expands and closes to form a double membrane-bound structure called autophagosome. Autophagosomes are transported to lysosomes (vacuoles in yeast cells) and fused with the lytic organelles. Lysosomal hydrolytic enzymes degrade the autophagic cargoes. In selective autophagy, specific cargoes such as protein aggregates, dysfunctional organelles, and invading pathogens are sequestered into autophagosomes, delivered to lysosomes, and degraded.

primarily mediates bulk degradation of many intracellular components.

Although autophagy is recognized as a nonselective catabolic pathway, numerous studies have revealed that it also acts in a selective mode (Fig. 1, lower part) [12,13]. This selective type of autophagy is induced in response to specific stimuli (triggers for activation) and requires signals (labels for substrate recognition). Mitophagy is one of the selective autophagy pathways that exclusively encapsulates damaged or excess mitochondria to degrade them [14–19]. Overview of this process is depicted in Fig. 2.

Autophagosome formation itself and mitophagy in general are complex processes involving many proteins that need to be tightly regulated. Studies in the last decades have identified such key proteins important for the degradation of mitochondria in various model organisms [19] (Fig. 3). Importantly, labeling of mitochondria is dictated by specific OMM (outer mitochondrial membrane) proteins called mitophagy receptors, or ubiquitin chains conjugated to multiple OMM proteins. The former recruit the autophagy machinery to mitochondria via direct interaction, whereas the latter do so via indirect interaction with adaptors that bind both ubiquitin and autophagy-related proteins. These labeling molecules establish mitophagy initiation sites and facilitate the generation of isolation membranes localized to mitochondria. Hence, mitophagy can be categorized into two modes: receptor-mediated mitophagy and ubiquitin-mediated mitophagy. In this review, we will focus on molecular mechanisms that promote mitochondrial clearance in yeast and mammalian cells and overview its pathophysiological roles.

Receptor-mediated mitophagy in yeast

Atg32 and other factors involved in yeast mitophagy

Receptor-mediated mitophagy in the budding yeast *Saccharomyces cerevisiae* is mostly dependent on Atg32, a single-pass transmembrane protein on the OMM, that has been identified in 2009 by two independent groups from their genome-wide screens using a nonessential gene deletion library [20,21]. This 59 kDa protein exposes its N-terminal region (43 kDa) to the cytosol and its C-terminal region (13 kDa) to the mitochondrial IMS (intermembrane space) [20,21]. The cytosolic region of Atg32 is critical for its binding

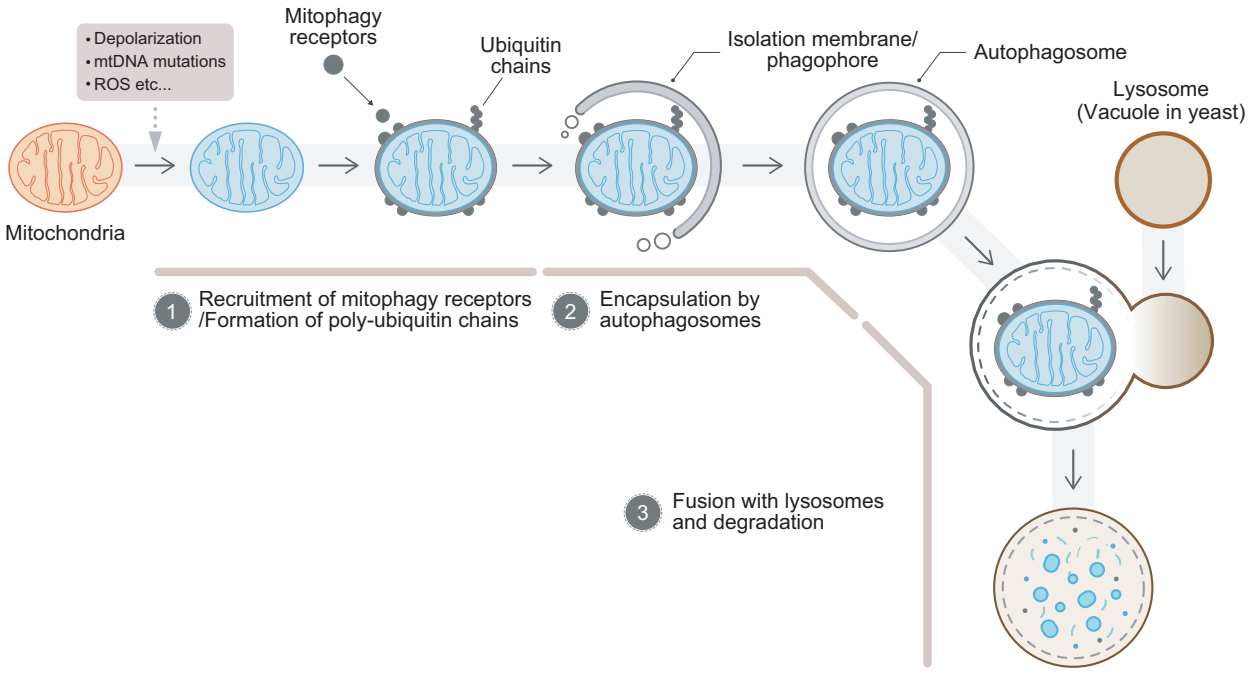

Fig. 2. Overview of mitochondria-specific autophagy (mitophagy). Upon mitochondrial stress (e.g., loss of membrane potential, accumulation of mtDNA mutations, and generation of ROS), mitophagy receptors or ubiquitin chains/autophagy adaptors label mitochondria. These degradation tags recruit the autophagy machinery to the mitochondrial surface and promote generation of isolation membranes/phagophores encapsulating mitochondria. The isolation membrane elongates and closes to form an autophagosome that completely engulfs the targeted mitochondria. Autophagosomes are transported and fuse with lysosomes (vacuoles in yeast). Lysosomal hydrolases then degrade mitochondria.

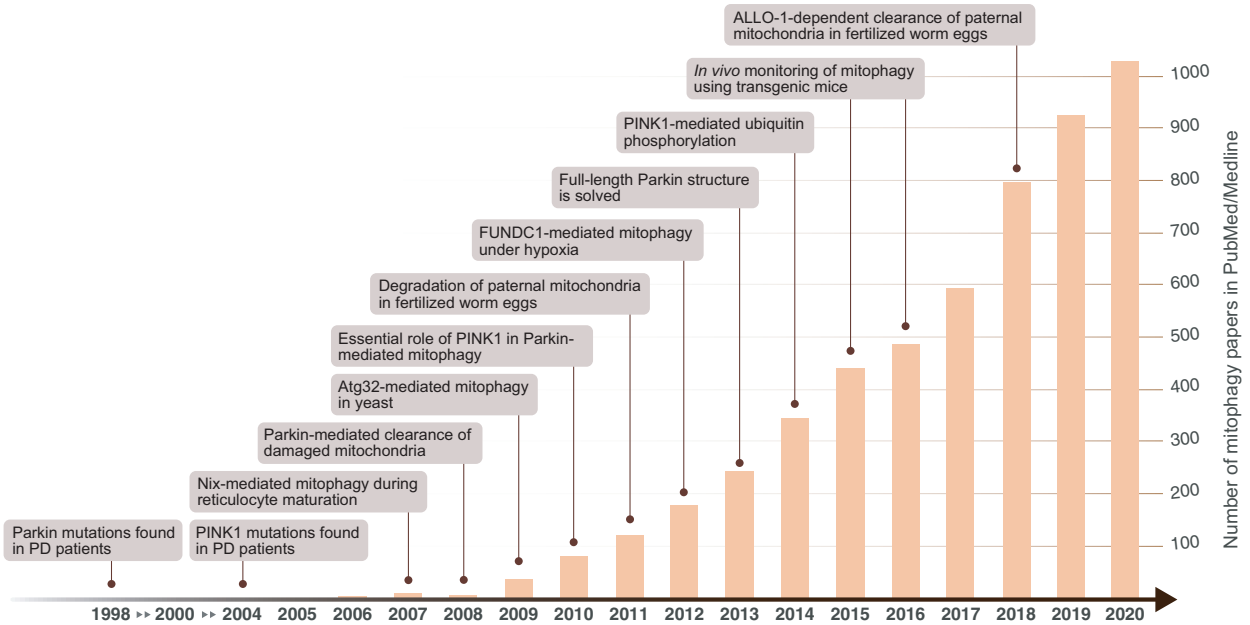

Fig. 3. Expanding histories of the mitophagy field. Spots on the timeline depict key discoveries and developments of experimental methods related to mitophagy. Note that all the important events cannot be included due to space limitations. Bar graphs demonstrate the number of papers published in the indicated year that are extracted from the PubMed/Medline database using the title/abstract keyword 'mitophagy'.

to Atg8 and Atg11 (Fig. 4), promoting formation of autophagosomes encapsulating mitochondria. Loss of Atg32 almost completely abolishes mitophagy, whereas other autophagy-related processes such as bulk autophagy, pexophagy, ER-phagy, and the Cvt (cytoplasm-to-vacuole targeting) pathway are hardly affected, suggesting that Atg32 is a mitophagy-specific receptor [20,21].

