Outstanding Questions in Mitophagy: What We Do and Do Not Know

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https://doi.org/10.1016/j.jmb.2019.06.032

Abstract

The elimination of mitochondria *via* autophagy, termed mitophagy, is an evolutionarily conserved mechanism for mitochondrial quality control and homeostasis. Mitophagy, therefore, has an important contribution to cell function and integrity, which extends to the whole organism for development and survival. Research in mitophagy has boomed in recent years, and it is becoming clear that mitophagy is a complex and multifactorial cellular response that depends on tissue, energetic, stress and signaling contexts. However, we know very little of its physiological regulation and the direct contribution of mitophagy to pathologies like neurodegenerative diseases. In this review, we aim to discuss the outstanding questions (and questions outstanding) in the field and reflect on our current understanding of mitophagy, the current challenges and the future directions to take.

What Is Mitophagy?

Mitochondria are intricate organelles within every nucleated eukaryotic cell and provide key functions that enable complex organism survival. Mitochondria are metabolic hubs: they are the main generators of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) as well as providers of key intermediates for fatty acid, hormone and amino acid biosynthesis [1]. Mitochondria are responsible for heme and iron–sulfur clusters, which are essential co-factors for many enzymes involved in diverse pathways from DNA repair to oxygen sensing. Mitochondria act as essential signaling platforms: not only do they control intracellular calcium levels [2], but also generate reactive oxygen species (ROS) [3], as well as regulate the innate immune response [4]. To top this off, mitochondria are also arbiters of cell death through the initiation of the intrinsic apoptotic pathway [5]. In order to carry out this plethora of functions, mitochondria are structured in dynamic networks where mitochondria biogenesis, fission, fusion, transport and elimination are harmoniously integrated [6,7]. Signaling pathways regulating mitochondria homeostasis are therefore critical for cell survival, and their dysfunction is associated with aging as well as the rise of major diseases ranging from cancer to cardiovascular and neurodegenerative diseases [8].

What is mitophagy then and how does it relate to mitochondria? The mitochondrial network is surveyed by several mechanisms of mitochondrial quality control and mitophagy is one such pathway. In its simplest sense, mitophagy is the autophagy of mitochondria—which we define as the delivery of mitochondria, or parts of mitochondria, to lysosomes for degradation. One such route to lysosomes is *via* the macroautophagy pathway, which involves the engulfment of a mitochondrion by a double-membraned autophagosome [9]. Autophagosomes containing the sequestered mitochondrial cargo can then fuse with lysosomes, either directly or *via* endosomal fusion to form intermediate amphisomes [10]. The end point is the formation of an autolysosome, where the mitochondrion is degraded and recycled. It is this pathway that is the focus of this review article. Alternatively, mitochondrial turnover also occurs by budding mitochondrial pieces from the outer mitochondrial membrane to form mitochondrial-derived vesicles (MDVs), which then

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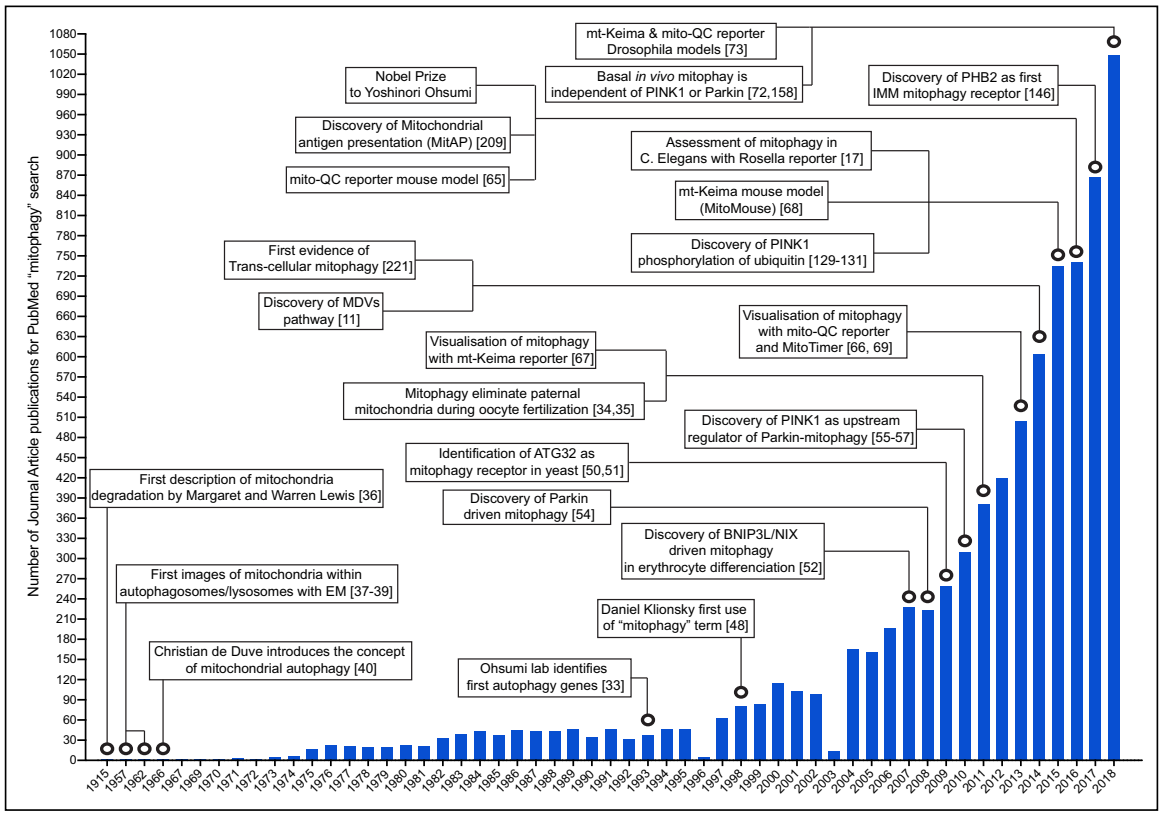

Fig. 1. A timeline for mitophagy research. The chart shows the rapidly increasing number of publications identified as a "journal article" in a PubMed search for the term "mitophagy." A selection of important contributions to the mitophagy field is highlighted across the timeline [33–35].

fuse with the endolysosomal system [11]. Ultimately though, as mentioned earlier, mitophagy is one of the several mechanisms for mitochondrial quality control. Mitochondrial network homeostasis may also involve proteasome-dependent degradation of mitochondrial proteins, mitochondrial proteases and chaperones or the mitochondrial unfolded protein response [12,13]. However, the molecular and physiological interplay between mitophagy and all these other mitochondrial quality control pathways remains poorly understood. Indeed, in *Drosophila melanogaster*, canonical autophagy only accounts for around one-third of basal mitochondrial protein turnover [14]. Furthermore, mitochondrial proteins examined in this study also exhibited different basal autophagic turnover rates, which supports previous observations of selective mitochondrial protein degradation [15,16]. How mitochondrial proteins are selectively sorted and regulated within the network for mitophagic elimination is indeed an intriguing puzzle. This also emphasizes our lack of understanding on how mitophagy harmonizes within a bigger picture of mitochondrial biogenesis, function and dynamics for ultimate cellular response and function. For example, the coordinated regulation between mitochondria biogenesis and clearance has been previously studied in *Caenorhabditis elegans* to preserve energy homoeostasis, renew mitochondria and regulate longevity [17,18]. However, this coordination remains poorly understood in mammalian systems.

Over the past 2 years, the state-of-the-art mitophagy research has been exhaustively reviewed in many excellent articles [12,18–32]. As discussed below, our knowledge of how mitophagy functions is far from complete but it is rapidly expanding, as is evidenced by the almost exponential increase in publications that mention this keyword (Fig. 1). Here, we aim to review the key questions that still remain regarding the molecular and physiological basis of mitophagy, emphasizing the most recent discoveries and challenges.

What Have We Learnt After 104 Years?

The idea of mitochondrial degradation (or "degeneration") was first proposed in the early work on mitochondrial dynamics by Margaret and Warren Lewis in 1915 [36] (see Fig. 1). Later in the century, observations using electron microscopy revealed the first instances of mitochondria within vesicles

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and lysosomes in rat tissues [37–39]. When Christian de Duve postulated the concept of cellular autophagy, the idea of mitochondrial autophagy flourished [40,41] and was further supported with reports describing mitochondrial autophagy in the muscle of metamorphosing *Antheraea polyphemus* [42], rabbit hearts under ischemia–reperfusion injury [43] and during rat erythrocyte maturation [44]. The first indications for selectivity in the autophagic elimination of mitochondria happened in the early 2000s, with reports indicating degradation of depolarized mitochondria [45–47]. To our knowledge, the term mitophagy was first used by Sidney Scott and Daniel Klionsky in 1998 [48] and became popularized by John Lemasters in 2005 [49].

The first mechanistic insights on how mitochondria were selectively targeted for mitophagy arose from landmark studies by the Klionsky, Ohsumi, Ney and Youle laboratories. The protein ATG32 was identified as a key mitophagy receptor in yeast [50,51]; meanwhile, BNIP3L/NIX was identified to play a critical role in mitophagy during mammalian erythrocyte differentiation [52,53]. In a distinct pathway, the ubiquitin E3-ligase Parkin was shown to regulate mammalian mitophagy following mitochondrial depolarization [54], and the Parkin link was further strengthened following the key discovery of PTEN-induced kinase 1 (PINK1) as an upstream regulator of Parkin-mediated mitophagy [55–59]. The connection between these two proteins, both of which can be mutated in familial Parkinson's disease (PD), sparked the hypothesis of defective mitophagy as a potential cause of neurodegeneration. This fostered intense research on how the PINK1/Parkin pathway works at all levels [32,60,61]. However, over the subsequent years, other mitophagy receptors and signal mechanisms have been identified. The contribution each of them makes to the global turnover of mitochondria within an organism, or the context in which they operate, is far from clear [29].