Mitophagy in yeast is strongly induced under respiratory or nitrogen starvation conditions [20–23]. When cells are grown in media containing nonfermentable carbon source (e.g., glycerol, ethanol, and lactate), they thoroughly rely on mitochondrial oxidative phosphorylation to generate ATP for their growth and survival. Under these conditions, Atg32 is tran scriptionally upregulated and localized to the OMM, forming a complex with Atg8 and Atg11. Atg8 is conjugated to PE (phosphatidylethanolamine), a major membrane lipid enriched in autophagosomes, and ensures maturation of autophagosomes. Atg11 acts as a scaffold in selective autophagy-related pathways and contains domains required for assembly of core Atg proteins such as Atg1 (a most upstream protein kinase for autophagy initiation) and Atg9 (a polytopic transmembrane protein embedded in vesicle-like structures) [24,25]. Thus, Atg32-Atg11 interactions are crucial to promote formation of autophagosomes. As the N-terminal cytosolic region of Atg32 interacts with Atg8 and Atg11, deletion of this domain significantly abolishes mitophagy under respiratory conditions, whereas deletion of the IMS domain has slight or almost no effects on mitophagy [26].

A previous study indicates a link between mitochondrial dynamics and mitophagy. Dnm1, a dynamin-related mitochondrial division GTPase localized to the mitochondrial surface via Fis1 (a tail-anchored OMM protein) and Mdv1 or Caf4 (a WD repeat adaptor for Dnm1 and Fis1) [27], interacts with Atg11 and associates with the Atg32-8-11 complex [28]. Atg11-Dnm1 interactions are critical for efficient mitophagy, and loss of Dnm1 causes significant defects in degradation of mitochondria. However, other studies have demonstrated that mitophagy is not significantly altered in the absence of Dnm1 [29,30], suggesting a previously unappreciated mechanism mediating mitochondrial fragmentation during mitophagy in some specific strains and/or under some specific conditions.

Domain structure of Atg32

Atg32 is predicted to consist of three modules: an N-terminal cytosolic domain (amino acid residues 1–388 a.a.), a single TM (transmembrane) domain (389–411 a.a.), and a C-terminal IMS domain (412–529 a.a.). The TM domain is required for targeting and insertion

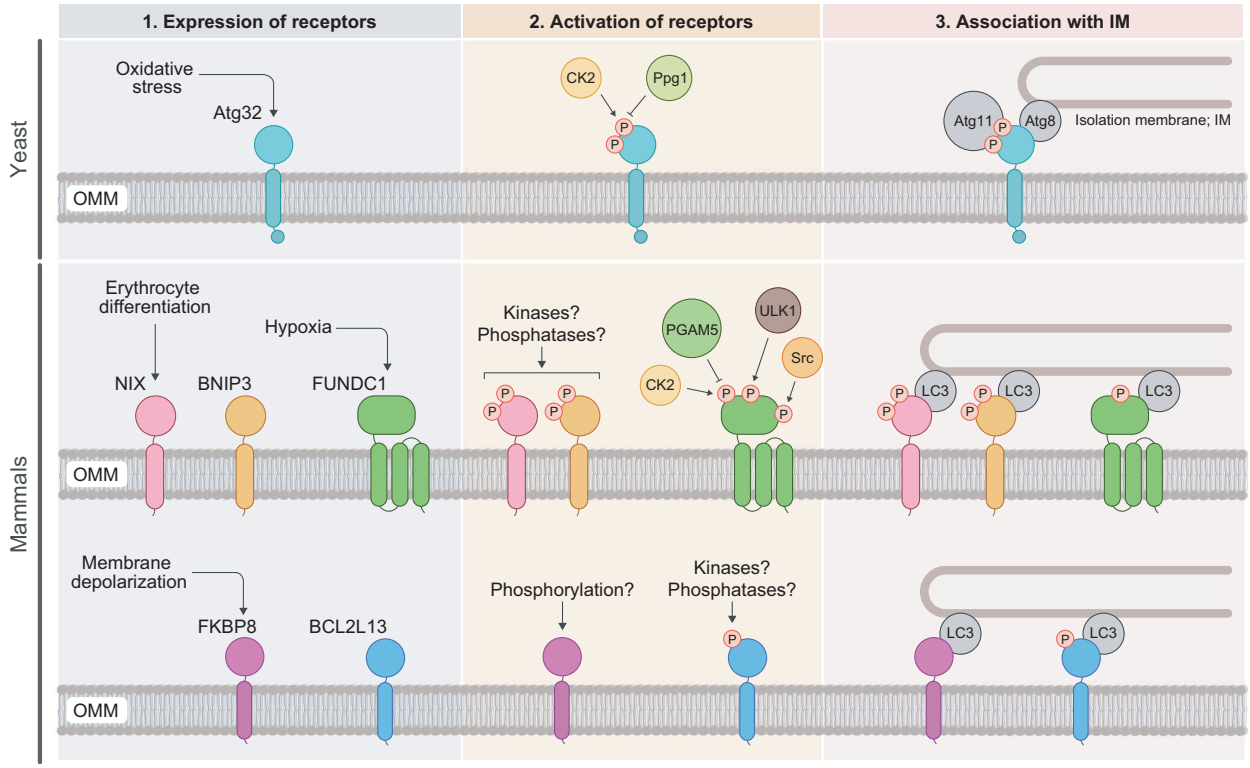

Fig. 4. Models for initial actions of mitophagy receptors. In yeast, the sole mitophagy receptor Atg32 is induced in response to oxidative stress and anchored to the OMM. Atg32 is then activated via CK2-dependent phosphorylation, which is reversed by the protein phosphatase Ppg1. Activated Atg32 interacts with Atg8 and Atg11, promoting formation of the isolation membrane. In mammalian cells, NIX, BNIP3, FUNDC1, FKBP8, and BCL2L13 seem to be induced in response to developmental cues and mitochondrial stress signals, activated via phosphorylation, and interact with LC3 on the OMM. Several kinases and phosphatases have been reported to regulate the activities of these mitophagy receptors.

of Atg32 to the OMM, as a variant lacking the TM domain is not properly localized to mitochondria but disperses throughout the cytosol and nucleus [26]. The N-terminal cytosolic domain contains two consensus motifs (86–122 a.a.) critical for the interaction of Atg32 with Atg8 and Atg11. Mutations in the AIM (Atg8-family interacting motif) affect Atg32-Atg8 interactions and cause a partial defect in degradation of mitochondria, suggesting that Atg32 binding to Atg8 contributes, at least to some extent, to mitophagy [26]. Moreover, a recent study suggests that Gyp1, a GAP (GTPase-activating protein) of Ypt1, has been identified as a novel Atg8 interaction partner. Loss of Gyp1, its GAP activity, or its first AIM; AIM1 (160–163 a.a.) causes a partial reduction in mitophagy, yet how Gyp1 contributes to autophagosome formation remains elusive [31].

An artificially OMM-anchored Atg32 cytosolic domain is sufficient to promote degradation of mitochondria. Remarkably, when ectopically anchored to peroxisomes, the Atg32 cytosolic domain is capable of promoting pexophagy, supporting the idea that the cytosolic domain of Atg32 acts as a degron-like module necessary and sufficient for the degradation of specific organelles [26]. In *S. cerevisiae*, ectopic targeting of the pexophagy receptor Atg36 to mitochondria can also activate mitophagy even in the absence of Atg32 [32]. Thus, mitophagy and pexophagy in yeast may share a common set of molecules coordinately acting downstream of their receptors to mediate formation of autophagosomes surrounding each organelle. Whether ectopically targeted Atg32 and Atg39/40 (receptors required for ER-phagy) can promote ER-phagy and mitophagy, respectively, remains to be investigated.

A recent study using NMR spectroscopy has revealed that Atg32 has a previously undescribed PsR (pseudo-receiver) domain (200–341 a.a.) [33]. Deletion of this region significantly reduces Atg32-Atg11 interactions and mitophagy activity during nitrogen starvation. A comparative analysis of protein structures indicates that this domain is well aligned to receiver

domains found in RRs (response regulators) known as a major family of signaling proteins in prokaryotes. Receiver domains in RRs accept phosphoryl groups transferred from specific protein kinases that sense environmental changes to transduce cellular signaling [34]. Despite their structural homology, Atg32 lacks the conserved residues that are essential for receiver domain function, thereby classifying Atg32 as a PsR domain family protein. The Atg32 PsR domain function is yet to be determined; however, it may contribute to activation of neighboring Atg32 domains or recruiting additional factors to mitochondria.

Transcriptional activation of Atg32

How Atg32 protein levels are increased upon mitophagy induction still remains obscure. Accumulation of oxidative stress caused by inefficient electron transport in mitochondria seems to be a primary signal that activates Atg32 transcription, as a treatment with NAC (N-acetylcysteine), a precursor form of the antioxidant glutathione and a scavenger of free radicals, suppresses Atg32 induction [21]. A study using *Pichia pastoris*, a methylotrophic yeast, has reported the roles of Tor (target of rapamycin) and its downstream *Pp*Sin3-*Pp*Rpd3 complex in expression of the ortholog *Pp*Atg32 [35]. Tor is a serine/threonine kinase that controls a variety of growth signals and connects nutrient availability with most of anabolic and catabolic processes. Inhibition of Tor by rapamycin induces *Pp*Atg32 expression, but can promote neither *Pp*Atg32 phosphorylation nor mitophagy. In addition, *Pp*Sin3 and *Pp*Rpd3, two orthologs that function downstream of Tor as a transcriptional repressor complex for Atg8 in *S. cerevisiae*, are also important for transcriptional repression of *Pp*Atg32. In cells lacking *Pp*Sin3 and *Pp*Rpd3, *Pp*Atg32 is induced without starvation signals, suggesting that the Tor-Sin3-Rpd3 axis contributes to downregulation of *Pp*Atg32 expression. Moreover, a recent study showed that loss of the Paf1 (RNA polymerase Ⅱ-associated factor 1) complex, a highly conserved complex involved in transcription elongation, leads to global upregulation of *ATG* genes including *ATG32* and *ATG11*, and induction of mitophagy [36].

A previous report has revealed that protein N-terminal acetylation, a co-translational modification conserved from yeast to humans, is required for efficient mitophagy [37]. During prolonged respiration, the *ATG32* mRNA and Atg32 protein levels are partially reduced in cells lacking NatA (N-acetyltransferase A), a heterodimeric complex that consists of the catalytic subunit Ard1 and the ribosomal adaptor subunit Nat1, or cells expressing a catalytically inactive NatA variant. Although NatA substrates critical for mitophagy in yeast have not yet been identified, protein N-terminal acetylation seems to be linked to the regulatory steps of Atg32 induction.

Phosphorylation of Atg32

Phosphorylation/dephosphorylation switch of Atg32 is a key regulatory step of mitophagy. Previous studies have demonstrated that Atg32 is phosphorylated under mitophagy-inducing conditions in a manner dependent on Ser114 and Ser119 [26,38,39]. Substitution of these residues with alanine significantly decreases Atg32 phosphorylation, Atg32-Atg11 interactions, and mitophagy. Importantly, these post-translational modifications are mediated by CK2 (casein kinase 2), a constitutively active serine/threonine kinase (Fig. 4) [39].

A recent study has revealed that the PP2A-like phosphatase Ppg1 dephosphorylates Atg32 and suppresses mitophagy (Fig. 4) [40]. Loss of Ppg1 and its interacting partners Far proteins enhances CK2-dependent Atg32 phosphorylation, Atg32-Atg11 interactions, and mitophagy. The Ppg1-Far complex binds to Atg32 on the OMM, and this interaction is reduced upon nitrogen starvation, indicating that dissociation of the Ppg1-Far complex from Atg32 is crucial for Atg32 activation [41]. Signals that perturb the association of the Ppg1-Far complex with Atg32 upon mitophagy induction remain unclear.

Moreover, excess activation of TORC1 (Tor complex Ⅰ) seems to destabilize Atg32-Atg11 interactions [42]. Loss of Npr2, a component of the SEACIT (Seh1-associated complex inhibiting TORC1) complex that suppresses TORC1 activity, significantly reduces mitophagy during prolonged respiration. Whether overactivation of TORC1 affects the phosphorylation status of Atg32 remains to be tested. Given that TORC1 may negatively regulate CK2-substrate interactions [43], it is conceivable that SEACIT-dependent inactivation of TORC1 could promote CK2-mediated Atg32 phosphorylation critical for Atg32-Atg11 interactions.

Relevance of phospholipids in mitophagy

Multiple studies establish that perturbation of phospholipid homeostasis in mitochondria or the ER could affect mitophagy. Psd1 and Psd2, two phosphatidylserine decarboxylases that synthesize PE from PS (phosphatidylserine), are required for efficient mitophagy [44]. Intriguingly, Psd1 is only required for nitrogen

starvation-induced mitophagy, while Psd2 is more critical for prolonged respiration-induced mitophagy but also partially for nitrogen starvation-induced mitophagy. Although detailed mechanisms underlying these defects are not well understood, loss of Psd1 seems to cause inefficient recruitment of Atg8 to the surface of mitochondria.

Loss of Opi3, a phospholipid methyltransferase localized in the ER, leads to suppression of Atg32 induction and a decrease in mitophagy during respiration [45]. Opi3 catalyzes conversion of PE to PC (phosphatidylcholine) in the ER membrane. Deletion of the *OPI3* gene causes aberrant elevation of glutathione levels that reduces cellular oxidative stress, thereby negatively affecting Atg32 induction and mitophagy [45]. These findings raise the possibility that respiring yeast cells coordinate phospholipid methylation and mitophagy via unknown mechanisms.

The ER stress induced by DTT (dithiothreitol) affects PE export from mitochondria to the ER, causing reduced PC levels in both organelles [46]. This ER stress-responsive PE transport is reduced in cells lacking Mdm10, a component of the ERMES (ER-mitochondria encounter structure), leading to more reduced levels of PC in the ER and mitochondria. Reducing mitochondrial PC levels could induce mitophagy [46]; however, the detailed mechanisms remain to be elucidated.

Regulation of Atg32-mediated mitophagy via ER factors

Exchange of phospholipids between the ER and mitochondria is mediated by the ERMES complex [47]. This multimeric tethering complex is localized as distinct foci where the ER and mitochondrial membranes are closely positioned. Loss of ERMES components strongly decreases starvation-induced mitophagy [48]. Under the same conditions, the ERMES component Mmm1 is colocalized with Atg8 and Atg32, supporting the idea that ERMES acts in formation of autophagosomes enclosing mitochondria.

A recent study has revealed a link between mitophagy and protein biogenesis in the ER. The GET (guided-entry of tail-anchored proteins) pathway mediates membrane insertion of TA (tail-anchored) proteins into the ER. Get1 and Get2 form a heterodimeric complex on the ER, acting as a receptor for TA proteins newly synthesized in the cytosol and promoting membrane insertion of TA proteins [49–51]. Loss of the Get1/2 complex strongly decreases mitophagy, whereas it only slightly affects other autophagy-related processes [52]. Further studies are needed to elucidate how this ER-associated complex acts in Atg32-mediated mitophagy.

Receptor-mediated mitophagy in mammals

In mammals, multiple mitophagy receptors/adaptors seem to promote selective clearance of mitochondria (Fig. 4). These receptors/adaptors function in certain specific cell types and under particular conditions, but they may also function in a partially redundant manner. Similar to Atg32, mammalian mitophagy receptors are transmembrane proteins anchored to the OMM and contain LIR (LC3-interacting region) motif that binds to LC3, a mammalian Atg8 homolog, thereby localizing autophagosome formation to mitochondria.

BNIP3 and NIX

BNIP3 (Bcl-2/adenovirus E1B 19 (nineteen)-kDa-interacting protein 3) is a ubiquitously expressed protein that has first been identified as a pro-apoptotic mitochondrial protein to increase permeability of mitochondrial membranes and release cytochrome *c* [53–55]. BNIP3 is expressed as an inactive monomer under normal conditions, but upon stress stimuli, it homodimerizes on the OMM in a manner dependent on its TM domain. Mutations in the TM domain, which disrupt BNIP3 homodimerization but not its mitochondrial targeting, reduce the number of autophagosomes [56]. BNIP3 contains a conserved LIR motif in its N-terminal region and interacts with LC3. Mutations in this region reduce the interaction between BNIP3 and LC3 [56]. Expression of a BNIP3 LIR mutant reduces colocalization of LC3 with mitochondria, indicating that LC3 targets to mitochondria via its interaction with BNIP3. Furthermore, substitutions of Ser17 and Ser24 near the LIR motif of BNIP3 decrease its interaction/colocalization with LC3B under mitophagy-inducing conditions [57]. In addition, overexpression of phosphomimetic BNIP3 mutants decreases mitochondrial protein levels. Collectively, these findings suggest that phosphorylation of BNIP3 promotes BNIP-LC3 interactions and mitophagy.