The gold standard method to investigate mitophagy and autophagy over the years has been through the use of electron microscopy [62]. However, the development of simple, fast, selective and reliable reporter systems for assessing mitophagy has revolutionized the field. Two mitochondrial reporters were initially established to study yeast mitophagy in 2008: (1) fusion of GFP with mitochondrial proteins, where mitophagy is analyzed by Western blot measuring the amount of cleaved GFP that happens in the vacuole [63], and (2) the Rosella reporter, which exploits the acid labile properties of fluorescence proteins GFP and DsRed (the former being sensitive to the acidic pH of the vacuole, while the latter is not). This assesses mitophagy as a change in color to DsRed-only fluorescing mitochondria, which occurs upon delivery to the vacuole [64]. A similar fluorescent pH-biosensor system was used to generate the *mito*-QC reporter for assessing mitophagy in mammalian cells *in vitro* and *in vivo* [65,66]. Another fluorescent probe system, called mt-Keima, also uses a pH-sensitive protein with a pH-dependent shift in fluorescence excitation to assess mitophagy in cells and tissues [67,68]. An alternative reporter system, mito-Timer, relies on the fluorescence shift of DsRed1-E5 fluorophore from green to red over time. Although it does not directly measure mitophagy, it provides a powerful tool to monitor age and biogenesis of mitochondria [69]. The recent advent of mouse models expressing mt-Keima, *mito*-QC and mito-Timer has now provided the opportunity for in-depth physiological analysis of mammalian mitophagy regulation and its direct implication in disease conditions [65,68,70,71].

Why Degrade Mitochondria through Autophagy?

Mitophagy is considered a cell survival mechanism responsible for clearing damaged, superfluous or aged mitochondria. It has the advantage over other degradation pathways in that a whole mitochondrion (including its membrane, proteins and nucleic acids) can be turned over in one go. While mitophagy has historically been considered a quality control mechanism to survey mitochondrial damage, we now know that cells degrade their mitochondria for multiple reasons and under different situations. For example, mitophagy has been observed under various circumstances ranging from the basal state to conditions of cell stress, and even during programmed cellular differentiation or cell death. Thus, mitophagy has evolved into a complex and multi-factorial cellular response that highly depends on the tissue, energetic, stress and signaling contexts.

Why do cells undergo basal mitophagy?

Mitophagy can occur under apparently normal conditions, in the absence of any overt stress. The exact function (or functions) of this so-called basal mitophagy is unclear, but the extensive nature of basal mitophagy across mouse and fly tissues was demonstrated using the fluorescent mitophagy reporters mt-Keima and *mito*-QC [65,68,72,73]. While the mainstream idea is that cells undergo basal mitophagy as a general everyday mitochondrial network housekeeping mechanism, this mitophagy is homogenous neither across tissues nor between cells within the same tissue [65,72]. One example is the levels of basal mitophagy within the adult mouse kidney cortex, where proximal convoluted tubules are highly mitophagic, displaying a level much greater than that of adjacent distal convoluted tubules, despite having a similar

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mitochondrial content [65]. This highlights an important context-dependent regulation of basal mitophagy, which remains unknown. More evidence of context-dependent basal mitophagy occurs in the eye. Mitophagy is surprisingly restricted in the retina, being localized to the outer nuclear layer (ONL), which is enriched in photoreceptor cell bodies [72,74]. Intriguingly, mitophagy in the ONL proceeds at similar rates during murine light or dark cycles [72]. In fact, we know very little about how the circadian clock regulates mitophagy, which has direct implications on autophagy and mitochondrial metabolic functions in general [75]. Could this basal mitophagy be selective or is it just a reflection of the level of macroautophagy in general? Using the *mito*-QC mouse and an almost identical autophagy reporter mouse expressing mCherry-GFP-MAP1LC3B, McWilliams *et al*. [74] compared basal mitophagy and total macroautophagy side by side across eye tissues. Surprisingly, high levels of total macroautophagy do not necessarily mean high levels of mitophagy, implying that there is indeed autophagic selectivity *in vivo*. For example, mitophagy accounted for a significant amount of the total macroautophagy in the ONL, which was in stark contrast to lens epithelium or corneal stroma, where minimal mitophagy was observed despite high levels of total autophagy. Furthermore, these differences also highlight the specificity of the two, almost identical, reporter systems themselves for mitophagy and autophagy.

The cellular triggers and the signaling driving basal mitophagy *in vivo* remain unclear, but metabolic demand and a baseline level of stress (as mentioned below) are likely to play a role. This is supported by the fact that greater levels of basal mitophagy exist in subsets of highly metabolic cells such as dopaminergic and retina photoreceptor neurons, cardiomyocytes or pancreatic acinar cells [72]. Ultimately, cellular signaling, tissue context (i.e., metabolism, nutrient/oxygen availability) and specific physiological functions will likely shape the requirement of certain cells to undergo basal mitophagy.

Why do cells trigger mitophagy upon distinct stresses?

Nutrient starvation, in particular amino acid starvation, is a well-established stress that activates autophagy. Therefore, cells facing prolonged starvation are thought to use autophagy to non-selectively degrade cellular components, including mitochondria to recycle and free-up amino acids [76]. However, under short periods of amino acid starvation, mitochondria are not degraded as they are required for energy production. In this case, mitochondrial fission is restrained and a more fused mitochondrial network is generated, which hinders mitophagy by preventing the break-up of the network into “bite-size” fragments that are readily autophagocytosed [77–79]. Are cells then selectively regulating mitophagy during starvation? In yeast, nitrogen starvation induces bulk autophagy and mitophagy under a fermentable carbon source. However, yeast cultured under a non-fermentable carbon source blocked mitophagy, but not autophagy in general, upon nitrogen starvation [63]. This would suggest that indeed different regulation exists between mitophagy and other forms of autophagy triggered under stress. In a similar way, mammalian cells forced to rely on mitochondrial OXPHOS metabolism were also unable to engage mitophagy following mitochondria depolarization or iron chelation [66,80–82]. It is of note that these treatments will affect the total mitochondrial pool, and mitophagy may be more permissive under OXPHOS conditions if only a subset of mitochondria are disrupted. Regardless, this implies that the cellular metabolic context could selectively modulate mitophagy under stress. Amino acid starvation was reported to increase ROS co-localizing with mitochondria, suggesting a potential correlation with increased mitochondria activity or dysfunction [83,84]. In a similar scenario, some reports claim that higher mitochondrial respiratory activity promotes mitophagy [85,86], which could be protecting cells from a greater intrinsic mitochondrial stress. It is also possible that mitophagy induced during prolonged starvation is used as an adaptive response to renew and optimize the mitochondrial population in response to the reduced nutrient environment, or to release resources that are contained within mitochondria.

Over the years, mitophagy has been studied as a mitotoxic damage response. Defective mitochondria can generate excessive amounts of ROS, consume ATP through the reversal of F1F0 ATPase activity, impair mitochondrial metabolic functions and trigger apoptosis [87]. Therefore, mitophagy aims to rapidly clear these dysfunctional mitochondria to protect cell integrity, and this is thought to be especially relevant in post-mitotic long-lived cells such as neurons and cardiomyocytes. These likely need mitophagy during their long lifetime to ensure mitochondria homeostasis in the absence of the ability to “dilute” damaged mitochondria through cell divisions [19,25]. The selective photodamage of mitochondria provided the first insight on the mitophagic response to mitochondrial depolarization, where the autophagosome marker LC3 was recruited to damage sites [45,88]. However, extreme mitochondrial damage is often studied using chemicals such as protonophores (i.e., CCCP, FCCP) or selective electron transport chain inhibitors (e.g., rotenone, antimycin A or oligomycin A), which impair mitochondrial respiration and/or depolarize mitochondria [89,90]. These tools have been extensively exploited to gain mechanistic insights on how mitochondrial damage activates mitophagy and to explore its relevance to pathologies like PD. Indeed, toxins that cause

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Parkinsonian phenotypes in animal models like Paraquat or 6-hydroxydopamine (6-OHDA) can also depolarize and damage mitochondria [90], although it is not clear whether they induce mitophagy.