NIX (Nip3-like protein X)/BNIP3L (BNIP3-like protein) has previously been identified with its high homology to BNIP3 (56% identical overall) and is required for efficient elimination of mitochondria during erythrocyte maturation [58–60]. Expression of BNIP3 can promote degradation of mitochondria in $Nix^{-/-}$ reticulocytes. Similar to BNIP3, NIX is a single-pass transmembrane protein and contains a canonical

LIR motif that promotes interactions with several Atg8 family proteins. Ser34 and Ser35, which are located near the LIR motif of NIX, seem to be phosphorylated and contribute to NIX-LC3 interactions [61]. NIX stably dimerizes on the OMM via its TM domain. This dimerization is required for efficient NIX-LC3 interactions and robust recruitment of the autophagy machinery to mitochondria, as disruption of the dimerization decreases LC3 puncta formation and augments mitochondrial loss under mitophagy-inducing conditions [62]. How developmental cues during reticulocyte differentiation induce NIX activation is currently unknown.

FUNDC1

Under hypoxic conditions and reduced availability of oxygen, mitochondrial mass and respiration are reduced [63]. FUNDC1 (Fun14 domain-containing protein 1), a ubiquitously expressed multi-spanning OMM protein conserved from *Drosophila* to humans, acts as a receptor for hypoxia-induced mitophagy [64]. FUNDC1 contains a LIR motif in its N-terminal region, and deletion of this motif reduces the FUNDC1-LC3 interactions and impairs mitophagy [64]. Mass-spectrometric analysis revealed that Ser13 and Tyr18 around the LIR motif are potential phosphorylation sites [64,65]. Two distinct kinases, CK2 and Src, contribute to phosphorylation of FUNDC1, thereby inhibiting FUNDC1-LC3 interactions and subsequent mitophagy [64,65]. Upon hypoxia, the mitochondrial protein phosphatase PGAM5 dephosphorylates FUNDC1 at Ser13 and stabilizes FUNDC1-LC3 interactions [65]. Moreover, ULK1, an Atg1 kinase homolog in mammals, localizes to mitochondria and phosphorylates Ser17 of FUNDC1 to promote FUNDC1-LC3 interactions [66]. This finding raises the possibility that mitophagosome formation could be regulated via the interaction between core Atg proteins and mitophagy receptors. Whether this is the case for other mitophagy receptors remains to be investigated.

During hypoxia, FUNDC1 protein levels are decreased by the ubiquitin-proteasome system [67]. FUNDC1 is ubiquitinated at Lys119 by the E3 ubiquitin ligase MITOL/MARCH5 (a mitochondrial RING finger E3 ligase) and subjected to the proteasome-mediated degradation [67]. Mutation of Lys119 leads to an increase in levels of FUNDC1 upon hypoxia, promoting targeting of LC3 to mitochondria. Thus, MARCH5-mediated FUNDC1 degradation may suppress hypoxia-induced mitophagy. It is still elusive whether other types of mitophagy receptors are ubiquitinated and degraded via the proteasome-dependent pathway.

BCL2L13

Although the mammalian homolog of Atg32 has not been identified, a previous study revealed that mitophagy in yeast lacking Atg32 could partially be restored by expression of BCL2L13 (BCL2-like 13), a mammalian mitochondrial protein containing a single TM domain and two LIR motifs [68]. Expression of a BCL2L13 mutant defective in LC3 interaction does not induce mitophagy in the *atg32*-null yeast, indicating that BCL2L13 could interact with Atg8 via its LIR motif to promote mitophagy in yeast [68]. Loss of Atg7, a core Atg protein required for Atg8 lipidation, abolishes BCL2L13-mediated mitophagy in yeast lacking Atg32, indicating that BCL2L13 promotes yeast mitophagy via the conventional autophagy machinery. The affinity of BCL2L13 for LC3 seems to be modulated via its phosphorylation, as a mutation of Ser272 near the second LIR motif reduces BCL2L13-LC3 interactions and mitophagy. Moreover, a recent study demonstrated that BCL2L13 recruits ULK1 to mitochondria [69]. Whether ULK1 directly phosphorylates BCL2L13 has not been elucidated. Further studies will clarify whether/how BCL2L13 activity is regulated under mitophagy-inducing conditions.

FKBP8

FKBP (FK506-binding protein) family members are evolutionally conserved intracellular receptors for the immunosuppressive drug FK506 and act in diverse cellular processes such as calcium signaling, transcription, and protein folding/trafficking [70]. FKBP8 (FK506-binding protein 8, also known as FKBP38) is highly expressed in brain, and a recent study suggests its roles in mitophagy under several stress conditions. FKBP8 is targeted to the OMM upon CCCP treatment and binds to LC3A via its N-terminal LIR motif [71]. Co-overexpression of ATG8 family proteins, especially LC3A, and FKBP8 induces mitophagy in a manner independent of Parkin. Moreover, loss of FKBP8 increases enlarged mitochondria and overexpression of FKBP8 causes mitochondrial fragmentation, suggesting that this protein also acts in mitochondrial dynamics. Since FKBP8 functions in diverse cellular processes, further experiments are necessary to clarify how FKBP8 promotes mitophagy under physiological conditions.

Ubiquitin-mediated mitophagy in mammals

In addition to mitophagy receptors described above, ubiquitin chains on the OMM serve as signals for damaged mitochondria. Although several E3 ubiquitin ligases have been reported to play a role during mitophagy in mammals, we will mainly focus on two key players, PINK1 and Parkin, that are known as causal genes for hereditary recessive Parkinson's disease (PD) with early onset [72,73]. Thus, elucidation of the mechanisms underlying PINK1/Parkin-mediated mitophagy will shed light on the pathogenesis of this neurodegenerative disease.

PINK1 activation and phosphorylation of ubiquitin

PINK1/Parkin-mediated mitophagy mainly acts in elimination of damaged mitochondria with lower membrane potential [74–78]. The most upstream factor of this pathway is PINK1 (PTEN-induced putative kinase 1), a mitochondrial serine/threonine kinase. Under conditions that mitochondrial membrane potential is properly maintained, PINK1 precursor is constantly imported to the IMM (inner mitochondrial membrane) through TOMM40 in the OMM and TIMM23 in the IMM. The MTS (mitochondria-targeting signal) of this precursor is processed by MPP (mitochondrial processing peptidase), and the TM domain is cleaved by the IMM protease PARL (Presenilins-associated rhomboid-like protein) (Fig. 5) [79–82]. After the cleavage by PARL, PINK1 loses its TM domain and is translocated back to the cytosol, where PINK1 is ubiquitinated by E3 ubiquitin ligases UBR1/2/4 and rapidly degraded in a proteasome-dependent manner [83]. Thus, PINK1 expression is maintained at a relatively lower level under steady state conditions. Upon membrane potential dissipation (by treatment with ionophores such as CCCP and valinomycin), PINK1 precursor is not translocated across the IMM and thus cannot be processed by PARL, and, instead, is accumulated on the OMM. Then, PINK1 forms a homodimer on the OMM and is autophosphorylated at Ser228 and Ser402 (Fig. 5) [84]. This autophosphorylation of PINK1 is required for its kinase activity.

In 2014, three independent groups have reported that PINK1 phosphorylates the Ser65 residue of ubiquitin on the OMM [85–87]. The resultant phospho-ubiquitin has a high affinity for cytosolic Parkin, an E3 ubiquitin ligase, thereby recruiting Parkin to the surface of depolarized mitochondria. Importantly, activation of Parkin is also required for its mitochondrial targeting, as a catalytically inactive Parkin mutant cannot localize to depolarized mitochondria [88–91]. PINK1 also phosphorylates Parkin at the Ser65 residue, inducing conformational changes and activation of Parkin [92–94].

PINK1 stability seems to be modulated via PKA (protein kinase A) and PKA-mediated phosphorylation of MICOS (mitochondrial contact site) components. The MICOS complex tethers the IMM and OMM to establish a connection between the two membranes [95]. MIC60, a component of the MICOS complex, transiently interacts with PINK1 upon CCCP treatment, and its downregulation reduces PINK1 localization to depolarized mitochondria, thereby decreasing Parkin mitochondrial targeting [96]. Moreover, PKA phosphorylates MIC60 to negatively affect Parkin translocation to mitochondria, likely through disrupting PINK1-MIC60 interactions and consequently destabilizing PINK1, without affecting mitochondrial cristae structures.

Parkin-mediated ubiquitination of OMM proteins

Similar to other RBR (RING-in-between-RING) E3 ligases, Parkin contains two RING finger domains and an IBR (in-between-RING) region, and the overall structure of Parkin has been solved in 2013 [97,98]. Parkin is ubiquitously expressed in mammalian tissues. After recruited to damaged mitochondria and phosphorylated by PINK1, Parkin builds ubiquitin chains on OMM proteins (Fig. 6). Several OMM proteins including mitofusin, Miro, and VDAC have been identified as putative Parkin substrates [99–106]. A recent study revealed that exogenous proteins (e.g., GFP and maltose-binding protein; MBP) artificially targeted to mitochondria can be ubiquitinated by Parkin [107]. Moreover, when Parkin is recruited to peroxisomes via its interaction with the peroxisome-targeted PINK1, proteins on the peroxisomal membrane are ubiquitinated by Parkin [108]. Collectively, these results suggest that Parkin does not have high substrate specificity, which may be optimal for efficient and quick ubiquitin coating of damaged mitochondria. These ubiquitin chains are further phosphorylated by PINK1 and act as additional receptors for Parkin mitochondrial targeting (Fig. 6).

Autophagy adaptors linking ubiquitin chains to autophagosomes

How are ATG proteins recruited to ubiquitin-coated mitochondria? Autophagy adaptors are important to link mitochondria to the autophagy machinery. These

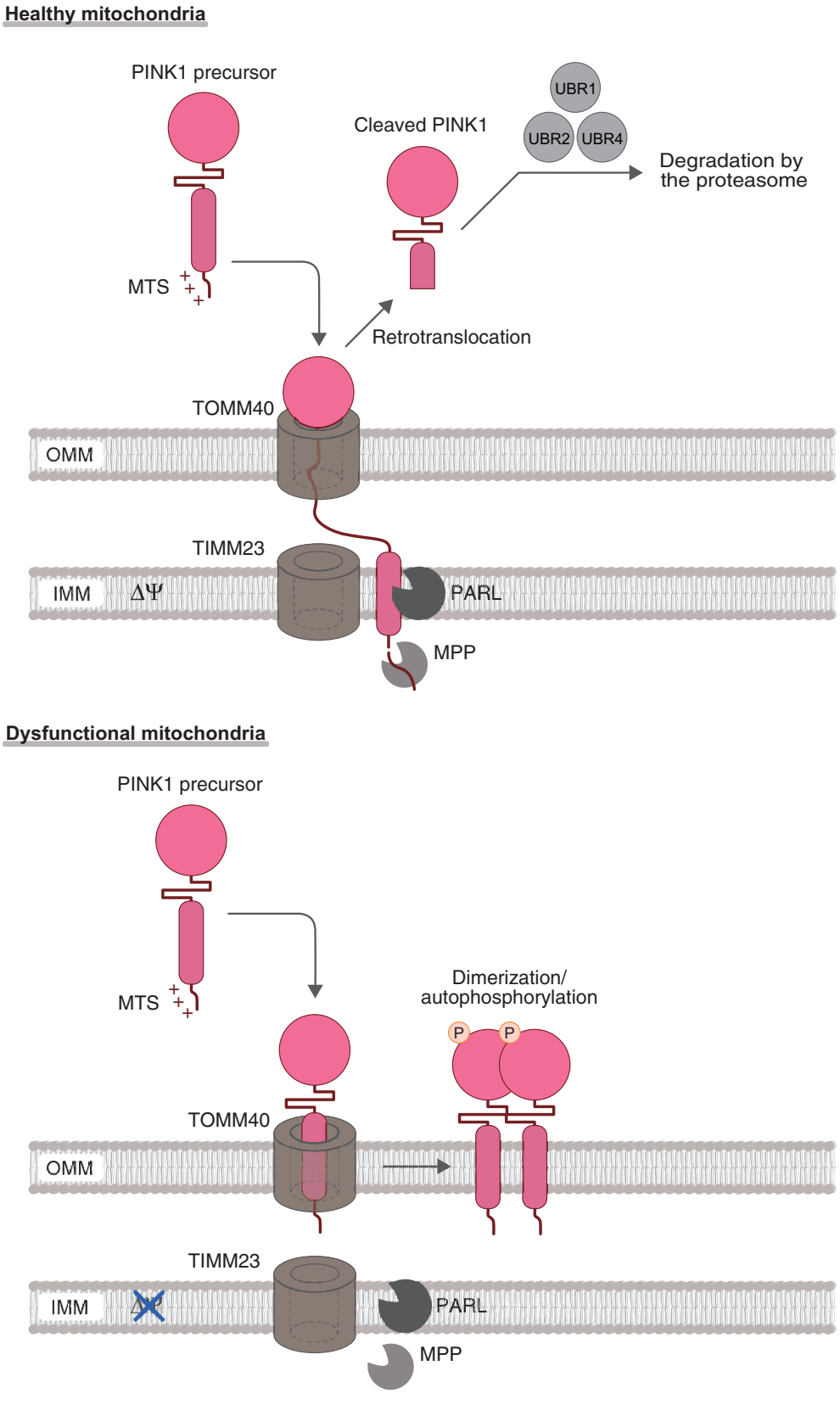

Fig. 5. PINK1 behaviors on healthy and dysfunctional mitochondria. On healthy mitochondria, a newly synthesized PINK1 precursor is imported into mitochondria in a membrane potential ($\Delta\Psi$)-dependent manner through the two translocator channels TOMM40 and TIMM23. MPP (mitochondrial processing peptidase) in the mitochondrial matrix cleaves the PINK1 MTS (mitochondrial targeting signal), and PARL (an IMM protease) cleaves the PINK1 TM domain. Processed PINK1 is retrotranslocated to the cytosol, subsequently recognized/modified by the E3 ubiquitin ligases UBR1/2/4, and degraded by the proteasome. Upon $\Delta\Psi$ dissipation, the PINK1 precursor is neither translocated across the IMM nor cleaved by PARL. Instead, it is stuck in the TOMM40 channel and inserted into the OMM, undergoing homodimerization and autophosphorylation to self-activate.

adaptors include p62/SQSTM1, NBR1, NDP52/CALCOCO2, TAX1BP1, and OPTN (optineurin), containing both a UBD (ubiquitin-binding domain) and a LIR motif that connect ubiquitin-coated mitochondria with autophagosomes [109–112]. All these receptors are recruited to depolarized mitochondria [113]. Among five autophagy adaptors, NDP52 and OPTN seem to be crucial for PINK1/Parkin-mediated mitophagy [113,114]. PINK1 kinase activity is required for localization of NDP52 and OPTN to depolarized mitochondria, further recruiting the upstream ATG proteins such as ULK1 and WIPI1 that induce autophagosome biogenesis. During phagophore elongation, PE-conjugated ATG8 proteins subsequently recruit more NDP52 and OPTN via their LIR motifs, forming a positive feedback loop to accelerate autophagosome biogenesis [115]. In addition, NDP52 associates with the ULK1 complex through its component FIP200, which is facilitated by the serine/threonine kinase TBK1 (TANK-binding kinase 1) [116]. Ectopic expression of NDP52 on the OMM is sufficient to recruit the autophagy machinery to mitochondria and initiate mitophagy in the absence of LC3, raising the possibility that NDP52 targets the autophagy initiation complex directly to the cargo. Moreover, OPTN interacts with ATG9 vesicles, a membrane source for phagophore elongation, and disruption of this interaction suppresses mitophagy [117]. Collectively, these results suggest that mitophagy adaptors associate with several core ATG proteins and promote formation of autophagosomes encapsulating damaged mitochondria.

TBK1-mediated phosphorylation of autophagy adaptors

TBK1 is activated via its phosphorylation at Ser172 upon mitochondrial depolarization. This activation requires OPTN and NDP52, and ubiquitin-binding activity of OPTN [114]. Loss of TBK1 reduces