Mitochondrial function is tightly linked to oxygen availability, in particular for OXPHOS metabolism. Under hypoxic conditions, cells activate hypoxia-induced factor 1 (HIF1) signaling, which regulates gene expression to drive stress and metabolic adaptation programs [91]. The lack of oxygen leads to inefficient mitochondrial respiration, mitochondrial stress and an energetic crisis [92]. For this reason, HIF1-signaling controls a metabolic switch to upregulate glycolytic genes, attenuate mitochondria respiration and trigger mitophagy [91,93,94]. In this context, mitophagy could be activated to refresh and adapt the mitochondrial network for the new hypoxic metabolic context. Alternatively, mitophagy could also be a protective mechanism against the mitochondrial stress caused during hypoxia, or the following the re-oxygenation process. For example, mitophagy has been suggested to contribute to brain and cardiac tissue protection following ischemia/reperfusion injuries [95,96]. Similarly, oxygen and glucose deprivation–reperfusion trigger mitophagy in cultured cortical neurons. Interestingly, this neuronal mitophagy is restricted to cell bodies and axonal mitochondria are transported here before undergoing mitophagy [97]. Similar observations *in vivo* showed how basal mitophagy primarily occurs in neuronal cell bodies, although whether autophagy was initiated in the axon for more distal mitochondria was not determined [65,72]. An alternative stress that mimics the hypoxia response, by stabilizing HIF1α, is iron chelation. Depletion of iron is one of the most potent inducers of endogenous mitophagy tested *in vitro* [67,97]. While iron chelation has extensive repercussions for cellular functions such as DNA replication or mitochondria respiration, loss of iron neither depolarized mitochondria nor produced extensive ROS, as compared to conventional protonophores or electron transport chain inhibitors [66]. Although the extent or type of mitochondrial damage caused by iron chelation is unclear, mitophagy could be activated as part of the metabolic reprogramming response induced by HIF1α signaling. Furthermore, it is possible that mitophagy could serve as a recycling response to iron deficiency by freeing iron stored within mitochondria. Intriguingly, *C. elegans* treated with an iron chelator or partial depletion of Frataxin (a protein involved in iron–sulfur-cluster biogenesis) induced mitophagy, leading to an adaptative response that increased lifespan [98].

Many forms of mitochondrial stresses have been associated with mitochondrial ROS production, which are natural products arising from mitochondrial oxidative metabolism. However, the unstable and variable nature of ROS makes their accurate measurement challenging and not straightforward [99,100]. Low levels of ROS perform important signaling roles in processes, such as skeletal muscle adaptive response to exercise, adipocyte differentiation, autophagosome formation and regulation of hypoxic and insulin signaling [3,84,101,102]. Excessive production and prolonged exposure to ROS can have severe consequences by increasing mitochondrial and nuclear DNA/RNA mutations, protein and lipid oxidation, and activation of cell death [8]. This raises the question as to whether ROS could trigger mitophagy directly. Some reports suggest that initiation of mitophagy was induced by mitochondrial ROS generated using the photoactivated dye KillerRed [103]. In yeast, mitophagy induced during post-log phase respiratory growth was partially suppressed in presence of the antioxidant *N*-acetyl cysteine [50]. In contrast, other reports in mammalian cells postulated that ROS may not be the direct trigger of mitophagy, as mitophagy induced with CCCP or iron chelation was unaffected by the antioxidant *N*-acetyl cysteine [54,66]. While the diverging observations could result from an organism-specific ROS response mechanism, the direct contribution of ROS in mitophagy remains a debatable question that requires further investigation.

The recent development of mouse models with the mitophagy reporters, mito-Timer, mt-Keima and *mito*-QC, has opened a new avenue to investigate how physiological stresses impact mammalian mitophagy *in vivo* [29]. For example, mice subjected to exhaustive exercise had an increase in skeletal and cardiac muscle mitophagy [104,105]. The impact of several stresses to liver mitophagy was assessed by Sun *et al*. [68] using the mt-Keima reporter, where increased mitophagy was observed in mice subjected to prolonged hypoxia or mitochondrial stress derived from the proofreading-deficient mitochondrial polymerase G ($Polg^{D257A}$). In contrast, in the same study, they reported that a high-fat diet caused a decrease in hepatic mitophagy. Temperature stress has also been shown to impact mitophagy. Mice subjected to chronic cold stress or beta-3 adrenergic receptor agonist stimulation induced the formation of mitochondrial-rich beige adipocytes. Once these stimuli were withdrawn, the thermogenic beige adipocytes underwent a transition to white adipocytes that triggered mitophagy to eliminate superfluous mitochondria and aid this transition [106–108]. Interestingly, cold temperature stress has also been recently shown to trigger mitophagy in human fibroblasts after returning to a normal physiological temperature [109]. Until today only a few studies have been conducted to assess mitophagy under physiological stresses, although we anticipate intense research in this matter during the coming years.

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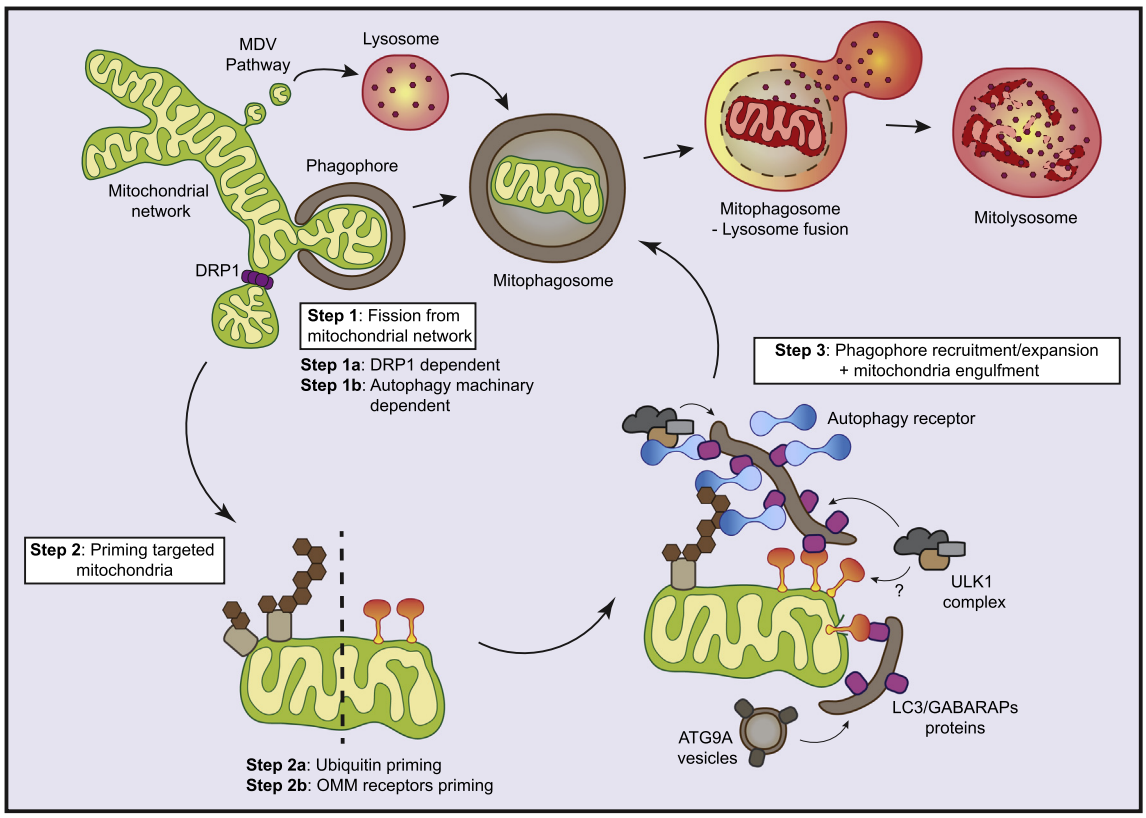

Fig. 2. The molecular steps for mitochondrial degradation. Cartoon depicting the three steps required for mitochondrial degradation *via* mitophagy. (A) Mitochondrion isolation or fission from the network, which can rely on DRP1 activity or the autophagy machinery. (B) Marking or priming targeted mitochondria, which is mediated by ubiquitin-dependent or independent mitophagy receptors. (C) Generation of the autophagosome and mitochondrion engulfment for degradation, which involves the recruitment of several factors such as autophagy receptors, the ULK1 complex and ATG9A-containing vesicles. Once the mitochondria are engulfed, the mitophagosome fuses with a lysosome to form the mitolysosome for the final elimination and recycling.

Why do cells require programmed mitophagy?

Induction of mitophagy is not just a mechanism for cells to combat mitochondrial stress or damage. Mitophagy also helps in eliminating and renewing mitochondrial populations for multiple developmental, metabolic and physiological proposes—this is termed programmed mitophagy [29]. There are instances where programmed mitophagy plays an important role during organism development. For example, during the later stages of erythrocyte maturation, the immature erythroblasts eliminate their mitochondria using mitophagy. After this process is complete, erythroblasts are enucleated and become mature erythrocytes [110]. The critical role of this mitophagy is highlighted by the fact that mice lacking the pro-apoptotic protein NIX/BNIP3L, which is essential for this process (see below), suffered from severe anemia, reticulocytosis and erythroid-myeloid hyperplasia [52,53]. Programmed mitophagy can also operate at the very beginning of organism development with the selective elimination of paternal mitochondria following oocyte fertilization by sperm (a process termed as “allophagy” [111]).

Cell differentiation often involves drastic metabolic reprogramming between glycolytic and OXPHOS metabolism from one cellular state and another [112]. In a similar way to hypoxia, these metabolic switches are thought to activate mitophagy for mitochondrial network remodeling and homeostasis in response to metabolic demands. One example was reported in cardiomyocyte maturation. In this study, cardiomyocyte mitophagy occurring in the perinatal heart was proposed to be instrumental in switching out fetal mitochondria, which are optimized for carbohydrate-driven OXPHOS, with more mature adult mitochondria that undergo fatty acid-driven OXPHOS [113,114]. This phenomenon may not be restricted to the heart and may occur during muscle differentiation in general [115]. Another example of metabolic switching occurs in stem cells where pluripotent and human embryonic stem cells heavily rely on glycolytic metabolism, while somatic and differentiated cells rely on OXPHOS metabolism. In

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this context, mitophagy and mitochondrial dynamics have emerged as an important factor for stem cell fate, differentiation and induced pluripotent stem cell generation [112,116–120]. However, the current understanding of how mitophagy is regulated in stem cells and induced pluripotent stem cell generation remains limited.