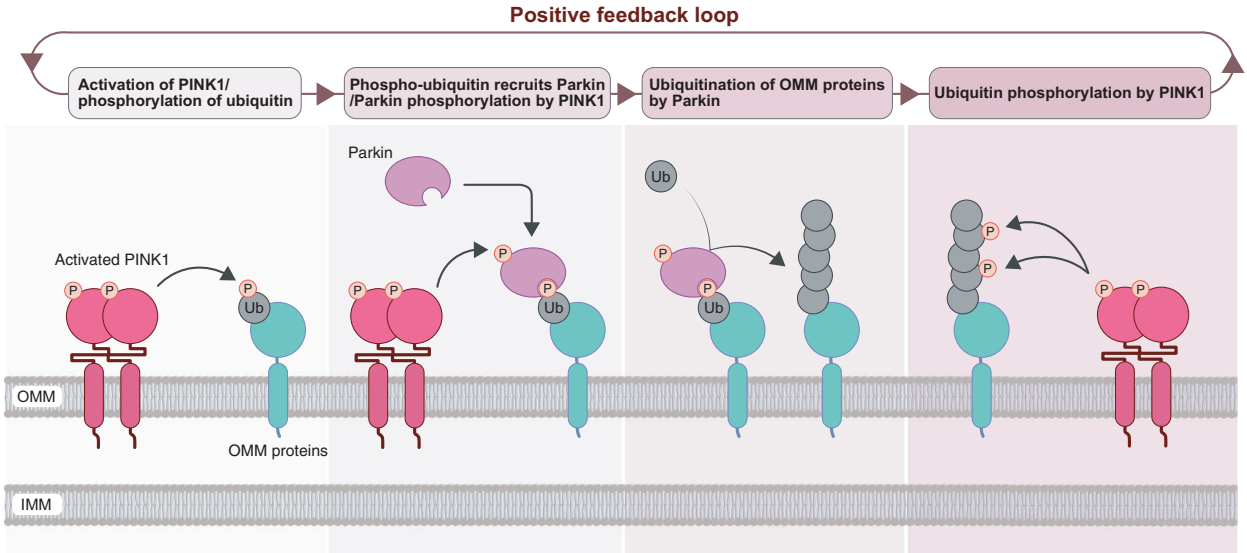

Fig. 6. Formation of positive feedback loop with Parkin-mediated polyubiquitination. Activated PINK1 phosphorylates ubiquitin on the OMM proteins. The resultant phospho-ubiquitin acts as a receptor for recruiting Parkin to damaged mitochondria. Parkin is activated via PINK1-dependent phosphorylation, promoting conjugation of ubiquitin chains to OMM proteins. These ubiquitin chains serve as additional substrates for PINK1, establishing a positive feedback loop to coat damaged mitochondria with massive ubiquitin chains.

recruitment of the autophagy adaptors such as NDP52, OPTN, and p62 to damaged mitochondria. Moreover, activated TBK1 mediates phosphorylation of OPTN at its UBAN domain to increase the binding to ubiquitin chains, and at Ser177 to stabilize its interaction with LC3, synergistically reinforcing OPTN association with ubiquitin-coated mitochondria and autophagosomes [114,118]. Notably, phosphorylation of OPTN further accelerates TBK1 activation, thereby promoting more recruitment of OPTN to depolarized mitochondria and forming another positive feedback loop to facilitate mitophagy [114]. In addition, a recent study suggests that Ca^{2+} increases interactions of a Ca^{2+}-binding protein TBC1D9 (Tre-2/Bub2/Cdc16 (TBC) 1 domain family member 9) with ubiquitin chains on damaged mitochondria, which are required for TBK1 activation and mitophagy [119].

Phagophore elongation during mitophagy

Ubiquitin chains formed by Parkin on the OMM are required for recruitment of RABGEF1, an upstream factor of the endosomal Rab GTPase cascade, to mitochondria [120]. RABGEF1 induces transient mitochondrial targeting of its downstream factors RAB5, MON1/CCZ1, and RAB7. RAB7 is inserted into the OMM and acts in assembly of ATG9-containing vesicles to damaged mitochondria [120]. TBC1D15 (TBC 1 domain family member 15) is a GAP for RAB7 and extracts RAB7 from the OMM. TBC1D15 is localized to the OMM via its interaction with Fis1, an OMM protein. Loss of Fis1 or TBC1D15 leads to massive accumulation of RAB7 on damaged mitochondria [120,121]. Taken together, these results suggest that proper endosomal RAB cycles are critical for efficient mitophagosome formation.

Deubiquitinating enzymes and phosphatases in PINK1/Parkin-mediated mitophagy

As described above, phosphorylation and ubiquitination on depolarized mitochondria are critical to promote selective clearance of mitochondria. These post-translational modifications are reversible, and several studies suggest that phosphatases and deubiquitinating enzymes (DUBs) also participate in the regulatory steps of PINK1/Parkin-mediated mitophagy. Two DUBs USP15 and USP30 remove ubiquitin chains on the OMM, thereby antagonizing PINK1/Parkin-mediated mitochondrial ubiquitin coating [122–125]. In contrast, USP8 detaches ubiquitin chains from autoubiquitylated Parkin, promoting Parkin recruitment to depolarized mitochondria and subsequent mitophagy [126]. Moreover, PTEN-L and PPEF2 (protein phosphatase with EF-hand domain 2) mediate dephosphorylation of phospho-ubiquitin and suppress mitophagy [127,128].

Other E3 ubiquitin ligases

Studies using transgenic mice to monitor mitophagy suggest that loss of PINK1 or Parkin does not seem to significantly reduce mitophagy levels *in vivo* [129,130]. In addition, PINK1- or Parkin-knockout mice do not exhibit severe disease-relevant phenotypes. It remains possible that Parkin may act in a mutually redundant manner with other mitochondrial E3 ubiquitin ligases to ubiquitinate mitochondrial proteins. The RBR family E3 ligase ARIH1/HHARI is widely expressed in cancer cells and promotes mitophagy in a manner dependent on PINK1, contributing to removal of dysfunctional mitochondria and protection from chemotherapy-induced cell death [131]. MUL1 (mitochondrial ubiquitin ligase 1; also known as MAPL, MULAN, and GIDE), an E3 ubiquitin ligase that promotes mitochondrial fragmentation through stabilization of Drp1 (a dynamin-related protein required for mitochondrial fission) and degradation of mitofusin (a protein required for OMM fusion), also acts in Parkin-independent mitophagy [132]. In addition, the autophagy adaptor p62 recruits a Cullin-RING E3 ubiquitin ligase Rbx1 to damaged mitochondria, building ubiquitin chains on the OMM in a manner independent of PINK1/Parkin [133]. MITOL/MARCH5 and HUWE1 (HECT, UBA, and WWE domain-containing E3 ubiquitin protein ligase 1) have also been suggested to act in Parkin-independent mitophagy [67,134].

Physiological relevance of mitophagy in cell/tissue homeostasis

Physiological roles of mitophagy in yeast

Atg32-mediated mitophagy contributes to the maintenance of mtDNA in yeast. During prolonged nitrogen starvation, under which mitophagy is induced and ROS generation is accelerated, cells lacking Atg32 exhibit surplus ROS damage and deletion of mtDNA, suggesting that mitophagy reduces mitochondrial ROS accumulation and mtDNA instability [135].

Interestingly, Atg32-mediated mitophagy helps a pathogen to grow and infect to host cells. *Candida glabrata*, a haploid budding yeast, causes severe systemic infections in humans. During systemic infections, iron-chelating proteins such as transferrin limit iron supply to *C. glabrata* cells and repress their infection. Under iron-depleted conditions, *Cg*Atg32-mediated mitophagy is induced in *C. glabrata*, and loss of Atg32 decreases the number of viable

C. glabrata cells [136]. Consequently, *C. glabrata* lacking *Cg*Atg32 exhibits reduced virulence in infected mice, suggesting that mitophagy is critical for their survival and efficient infection in the host organism. How *C. glabrata* cells detect decreased iron availability and induce *Cg*Atg32-mediated mitophagy remains elusive.

Programmed mitophagy in development and differentiation

Mitophagy plays key roles in metabolic adaptation during differentiation and development. This type of mitophagy is called 'programmed mitophagy'. NIX is critical for removal of mitochondria during erythrocyte differentiation [58,59]. Deletion of NIX in mice, as well as ATG7, leads to accumulation of mitochondria in erythrocytes and elicits anemia with increased immature erythrocytes. NIX also contributes to RGC (retinal ganglion cell) differentiation [137]. Mitochondrial mass decreases during embryonic retinal development and this seems to be dependent on NIX-mediated mitophagy, as NIX-KO mice exhibits increased mitochondrial mass and a reduced number of RGCs. Mitophagy may be important to degrade mitochondria and helps a shift to glycolytic metabolism, which is required for efficient cell differentiation [137]. Furthermore, NIX acts in mitochondrial elimination required for pluripotency of iPSCs (induced pluripotent stem cells), although the detailed molecular mechanisms remain to be elucidated [138].

Paternal mitochondria provided by sperm are selectively degraded via programmed mitophagy in *Caenorhabditis elegans* fertilized embryos [139,140]. This autophagy-dependent process, named allophagy (allogeneic organelle autophagy), is mediated by ALLO-1, a cytosolic protein that contains a LIR motif for binding to LGG-1 (an Atg8 homolog in worms), similar to mammalian autophagy adaptors [141]. In addition to ALLO-1, IKKE-1, a worm homolog of mammalian TBK1/IKKε kinases, is required for elimination of paternal mitochondria [141]. Mass-spectrometric analysis revealed that ALLO-1 is phosphorylated at Thr74, and this phosphorylation is required for efficient elimination of paternal mitochondria. Knockdown of the sole E1 ubiquitin-activating enzyme UBA-1 in worm reduces ALLO-1 localization to paternal organelles, indicating an involvement of ubiquitin in this process [141]. However, in *pink-1* and *pdr-1* (a Parkin homolog in worms) deletion mutants, paternal mitochondria seem to be eliminated at near wild-type levels, raising the possibility that PDR-1 may not be essential for this process, or several E3 ubiquitin ligases may act in a redundant manner with PDR-1 [141]. Further studies are required to elucidate whether and how paternal mitochondria are ubiquitinated.

A recent report has demonstrated that PINK1/Parkin-mediated mitophagy is important for adipocyte differentiation [142,143]. Among adipocytes, beige cells contain large amounts of mitochondria and produce heat during adrenaline and cold stimulation. Upon withdrawal of these stimuli, beige cells reduce mitochondrial mass and are converted to white cells [144]. In Parkin-deficient mice, this conversion does not progress as efficiently as that in wild-type mice. These findings support the idea that Parkin contributes to cell-fate decision of adipocytes, possibly via mitophagy, *in vivo*.

Mitophagy and inflammation

In a model of polymicrobial sepsis, loss of PINK1 or Parkin elicits increased sensitivity and aberrant activation of inflammasome, suggesting the roles of PINK1/Parkin in host protection during inflammation [145]. Moreover, PINK1 and Parkin have been suggested to suppress release of mtDNA from mitochondria and thus inhibit excess inflammation [146]. When exposed to exhaustive exercise, mice lacking PINK1 or Parkin exhibit increased secretion of cytokines, an indicative of excess inflammation. These phenotypes are reversed by concurrent loss of the NLRP3 inflammasome, indicating that mtDNA release in PINK1- or Parkin-deficient mice leads to activation of inflammasomes [146]. mtDNA could induce inflammatory responses in the cytosol in a manner dependent on the cGAS-STING pathway [147–149]. Loss of STING in these mutant mice almost completely abolishes inflammatory responses [146]. Collectively, these results support the idea that PINK1 and Parkin may prevent an excess inflammatory response via the STING pathway. However, it should be noted that whether this aberrant activation of the STING pathway occurs in other species is not currently clear, as *Drosophila Sting* mutants do not seem to display suppressing effects on the locomotor deficits or mitochondrial disruption in *Pink1* or *parkin* mutants [150]. Moreover, kidney-specific knockout of TFAM (a mitochondrial matrix protein required for packaging and maintenance of mtDNA) or a nephrotoxic reagent-induced mtDNA release to the cytosol, induces STING pathway-dependent activation of inflammation and renal damage [151,152]. Although the precise relationship between this phenomenon and mitophagy in kidney remains unclear, it seems likely that proper maintenance of mtDNA is important for kidney homeostasis.

PINK1 and Parkin have also been suggested to be linked to a potential autoimmune mechanism that could lead to neurodegeneration, suppressing transport of mitochondrial proteins via the MDV (mitochondria-derived vesicle) pathway and MitAP (mitochondrial antigen presentation) on the surface of antigen-presenting cells [153]. Although how PINK1 and Parkin contribute to suppression of MDV formation under immune-stimulating conditions is not fully understood, these findings imply the potential involvement of PINK1 and Parkin in autoimmune mechanisms. Moreover, a recent study has reported that genetic ablation of PINK1 induces MitAP and leads to establishment of cytotoxic mitochondria-specific $CD8^+$ T cells in the periphery and in the brain in response to intestinal infection with gram-negative bacteria [154]. Notably, *Pink1*$^{-/-}$ mice infected with these bacteria exhibit a decrease in dopaminergic axonal varicosities in the striatum and locomotor dysfunction.

Mitophagy seems to be modulated upon viral infection. Upon viral infection, RIG-I (retinoic acid-inducible gene-I) and MDA-5 (melanoma differentiation-associated gene 5) recognize RNA derived from the invading viruses, and promote oligomerization of MAVS (mitochondrial antiviral signaling protein) on mitochondria, which is required for induction of downstream cascades to activate transcription of IFN (interferon) genes and repress virus replication [155]. HHV-8 (human herpesvirus 8) encodes a number of proteins, such as vIRF-1 (viral IFN regulatory factor 1), that are critical to inhibit IFN-activated antiviral responses in the host cells. vIRF-1-expressing cells exhibit reduced number of mitochondria during lytic reactivation, and deletion of vIRF-1 maintains mitochondrial content [156]. NIX interacts with vIRF-1, and loss of vIRF-1 reduces virus replication-promoted mitochondrial localization of NIX. Further studies are needed to investigate whether and how NIX-vIRF-1 interactions contribute to degradation of mitochondria upon viral infection.

Mitophagy and neurodegeneration

Mitochondrial quality control is important especially for nondividing cells, such as neurons, and a failure in mitophagy could lead to neurodegenerative diseases. Indeed, accumulation of damaged mitochondria is a hallmark of age-related neurodegeneration, including PD (Parkinson's disease), AD (Alzheimer's disease), and ALS (amyotrophic lateral sclerosis).

PD is a neurodegenerative disease caused by loss of dopaminergic neurons in the midbrain *substantia nigra* [157]. Mitochondrial dysfunction has been considered to be involved in the pathogenesis of PD, based on the findings that the activity of the mitochondrial electron transfer system is reduced in PD patients and that inhibitors of the system induce PD-like symptoms. Pink1 and Parkin genes are mutated in PD patients. Although loss of PINK1 or Parkin does not exhibit PD-like phenotypes, its combination with mtDNA mutator mice leads to significant mitochondrial dysfunction and impairs dopaminergic neurons, supporting the idea that Parkin preserves mitochondrial fitness and contributes to neuroprotection [158]. As PINK1 and Parkin act in the immune responses, it should be noted that phenotypes in PD could be caused by multiple losses of PINK1/Parkin functions.

In addition to Pink1 and Parkin, LRRK2 (leucine-rich repeat kinase 2) is also mutated in PD patients and related to elimination of damaged mitochondria [159]. LRRK2 acts in removal of Miro, a mitochondrial transmembrane GTPase that anchors mitochondria to motor proteins and microtubules, and modulates mitochondrial motility in the early step of mitophagy [160]. LRRK2 forms a complex with Miro upon mitophagy induction, thereby promoting dissociation of Miro from mitochondria and suppressing mitochondrial mobility. The pathogenic LRRK2 G2019S mutation causes delayed mitochondrial arrest and inefficient mitophagy [160]. Moreover, recent studies reveal that the G2019S mutation leads to hyperactivation of LRRK2, thereby reducing PINK1/Parkin-mediated mitophagy, and that inhibition of the LRRK2 kinase activity restores mitophagy in cells expressing LRRK2-G2019S [161,162]. By contrast, two earlier studies report that expression of LRRK2-G2019S increases mitophagy [163,164]. Further studies are necessary to reconcile these discrepancies and elucidate precise roles of LRRK2 in mitochondrial clearance.

Deficits in mitophagy have also been associated with AD (Alzheimer's disease), the most common cause of dementia characterized by loss of memory and other cognitive abilities. Immunohistochemistry revealed that reduced colocalization of mitochondrial proteins and lysosomes in hippocampus of AD patients, indicating decreased mitophagy-like events [165]. In addition, expression levels of several mitophagy-related proteins are decreased in human AD samples and AD iPSC-derived neurons.

Mutations in OPTN and its upstream kinase TBK1 have been identified in patients with ALS, a disease hallmarked by degeneration of motor neurons, which leads to muscle weakness and paralysis [166–170]. OPTN-ubiquitin chain interactions on damaged

mitochondria are disrupted by E478G, an ALS-linked mutation in the UBAN domain of OPTN [171]. Several ALS-linked mutations in TBK1 have also been identified by whole-exome sequencing. A disease-associated mutation in the C-terminal coiled-coil domain renders TBK1 unable to interact with OPTN, possibly interfering encapsulation of damaged mitochondria into autophagosomes [168].

Mitophagy and tumorigenesis

Accumulation of defective mitochondria and excessive ROS production has been linked to tumor formation [172]. Thus, mitophagy could inhibit tumor formation by removing defective mitochondria. Bnip3 expression is reduced during malignant tumor development, and Bnip3-deficient mice develop tumors more rapidly than wild-type mice [173]. In addition, Parkin expression is decreased in breast cancer cells, and Parkin overexpression mitigates abnormal cell proliferation, suggesting that Parkin has an antitumor function [174]. Together, these findings imply that inefficient degradation of mitochondria could be linked to tumorigenesis and progression, yet whether these phenotypes are due to deficits in Bnip3- and Parkin-mediated mitophagy or other functions of Bnip3 and Parkin independent of mitophagy remains elusive. Furthermore, mice lacking FUNDC1 in the liver exhibit increased susceptibility to HCG (hepatocarcinogenesis) and enhanced inflammatory responses, suggesting the protective role of FUNDC1 against tumorigenesis in the liver [175]. It should, however, be noted that the contribution of mitophagy receptors to tumorigenesis seems to differ in cellular subtypes and/or cancer stages. Further studies are required to dissect the precise roles of mitophagy in tumor suppression.