In addition to associating with metabolic switches, developmentally programmed mitophagy may also drive them, in particular when promoting a shift toward glycolysis. This was shown to happen when retinal ganglion cells differentiated from neuroblasts following hypoxia or in macrophage differentiation [27,121]. In a different context, mitophagy can also contribute to cancer cell survival under mitotic arrest. When mitosis is blocked, cancer cells use mitophagy to clear their mitochondria and create an ATP-deficiency crisis, which leads to AMPK activation and upregulation of glycolysis [122].

Can cells use mitophagy to die?

Autophagy and mitophagy are generally known by their protective and cell survival functions. However, autophagy has also been implicated in inducing cell death, with important functions during embryo development and cancer [123]. While autophagic cell death has been extensively studied, the contribution of mitophagy to this, termed as lethal mitophagy, remains enigmatic and its physiological contribution poorly understood. One of the few instances of lethal mitophagy suggested that ceramide, a bioactive sphingolipid, is responsible for mediating caspase-independent cell death *via* excessive mitophagy [124]. Interestingly, there are hints for a potential tumor suppressor role of lethal mitophagy, as the growth of xenograft-generated tumours in mice was suppressed after overexpressing CerS1, which is involved in the synthesis C-18 ceramide [124].

How Are Mitochondria "Eaten" during Mitophagy?

Multiple signaling events converge for the execution of mitophagy. On one hand, this requires the mitochondria to be specifically marked or primed in some way depending on the cellular need, stimuli or damage. This could be considered an "eat-me" signal. On the other hand, mitochondria also need to be isolated from the network, to either decrease their size to be engulfed by the autophagosome or protect the rest of the network integrity to avoid further damage. Therefore, the initial stages of mitophagy comprise three main steps: (a) mitochondrion isolation or fission from the network, (b) marking or priming this isolated mitochondrion and (c) recruitment of phagophore membranes to engulf the primed mitochondrion (Fig. 2). Whether these are sequential events or happen in concert is not yet clear. For example, mitochondrial fission from the network is required for mitophagy and this can be driven by DRP1-dependent machinery prior to engulfment [29], but in contrast, the forming phagophore can also mediate mitochondrial isolation directly, independently of DRP1 [125].

What are the mitochondrial "eat-me" signals?

The so-called autophagy cargo receptor proteins are key in determining how a cellular component is engulfed by a forming autophagosome and the reader is referred to recent reviews for a more in-depth perspective of this class of proteins [21,30]. It is therefore no surprise that these proteins are essential for mitophagy. In yeast, the mitochondrial protein ATG32 is currently the only mitophagy receptor identified to selectively prime mitochondria for degradation. When mitophagy is stimulated, either through nitrogen starvation or long-term respiratory growth, this single-pass OMM protein is upregulated and recruits the nascent autophagosome *via* direct interaction with the selective-autophagy adaptor ATG11 and ATG8 [50,63,126]. In metazoans, the number and type of mitophagy receptors have evolved to allow fine-tuning of mitophagy to the different stimuli and cellular contexts mentioned above. In general, forming autophagosomes (or phagophores) are recruited to mitochondria through two main types of "eat-me" signals that depend on the requirement of the receptor to bind ubiquitin or not (Fig. 3). Whether each type of receptor functions in a distinct pathway or there is co-operation between them remains to be determined.

Ubiquitin-dependent mitophagy receptors

Classically, ubiquitin-dependent mitophagy has primarily referred to the PINK1/Parkin mitophagy pathway. This pathway requires the mitochondrial-associated Ser/Thr kinase PINK1 and the RBR E3-ubiquitin ligase Parkin, both of which are mutated in some forms of hereditary PD. This is the most studied and understood mitophagic pathway, at least in terms of mechanism. This area has also been extensively reviewed (see Refs. [12,30,32]) and is summarized briefly here (see Fig. 3A). When mitochondria are depolarized, PINK1 is stabilized and accumulates in the outer mitochondria membrane (OMM) [55,56]. This stabilization results in activation of PINK1 that in turn drives mitophagy through two parallel processes: (a) phosphorylation of ubiquitin at serine 65, present at a basal level at the mitochondrial surface, and (b) mitochondrial recruitment and phosphorylation of Parkin (also at serine 65) [127–131]. Phosphorylated Parkin and its

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interaction with phospho-ubiquitin allow Parkin to achieve a fully active conformation [132,133]. This drives ubiquitylation of multiple substrates in the OMM [134]. The continuous PINK1-dependent phosphorylation of ubiquitin chains creates a feed-forward loop that recruits autophagy receptors to the mitochondria [135–138]. This ubiquitylation of mitochondrial surface proteins does not exclusively rely on Parkin, as other E3-ubiquitin ligases, such as MUL1, ARIH1, SIAH1, SMURF1 and Gp78, have been described to cooperate with, or act alternatively, to Parkin activity downstream of PINK1 [25]. The prevailing model for how autophagy receptors function proposes that they bridge ubiquitylated cargos with forming autophagosomes *via* their ubiquitin-binding domains and LC3-interacting region (LIR) motifs, respectively [30]. Three receptors, NDP52/CALCOCO2, Optineurin (OPTN) and

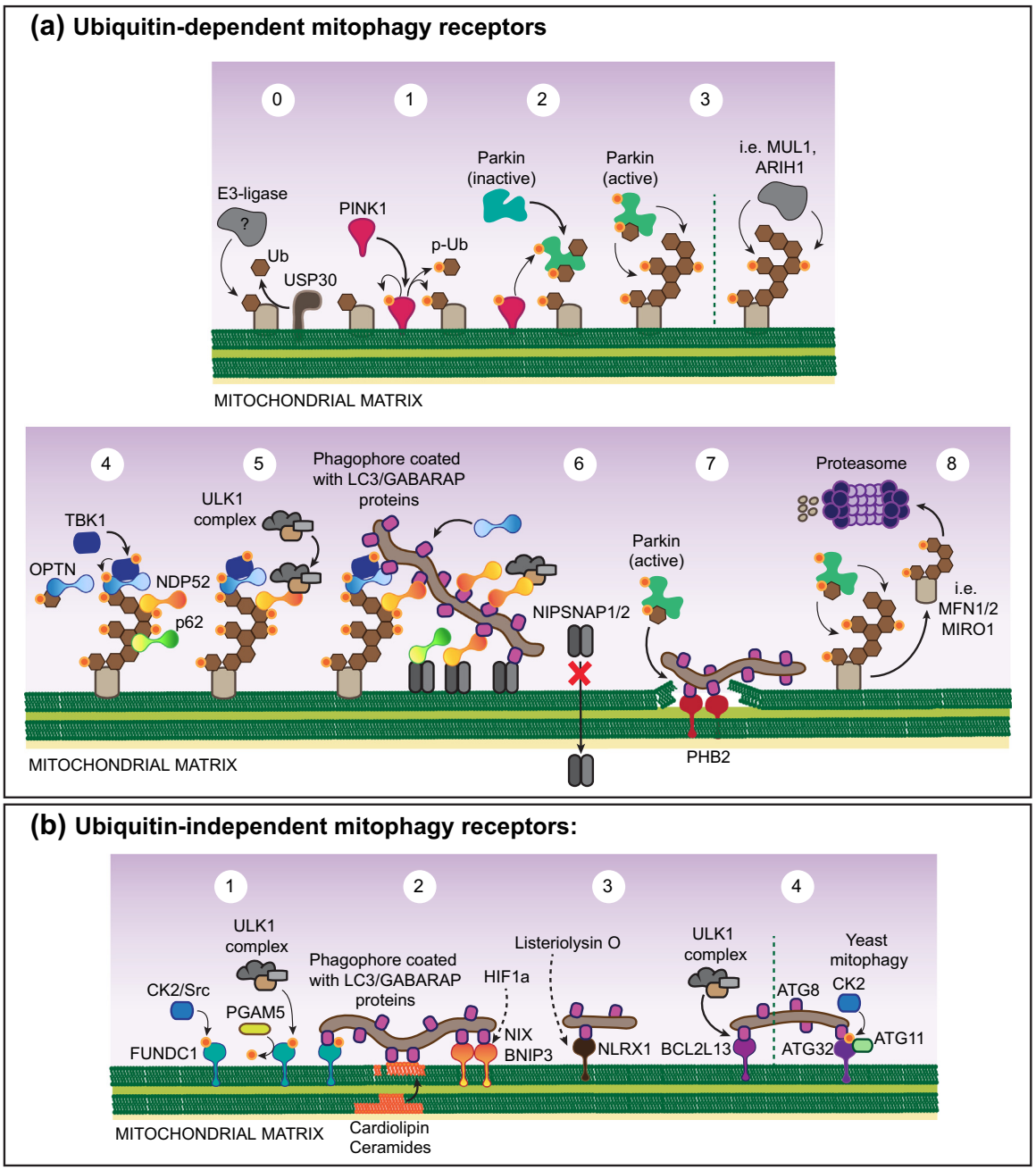

Fig. 3 (*legend on next page*)

partially TAX1BP1, were shown to play an essential role during PINK1/Parkin mitophagy [136]. Interestingly, the phospho-ubiquitin signature generated by PINK1 was sufficient to recruit NDP52 and OPTN receptors to mitochondria independently of Parkin [136]. In parallel, receptor binding to mitochondrial ubiquitin chains is enhanced by TANK-Binding kinase 1 (TBK1) phosphorylation, which interacts with OPTN and NDP52 to establish a second feed-forward mechanism [137]. The role of the archetypal autophagy receptor, p62/SQSTM, in PINK1/Parkin mitophagy is less clear as contradictory evidence suggest that it is dispensable [136] or required [57,139]. In contrast, p62 has been also reported to act upstream of the ubiquitylation cascade to mediate basal hepatocyte mitophagy independently of Parkin [140]. In DRP1 knock-out hepatocytes, p62 accumulated in mitochondria and promoted mitochondria ubiquitylation by interacting with Keap1 and recruiting the E3-ligase RBX1 [140].

In addition to receptor recruitment, ubiquitylated OMM proteins are also targets for proteasomal-mediated degradation, a process termed as outer mitochondrial membrane-associated degradation (OMMAD) [141]. The selective elimination of mitochondrial proteins plays an important role during mitophagy induction to regulate mitochondria dynamics such as transport and fusion. For example, following mitochondria damage and PINK1/Parkin activation, Mitofusin 1 and 2 (MFN) are rapidly ubiquitylated by Parkin to mark them for proteasomal degradation. This has important implications for Parkin-dependent mitophagy: (a) it inhibits damaged mitochondria fusion with the network, facilitates fragmentation and activation of mitochondria fission [142], and (b) it destroys mitochondria–ER contact sites mediated by MFN2 tethering functions and thus facilitates mitophagy [143]. In a similar way, the mitochondrial transport regulator MIRO1/RHOT1 is also degraded after PINK1 activation to impair targeted mitochondrial motility [144].

OMMAD may also play a more direct role in mitophagy activation. This large-scale extraction of ubiquitylated proteins is thought to lead to OMM rupture and damage [145]. This in turn leads to the exposure of inner mitochondrial proteins that can signal mitophagy. For example, proteasome-dependent OMM rupture during Parkin-mediated mitophagy facilitates the exposure of the inner mitochondria membrane mitophagy receptor, Prohibitin 2 (PHB2). Following OMM breakage, PHB2 can interact with the forming phagophore *via* its LIR motif [146]. While PHB2 function suggests a dual model of phagophore membrane recognition with the cooperation of both OMM and IMM receptors, it could also serve as an alternative stress-response signal responsible for monitoring internal mitochondria integrity. Related to this, recent work has shown that the normally mitochondrial matrix localized NIPSNAP1 and NIPSNAP2 proteins are essential for Parkin-mediated mitophagy and play a key role in receptor recruitment [139]. Taken together, this raises the intriguing possibility that the function of mitochondrial protein ubiquitylation may be to cause OMM damage in order to reveal the receptor-binding “eat-me” signals.

Ubiquitylation is reversible through the action of deubiquitylase enzymes (DUBs), and these, such as USP30, USP35 and USP15, have been linked to mitophagy. USP30 is the only DUB constitutively

Fig. 3. Molecular mechanisms of mitophagy “eat-me” signals. Schematic cartoon summarizing the main signaling events mediated by ubiquitin-dependent or independent mitophagosome recruitment. (A) Ubiquitin-dependent mitophagy receptors (PINK1/Parkin mitophagy). (0) Basal levels of mitochondrial ubiquitylation is regulated by USP30 and an unknown E3-ligase. (1) Following mitochondrial depolarization, PINK1 is stabilized in the mitochondrial OMM and activated. This leads to phosphorylation of ubiquitin found on the OMM. (2) Parkin is recruited to the mitochondrion and activated by phospho-ubiquitin and PINK1 phosphorylation. (3) Activated Parkin starts ubiquitylating OMM proteins. Other E3-ligases have been described to cooperate or work independently of Parkin at this step. (4) Generation of ubiquitin chains lead to the recruitment of autophagy receptors like NDP52, OPTN or p62. Phospho-ubiquitin can also directly recruit autophagy receptors. In parallel, TBK1 is recruited and activated by an unknown kinase. TBK1 phosphorylates OPTN, which strengthens the interaction with ubiquitin chains. (5) ULK1 complex and other autophagosome biogenesis machinery are recruited by autophagy receptors. Autophagy receptors interact with LC3/GABARAP proteins coating the forming phagophore. (6) Upon mitochondria depolarization, NIPSNAP1/2 are no longer translocated to the mitochondrial matrix and mediate direct recruitment of autophagy receptors. (7) Downstream of Parkin activity, the OMM is broken to expose PHB2 from the inner mitochondria membrane (IMM). PHB2 acts as mitophagy receptor and interacts with LC3/GABARAP proteins. (8) OMMAD mediated by Parkin and other E3-ligases trigger the proteasomal degradation of mitochondrial proteins including MIRO1 or Mitofusin1/2 (MFN), affecting mitochondria fusion and transport. (B) Ubiquitin-independent mitophagy receptors, which recruit the phagophore by direct interaction with LC3/GABARAP proteins from the mitochondrial OMM. (1) FUNDC1 function is inhibited by CK2 and Src kinases phosphorylation of its LIR motif. Once mitophagy is triggered, PGAM5 phosphatase dephosphorylates FUNDC1 to facilitate its interaction with LC3. ULK1 also phosphorylates FUNDC1 to strengthen the interaction with the phagophore. (2) Cardiolipin and ceramides translocate OMM to interact with LC3. NIX and BNIP3 are upregulated by HIF1α and accumulate in the OMM to mediate mitophagy. (3) In macrophages, NLRX1 mitochondrial receptor is activated by listeriolysin O virulence factor after *Listeria* infection. (4) BCL2L13 interacts with ULK1 complex and LC3 proteins to mediate mitophagy. In yeast, the mitophagy receptor ATG32 is phosphorylated by CK2 to promote ATG11 interaction, which leads to association with ATG8 and recruitment of mitochondria to the phagophore assembly site (PAS).

Please cite this article as: L. Montava-Garriga and I. G. Ganley, Outstanding Questions in Mitophagy: What We Do and Do Not Know, Journal of Molecular Biology, https://doi.org/10.1016/j.jmb.2019.06.032

associated with the mitochondrial surface, where it deubiquitylates Parkin substrates to inhibit Parkin-dependent mitophagy [147,148]. Once Parkin is activated, USP30 activity is overwhelmed and impaired by phospho-ubiquitin chains [149]. Interestingly, USP30 has recently been shown to regulate basal mitophagy [150]. Depletion of USP30 up-regulates basal mitophagy, which is dependent on PINK1 function but not Parkin. This suggests that a basal level of mitochondrial ubiquitylation is indeed a key signal for mitophagy regulation. USP30 has also been reported to localize at peroxisomes and to regulate basal and stress-induced pexophagy. Peroxisome function and biogenesis are closely interlinked with mitochondria [151] and ubiquitylation of peroxisomal proteins also occurs during pexophagy [152]. Therefore, it could be speculated that the selective degradation of both organelles is co-regulated, and USP30 is potentially a common regulatory node. Another example of organelle communication during mitophagy occurs between ER and mitochondria. BCL2 and FKBP8 were reported to translocate from mitochondria to the ER upon mitochondrial damage and mitophagy induction, thus escaping lysosomal turnover where they can initiate an anti-apoptotic response [153]. FKBP8 itself is interesting as it can serve as a ubiquitin-independent mitophagy receptor despite translocating to the ER [154]. It is well known that organelle dynamics and functions are interlinked; however, it remains largely unexplored how mitophagy signaling interplays with other forms of selective autophagy or organelle functions to coordinate cell responses.

The PINK1/Parkin pathway has, quite rightly, dominated the field of mitophagy over recent years, and there is no doubt that this pathway can trigger mitophagy under the right conditions. It is also evident that PINK1 and Parkin clearly perform important cellular functions given that their mutation results in PD. However, the way that this pathway has been primarily studied over the years (in transformed cell cultures overexpressing vast amounts of Parkin and coupled with harsh chemicals that depolarize mitochondria to cause dramatic mitochondrial damage) has raised debate as to when such a scenario may be relevant physiologically [19,155,156]. Mainly strong depolarizing agents have been reported to promote PINK1 activation, in contrast to Parkinsonian neurotoxins [127]. Although the accumulation of misfolded protein in the mitochondrial matrix has also been shown to trigger PINK1/Parkin mitochondrial recruitment without apparent mitochondrial depolarization [157]. This may represent a more physiological stimulus. While these *in vitro* experiments have been instrumental for determining the mechanism of PINK1/Parkin-driven mitophagy, solid evidence for when this pathway operates *in vivo* is still in short supply. However, it has become clear in recent years that it is not the major mitophagy pathway operating *in vivo* under normal laboratory-based conditions. For example, loss of PINK1/Parkin pathway in flies and mice expressing either mt-Keima or *mito*-QC reporters failed to show any notable difference in steady-state basal mitophagy [72,73,158]. The pathway or pathways responsible for these instances of mitophagy remain to be determined. Likewise, loss of the key PINK1 phosphorylation site for Parkin activation, serine 65, resulted in the loss of endogenous Parkin activity in cultured cortical neurons and selective locomotor impairments with mild mitochondrial defects in mice [158]. However, neither basal mitophagy *in vivo* nor mitochondrial depolarization-induced mitophagy in patient-derived fibroblasts was altered. Using a more general mitochondrial stress, Sterky *et al*. [159] crossed Parkin knock-out mice with mice lacking Tfam in dopamine neurons (MitoPark model). While MitoPark mice exhibit parkinsonism phenotype, the Parkin knock-out did not exacerbate the phenotype. In addition, overexpressed Parkin in dopamine neurons *in vivo* failed to recruit to mitochondria despite the mitochondrial stress. In contrast to this study, Pickrell *et al*. [160] used the mutator mouse model ($Polg^{D257A}$) to drive mitochondrial DNA mutations and increase mitochondrial stress. While dopamine neurons indeed degenerated with age in the absence of Parkin and the mice had neurodegenerative phenotypes, it was unclear from the study whether the defective mitophagy was the root of the problem. Likewise, in the heart, depletion of Parkin has been shown to increase mouse susceptibility to myocardial infarction, and under these conditions, the mitochondria exhibited an abnormal morphology that is consistent with a mitophagy defect [161]. More recently, using the mt-Keima reporter in PINK1 knockout mice, Sliter *et al*. [105] showed that although mitophagy still occurred, its levels were diminished in the heart following extreme exhaustive exercise (though it was uncertain whether mitophagy levels in nigrostriatal dopamine neurons were also altered). Thus, it seems possible that PINK1/Parkin mitophagy only becomes relevant under specific high-stress contexts. Overall, the physiological significance of the PINK1/Parkin pathway and its contribution to mitophagy *in vivo* remains a very important challenge to clarify in the coming years, in particular in determining what these physiological stimuli are. Perhaps, with respect to PD, mitophagy-independent functions of PINK1 and Parkin could also play a critical role.