Mitophagy and aging

Similar to autophagy, mitophagy could contribute to suppression of aging. Excessive accumulation of mtDNA mutations is a cause of accelerated mammalian senescence, and appropriate removal of mitochondria containing high levels of mtDNA mutations by mitophagy may be one of the ways to prevent senescence [176]. A study using yeast as a model has revealed that under longevity-extending conditions, loss of Atg32 leads to accumulation of dysfunctional mitochondria and aberrant mitochondrial networks, resulting in shortened lifespan [177].

Establishment of several fluorescent probes enabled *in vivo* analyses of mitophagy levels during aging in higher eukaryotes. A study using transgenic mice expressing mt-Keima (for details, see Fig. 7) revealed that in some brain regions, mitophagy activity decreases during aging [178]. On the other hand, a study using *Drosophila* expressing mt-Keima demonstrated that mitophagy is increased in aged flight muscle [179]. These findings raise the possibility that age-related fluctuation of mitophagy levels differ in some species and/or tissues. Moreover, loss of Parkin or PINK1 function causes muscle tissue collapses and decreased motor function in flies, resulting in shortened lifespan [180–184]. Whether and how PINK1/Parkin-dependent mitophagy directly contributes to these observed phenotypes await detailed analyses of the relationship between individual senescence and mitophagy.

Fluorescent reporters to monitor mitophagy *in vivo*

Monitoring mitophagy in normal physiology and various disease states still remains a major challenge. Classical methods such as electron microscopy can visualize mitochondrial remnants engulfed in the autophagosome-like structures in only a small section of cells and tissues, thereby making it difficult to quantify mitophagy levels in a whole cell and tissue. To overcome this issue, several quantitative methods using fluorescent reporters have been developed to evaluate mitophagy and mitochondrial status *in vivo* (Fig. 7) [185].

mt-Keima is excited by light peaking at 438 nm under cytosolic conditions and increases to 550 nm at acidic pH under lysosomal conditions, which enables dual-excitation ratiometric imaging [186,187]. This acid-stable probe provides end-point readouts of mitophagy in mammalian cells, tissues, flies, and mice [122,188–195]. *Mito*-QC is a tandem mCherry-GFP fluorescent tag fused with the TM domain of the OMM protein Fis1 [196,197]. Upon delivery to lysosomes, only GFP, but not mCherry, signals are rapidly quenched under the low pH environment. Mito-SRAI (signal retaining autophagy indicator) is a mitochondrially targeted fusion protein of YPet and TOLLES [198]. TOLLES (tolerance of lysosomal environments) is a newly established fluorescent protein that is stable under lysosomal pH. FRET (fluorescence resonance energy transfer) occurs from TOLLES to YPet, a relatively less pH-sensitive YFP. Only YPet is irreversibly acid-denatured upon delivery to lysosomes, leading to dequenching of TOLLES. MitoTimer is a redox-sensitive fluorescent protein (DsRed1-E5) targeted to the mitochondrial matrix [199], and its green-to-red fluorescence transmission can be used to assess

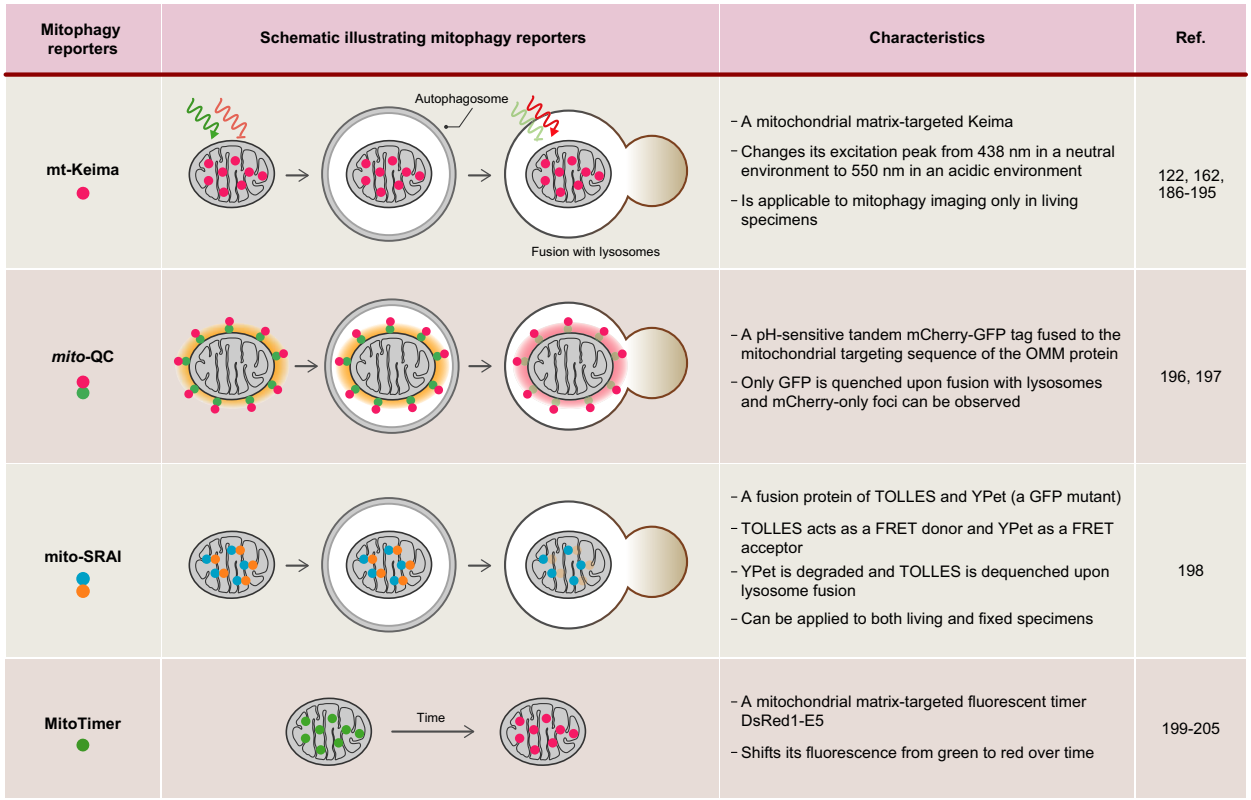

Fig. 7. Lineup of mitophagy reporters. Also see the text for further details.

mitochondrial structure, oxidative stress, and mitophagy *in vivo* [200–205].

Potential interventions to modulate mitophagy

Defects in mitophagy, which lead to accumulation of dysfunctional mitochondria and imbalance between energy supply and demand, are associated with pleiotropic pathological consequences. Thus, interventions modulating mitophagy may become potential therapeutic approaches [206]. UA (Urolithin A) is a natural compound that promotes induction of mitophagy both *in vitro* and *in vivo* [207]. In *C. elegans*, UA treatment prevents accumulation of dysfunctional mitochondria with age and extends lifespan. These phenotypes seem to require mitophagy activity, as *pink-1* and *dct-1* (a homolog of BNIP3 and NIX in worms) mutants treated with UA do not exhibit lifespan expansion [207].

Moreover, a recent study has established AUTAC, an autophagy-targeting chimera that contains S-guanylation-inspired degradation tag for selective autophagy [208]. Artificial targeting of AUTAC to mitochondria accelerates both selective clearance of fragmented mitochondria and biogenesis of functionally normal mitochondria in cells from Down syndrome patients. These events seem to be independent of Parkin, as AUTAC-mediated degradation of mitochondria can be observed in HeLa cells expressing undetectable levels of endogenous Parkin. AUTAC could induce degradation of disease-associated cytosolic substrates, opening a novel autophagy-based approach against various human disorders.

Conclusions and future perspectives

In this article, we reviewed the fundamental principles of selective degradation of mitochondria via autophagy. Combined with the molecular understanding of mitophagy, studies using model organisms deficient in the key molecules of mitophagy have revealed the diverse physiological functions of mitophagy. Despite these advances, many questions remain unanswered regarding mitophagy. Specifically, (1) Is there a signaling pathway that regulates the balance between degradation and biogenesis of mitochondria?, (2) Is mitophagy promoted cell nonautonomously across organs and tissues?, (3) What proteins regulate the basal mitophagy?, (4) Are there different organ- and tissue-specific mitophagy receptors?, and (5) Do organelles other than mitochondria act in induction, promotion, and suppression of mitophagy? Finally, (6)

among more than 8 million species of eukaryotes inhabiting the earth, how common is mitophagy and what are the variations in its molecular mechanisms and physiological functions? Future attempts with a combination of biochemical, cell biological, and genetic approaches, together with *in vivo* analyses using model and nonmodel organisms, will unveil these secrets of mitophagy.

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