Ubiquitin-independent mitophagy receptors

A certain class of cargo receptors bypass the need for ubiquitin to link the forming autophagosome with its cargo. For mitophagy, these ubiquitin-independent receptors are OMM proteins containing

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LIR motifs that directly recruit the autophagosomal membrane (see reviews Refs. [25,26,29] and Fig. 3B). ATG32 is the only mitophagy receptor in yeast, and its signaling mechanism is somewhat evolutionary conserved in mammals where there are currently several proteins identified: BNIP3, NIX/BNIP3L, FUNDC1 and BCL2L13. The primary mode for these proteins to mediate mitophagy is thought to be in mediating the direct interaction of the mitochondrion with the nascent autophagosome. The process is modulated at two different levels: (1) Protein abundance: For example, BNIP3 and NIX are under transcriptional control by upstream mitophagy signaling [93]. Once a cue for mitophagy happens, these receptors are upregulated several-fold and increase their presence in the OMM. It remains unclear how (or if) selectivity, in terms of mitochondrial targeting of receptors, occurs in these instances. (2) Post-translational modifications: mitochondrial receptor function is modulated primarily by phosphorylation events that facilitate or enhances the receptors' capacity to interact with LC3/GABARAP proteins to drive mitophagy. For example, ATG32 is phosphorylated near the LIR motif, potentially by CK2, to facilitate the key interaction with the adaptor protein ATG11 [162,163]. Similarly, NIX is also phosphorylated by an unknown kinase to strengthen the interaction with LC3/GABARAP proteins [164]. In contrast, FUNDC1 is inhibited under basal conditions by CK2/Src phosphorylation of the LIR motif [165,166]. In this case, mitophagic activity is facilitated by PGAM5 phosphatase activity and the FUNDC1–ATG8 interaction is strengthened by ULK1 phosphorylation [167]. FUNDC1 can also be ubiquitylated and proteasomally degraded to limit the mitophagy response [168]. Although phosphorylation is currently the main mechanism of regulation, other still uncharacterized post-translation modifications could be important for fine-tuning receptor activity. Overall, the upstream regulation of mitophagy receptors and the inherent selectivity for marking mitochondria remain poorly understood.

In terms of mitophagy pathways, ubiquitin-independent mitophagy receptors have been described to act in different contexts and stimuli, including programmed mitophagy or stress-induced mitophagy (during hypoxia or mitochondrial damage with depolarizing agents [29]). For example, as mentioned earlier, NIX is important in regulating programmed mitophagy during erythrocyte maturation [52,53,169] or to drive metabolic reprogramming during retinal ganglion cells or macrophage differentiation [121]. In parallel, FUNDC1, NIX and BNIP3 are known to be upregulated during hypoxia and regulate hypoxia-induced mitophagy [93,165]. Strikingly, mitophagy receptors may be exploited by the intracellular bacterial pathogen *Listeria monocytogenes* for survival. The virulence factor listeriolysin O triggers mitophagy in infected macrophages by inducing oligomerization of a new mitophagy receptor, Nod-like receptor X1 (NLRX1), which localizes to mitochondria and contains canonical LIR motifs [170]. It is thought that the bacterial-induced mitophagy reduces ROS production to aid its intracellular survival.

The regulation of mitophagy by mitochondrially-associated receptors is evolutionarily conserved from yeast to humans and physiologically relevant in mammals under different scenarios [29]. Thus, it could be hypothesized that mitophagy receptors are the ancestral mechanism for mitophagy, while the PINK1/Parkin pathway emerged later as direct on-site, fast and selective response mechanism—potentially in parallel with xenophagy [21]. Interestingly, when NDP52 is forced to act as canonical ubiquitin-independent mitophagy receptor, by ectopically tethering it to mitochondria with chemical inducible dimerization, the receptor can then bypass the PINK1/Parkin signaling cascade and directly recruit ULK1 complex to trigger mitophagy [171]. Furthermore, mitochondrial depolarization in PINK1/Parkin-deficient cells can still be rescued by NIX-mediated mitophagy, as overexpression of NIX restored CCCP-induced mitophagy in human fibroblast-derived from Parkin or PINK1-related PD patients [172]. As discussed, while ubiquitin dependent and independent receptors have been shown to mediate mitophagy in different contexts and stresses, the potential cooperation and crosstalk between both mitophagy signaling mechanisms require further study.

An alternative mechanism of mitochondrial priming utilizes lipids instead of proteins as mitophagy receptors, and this situation may be analogous to the PHB2/NIPSNAP mechanism mentioned above. Cardiolipin (CL) is a lipid exclusively found in the IMM with important roles in regulating mitochondrial respiration and dynamics [173]. Mitochondrial damage can result in translocation of CL from IMM to OMM, or at least allow exposure to the cytosol. On one hand, translocation to the mitochondrial surface leads to CL oxidation, which facilitates mitochondrial membrane permeability, cytochrome C realize and apoptosis [174]. On the other hand, the negative-charged head group of CL can interact with the basic amino acids in the N-terminal domain of LC3 proteins decorating autophagosomes [173], priming mitochondria for mitophagy. It was reported that the externalization of CL could be regulated by the IMS enzyme nucleoside diphosphate kinase NDPK-D, suggesting that this mitophagy pathway could be further regulated [175]. However, it remains uncertain if CL-mediated mitophagy is a parallel pathway or cooperates with other mitophagy signaling events mediating phagophore recruitment and expansion. CL may not be the only mitophagy receptor lipid as lethal mitophagy, mentioned earlier, is regulated by sphingolipids [124]. During lethal mitophagy, C18

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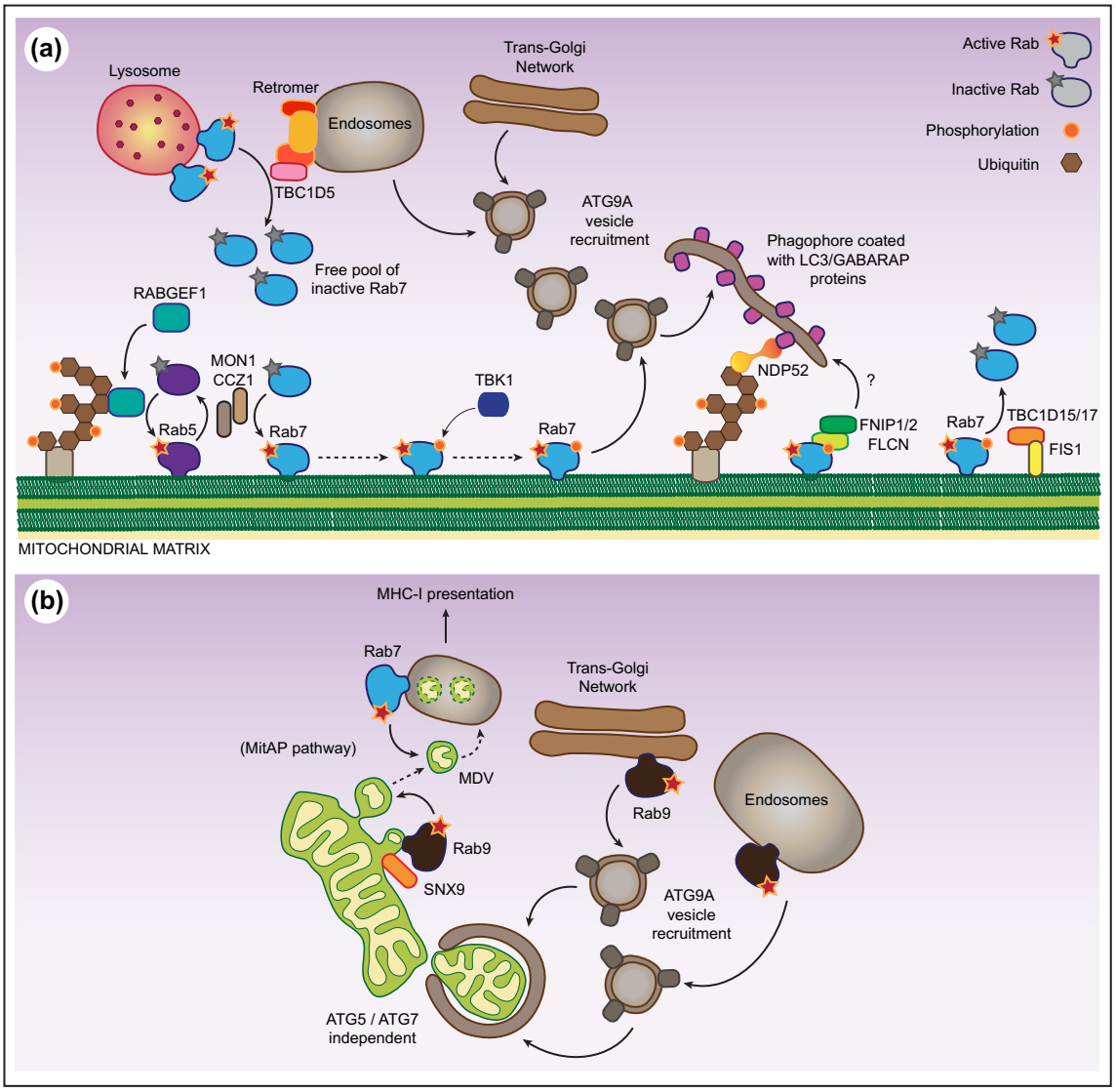

Fig. 4. Mitophagy-related roles of the Rab7 GTPase. (A) Rab7 recycling from lysosomes is mediated by retromer complex, providing an accessible pool of inactive Rab7 which can then be recruited to the OMM following Parkin activation. This is achieved by binding of the Rab5 GEF (RabGEF) to ubiquitylated substrates in the OMM, which triggers the canonical Rab5-Rab7 conversion cascade that results in Rab7 localization. Active mitochondrial Rab7 is phosphorylated by TBK1, which facilitates the recruitment of ATG9A vesicles for phagophore expansion. Active Rab7 also recruits FNIP1/2 and FLCN, which are required for mitophagy through an unknown function. Mitochondrial Rab7 is inactivated by TBC1D15/17, which are recruited to mitochondria by Fis1. (B) Rab9, SNX9 and Rab7 facilitate mitochondria antigen presentation (mitAP) *via* MDV vesicles. Rab9, localized at the TGN and late endosome, regulates the formation of ATG9A positive vesicles to form the phagophore independently of the ATG8 conjugating system.

ceramides follow a similar mechanism to CL and recruit phagophore membranes to the OMM by direct interaction with LC3. It is possible that the use of mitochondrial lipids in mitophagy is more widespread; however, how, when and which lipids in the mitochondrial double membrane system could directly contribute to mitophagy, outside of the two mentioned, has been largely overlooked. For example, Ivatt *et al.* [176] reported in flies and human cells that PINK1 stabilization was impaired under the loss of sterol regulatory element binding proteins (SREBPs). SREBPs are involved in the regulation of cholesterol and phospholipid synthesis, suggesting that defective lipid synthesis could lead to abnormal mitochondrial lipid composition and impaired PINK1-dependent mitophagy, although off-target consequences of depleting lipid synthesis cannot be discarded at this point. Of interest, genome-wide association studies identified SREBF1 as a risk locus for idiopathic PD [177].

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How is the autophagy machinery engaged to eat mitochondria?

The ultimate goal for any mitophagy priming mechanism is to recruit the autophagy machinery to nucleate the mitochondrion-engulfing autophagosome. Recruitment and activation of the ULK1 protein kinase complex (comprising of ULK1, ATG13, FIP200 and ATG101) is one of the earliest and essential events in triggering autophagosome formation, and it is thought that this is also a key requirement for mitophagy initiation [9,136,171,178–180]. For example, ULK1 knockout mice accumulated mitochondria in erythrocytes due to impaired mitophagy [178]. How then is the ULK1 complex recruited to mitochondria during mitophagy? It appears that the receptor proteins could play an essential role in this mechanism.

During PINK1/Parkin mitophagy, the mitochondrial receptors NDP52 and OPTN were shown to recruit the ULK1 complex and early autophagy markers such as WIPI1 and DFCP, which were proposed to nucleate autophagosome formation [136]. For NDP52 at least, ULK1 recruitment is mediated by direct interaction with the ULK1 complex member FIP200, which in turn is facilitated by TBK1 activity [171]. As mentioned above, the forced recruitment of NDP52 to mitochondria is sufficient to directly recruit the ULK1 complex and trigger mitophagy [171]. Although OPTN and TAX1BP1 are also important for PINK1-mitophagy, it is unclear whether they can also recruit ULK1. As with the ubiquitin-dependent receptors mentioned above, ULK1 has also been shown to interact directly with "ubiquitin-independent" FUNDC1 and BCL2L13 on the OMM to initiate autophagosome formation [167,181]. However, whether other receptors such as NIX or BNIP3 also directly recruit the ULK1 complex remains to be determined.

Over the years, it was thought that the primary function of autophagy and mitophagy receptors was to interact with ATG8/LC3/GABARAP proteins, using their LIR motifs, and in this way recruit the nascent autophagosome. However, the actual role of ATG8/LC3/GABARAP proteins in autophagosome formation at mitochondria (a mitophagosome) may be more complex. First, the LC3/GABARAP proteins are not essential for all forms of mitophagy, as mice lacking core genes for the ATG8 conjugating system, such as ATG5 and ATG7, can still clear mitochondria during erythrocyte maturation [182,183]. Furthermore, in cells depleted of all six mammalian LC3/GABARAP proteins, mitochondria can still be incorporated into forming autophagosomes, although the process is somewhat impaired. Interestingly, this study revealed that GABARAP was required at a later step for the fusion of the mitochondrion-containing autophagosomes with lysosomes [184], which has also been described for autophagosomes in general [185].

Although mitophagy receptors can interact with both ULK1 and LC3/GABARAP proteins, they may be recruited independently to mitochondria during mitophagy [125,171,179]. Recent evidence suggests that the canonical function of LIR motifs in autophagy receptors is perhaps not to selectively link ubiquitylated OMM proteins to LC3/GABARAP proteins on the forming autophagosome [186]. In the proposed model, the LC3/GABARAP proteins recruit receptors to form autophagosomes independently of ubiquitylation, which results in an LC3/GABARAP-dependent positive feedback loop to amplify the Parkin-driven mitophagic response. It is possible that a similar mechanism also occurs with ubiquitin-independent receptors, but further work is needed to confirm this. Intriguingly in *Arabidopsis*, ATG8 contains a conserved ubiquitin-interacting motif on the opposite face from the canonical ATG8-interacting motif [187]. This opens an avenue for potential new mitophagy receptors or alternative mechanisms of regulation [187].

How does Rab GTPase signaling regulate mitophagy?

As discussed above, autophagosomes fuse with the endocytic system and ultimately lysosomes. This process of autophagy mirrors many aspects of the classical endocytosis and phagocytosis pathways and has been reviewed previously [10,188]. Once an autophagosome has formed, it can utilize the same machinery as endosomes, namely tethering complexes and SNAREs to ultimately regulate fusion with lysosomes. The GTPase Rab7 plays a key role here [10]. The Rab family of small GTPases, of which there are close to 70 members in humans, are fundamental organizers of intracellular membrane trafficking. They confer membrane identity and ensure that membrane cargoes are transported, docked and fused to the correct destination [189,190]. Rab GTPases work as molecular switches relying on their capacity to bind GTP and GDP; they are inactive when bound to GDP (with a primarily cytosolic localization) and active, and able to bind effector proteins, when bound to GTP (with a membrane localization). This Rab cycle is tightly controlled by Rab-GEFs and Rab-GAPs. The guanine–nucleotide exchange factors (GEFs) regulate Rab activation by promoting the exchange of bound GDP for GTP; meanwhile, the GTP-hydrolysis activating proteins (GAPs) inactivate Rabs by stimulating their ability to hydrolyse GTP into GDP.

It has now become evident that Rabs can regulate autophagy, and hence mitophagy, at multiple points, prior to the lysosomal fusion mentioned above. Intriguingly, Rab7 may also play a role in mitophagy initiation during Parkin-mediated mitophagy (Fig. 4A). These studies have built up two

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complimentary models to describe how incoming ATG9a-positive vesicles contribute to autophagosome expansion [191–195]. 1) Ubiquitylation of OMM proteins, downstream of PINK1 and Parkin activation, leads to the direct recruitment of RABGEF1 (a Rab5-GEF). This creates a signaling platform to initiate the classical Rab conversion pathway observed during endosome maturation [196]. Recruitment of Rab5 engages the Rab7 effector complex MON1/CCZ1, which in turn leads to the recruitment and activation of Rab7. Then, Rab7 functions to direct ATG9a vesicles to the primed mitochondria, which provides essential membranes to mediate mitophagy [191]. This Rab cascade and Rab7 activity are negatively regulated by the Rab7-GAPs, TBC1D15 and TBC1D17, which are recruited to mitochondria by interacting with Fis1 [192]. These Rab7-GAPs also interact with LC3/GABARAP proteins coating the autophagosomes *via* a LIR motif, and regulate Rab7 activity to constrain phagophore formation to the target mitochondria [192]. Depletion of TBC1D15 and TBC1D17 leads to Rab7 accumulation on mitochondria and an abnormal accumulation of autophagosome-like structures that delays and impedes the clearance of depolarized mitochondria [191,192]. 2) The retromer complex, an endosomal protein recycling complex, consisting of a trimer of VPS26, 29 and 35 as well as a dimer of distinct sorting nexins, also has a pivotal role in regulating Rab7 localization to mitochondria for effective Parkin-dependent mitophagy [194]. The interaction of VPS29 with the Rab7-specific GAP, TBC1D5, enables the retromer complex to control Rab7 localization, activity and mobility. TBC1D5 activity inactivates Rab7 to release it from endo-lysosomal membranes and generate pools of inactive Rab7 that can re-localize to mitochondria, endosomes or Trans-Golgi network for other functions, including mitophagy. This Rab7 regulation by retromer during Parkin-mediated mitophagy is independent of the retromer's classical cargo sorting function and is thought to allow the proper sorting of ATG9a vesicles to autophagosome formation sites around primed mitochondria. When TBC1D5 and retromer complex functions are lost, hyperactivated Rab7 clusters around lysosomes and is unable to regulate mitophagy. In this case, ATG9a accumulates in the Trans-Golgi network, as is also observed in VPS35 PD-related mutants [197]. Rab7 function during Parkin-mediated mitophagy is also regulated *via* its phosphorylation at serine 72 by TBK1. While this phosphorylation is critical to regulate Rab7-dependent recruitment of ATG9a vesicles to depolarized mitochondria, it also facilitates the recruitment of FLCN/FNIP1 complex (a proposed Rab-GEF). Although FLCN/FNIP1 appears necessary for mitophagy, it does not regulate ATG9a-positive vesicle recruitment. Interestingly, HeLa cells lacking TBK1 have a delayed induction of Parkin-driven mitophagy [171]. Considering Rab7-regulation of autophagosome formation by TBK1, it could be speculated that this delay is caused by defective establishment and expansion of the phagophore at the mitophagic site. It remains to be clarified if this Rab7-dependent mitophagosome expansion also occurs in other mitophagy signaling contexts independently of PINK1 or Parkin.

Intriguingly, Rab7 has also been reported to regulate mitochondrial fission *via* lysosome–mitochondria contact sites. These contact sites were untethered under hyperactive Rab7 conditions, which blocked mitochondrial fission. However, TBC1D15 GAP activity could downregulate Rab7 activity and preserve lysosome–mitochondria contact sites, which facilitated mitochondrial fission. Although mitochondrial fission is a requirement for and often precedes mitophagy, the mitochondria contacting lysosomes were not observed to take part in mitophagy [198]. Mitochondrial fission and dynamics have also been associated with Rab32 and the SNARE protein Syntaxin-17 (STX17), specifically at ER–mitochondria contact sites, where they regulate DRP1 activity [199]. STX17 has further links to mitophagy given that it has been implicated in regulating fusion of autophagosomes with lysosomes [10,200], as well as mediating –ysosomal fusion [201]. STX17 may also regulate earlier stages of mitophagy as it has been recently linked with autophagosome formation following TBK1 phosphorylation [202]. In this context, STX17 has been reported to regulate Parkin dependent and independent mitophagy from ER–mitochondrial contact sites [203,204].

In addition to Rab7, other Rabs have also been directly implicated in mitophagy. Rab35 was shown to be recruited to depolarized mitochondria downstream of Parkin to aid in NDP52 receptor recruitment, which was enhanced by TBK1 and negatively regulated by the Rab-GAP TBC1D10A [205]. Another example is the role of Rab11a in recruiting WIPI2 at endosomes to regulate the ATG8-conjugation system for phagophore expansion. Although this was not shown to be specific for mitophagy, Rab11a-positive membranes were found to engulf mitochondria during iron chelation-induced and depolarization-induced mitophagy [206]. Furthermore, Rab8a/b and Rab13 can be indirectly phosphorylated by PINK1 after mitochondrial depolarization at ser111 to promote Rab inactivation [207]. The kinase or phosphatase downstream of PINK1 is still unknown, and it remains unclear whether PINK1 regulation of these Rabs contributes to mitophagy or other functions.

Another example of mitophagy regulation by Rabs occurs during ATG8-independent autophagy and mitophagy (Fig. 4B). In cells lacking the core ATG8 conjugating machinery, Rab9 (as well as ULK1) was

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shown to regulate autophagosome formation by fusing isolated membranes with vesicles derived from Trans Golgi Network and late endosomes. This Rab9-dependent autophagic pathway was implicated in programmed mitophagy during erythrocyte maturation [182]. More recently, Saito *et al*. [208] used the mt-Keima mouse model to show that mitophagy induced in cardiomyocytes during ischemia or nutrient starvation was regulated by a ULK1/Rab9 signaling axis independently of ATG7. However, it is unclear if Rab9 function is mediated *via* ATG9a vesicle sorting and if Rab7 also contributes to mitophagy in this context. Interestingly, Rab9, together with SNX9, can regulate the formation of heat stress or LPS-induced MDVs to deliver mitochondrial cargo into the endo-lysosomal system. In contrast to ROS-induced MDVs that require PINK1 and Parkin [11], these Rab9-mediated MDVs were inhibited by PINK1/Parkin pathway activation. It is thought that this pathway is key in generating mitochondrial antigens (MitAP) to drive T-cell activation *via* MHC-I presentation [209].

Is Mitophagy a Viable Therapeutic Target?

The important power of mitophagy as a quality control mechanism for mitochondrial network homeostasis, cell integrity and organism development, comes as well with great responsibility. It is therefore not surprising that defects in mitophagy signaling have been associated with a plethora of common disorders with important social, medical and economic impact, for example, neurogenerative diseases, cardiovascular diseases and cancer [25,29]. Below we discuss the recent advances regarding mitophagy dysfunction in a neurogenerative context, in particular in relation to PD. However, our understanding of the physiological roles and implication of mitophagy in diseases, in general, remains limited.

Over the past decades, the dysfunction of mitochondria has emerged as a potential common feature across multiple neurodegenerative diseases [210–213]. The low regenerative ability, high-energy demand and large size of post-mitotic neuronal cells make them especially sensitive to loss of quality control mechanisms like autophagy or mitophagy [19,214,215]. Indeed, the decline of autophagic and proteasomal activity are hallmarks of aging. Disruption of mitophagy, as well as the accumulation of dysfunctional mitochondria, has been hypothesized as one of the potential triggers of PD (and potentially AD [216]). This has been fostered by the familial forms of PD involving mutations in genes directly linked to mitophagy itself, such as PINK1 and Parkin. The majority of PD-related PINK1 and Parkin mutations challenge their activity or activation mechanism. For this reason, PINK1 and Parkin activators, or USP30 DUB inhibitors, have emerged as potential therapeutic strategies to compensate for the loss of mitophagy [217]. However, the physiological contribution of PINK1 and Parkin in mitophagy is still not clear, as previously discussed in this review. Many studies on this pathway have been carried out in mice though, and it should be noted that PINK1 or Parkin knockout mice do not develop a classical neurodegenerative PD phenotype [218]. This is in contrast to rats lacking PINK1, which do exhibit age-dependent PD-pathological phenotypes [219]. Hence, PINK1/Parkin function with respect to PD could differ in mouse *versus* humans or rats. We note that non-human primate models for PINK1 may also provide important validation for this pathway [220]. It is therefore essential in the coming years to clarify the direct contribution PINK1 and Parkin in PD pathology and the contribution of mitophagy in general. It is possible that only a small subset of mitochondria needs to be turned over; hence, any therapy must ensure that it is only these “unhealthy” mitochondria that are degraded to ensure exacerbation of the phenotype does not occur. Likewise, we currently do not know if a specific mitophagy pathway targets certain types of mitochondria; therefore, it will be essential to activate the right mitophagy pathway at the right time. With respect to PD, it is also noteworthy that several other mutated genes such as LRRK2, VPS35 or Rab7L contribute to regulating membrane trafficking at various levels and may also impact upon mitophagy. Thus, the PINK1-Rab signaling axis could harbor potential crosstalk with other endocytic-lysosome pathways with relevant contributions to the disease beyond mitophagy.

New avenues of research indicate that loss of Parkin and PINK1 could drive PD pathogenesis through a neuroinflammation response. Matheoud *et al*. [209] unraveled a new mechanism, independent of classical mitophagy, where MDVs deliver mitochondrial proteins for turnover and MHC-I mitochondrial antigen presentation (MitAP). Although the physiological and pathological contribution of MitAP to disease *in vivo* remains to be confirmed, it was speculated that MitoAP is activated upon loss of PINK1 or Parkin and DA neurons become exposed to immune system-mediated elimination [209]. On a more classical mitophagy note, Sliter *et al*. [105] revealed that STING-mediated type 1 interferon response leads to stress-induced neurodegeneration in mice lacking of PINK1 or Parkin. A failure to clear damaged mitochondria, caused by loss of PINK1 or Parkin, resulted in an increase in cytosolic and circulating mtDNA that triggered this inflammatory response. This inflammatory phenotype was only observed under dramatic acute and chronic stress, while depletion of PINK1 or Parkin failed to display an inflammatory response

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in mice at basal conditions [105]. Although the links between mitochondrial stress-driven inflammation and PD are important steps in the underlying pathology of PD, further research is still required to fully clarify the contribution of mitophagy during neuroinflammation and whether it is neuronal mitophagy that is most relevant. Indeed glial cells, such as microglia and astrocytes, which perform critical roles in brain homeostasis and neuroinflammation, undergo basal mitophagy *in vivo* [72]. Furthermore, astrocytes in the optic nerve were shown to coordinate transcellular mitophagy, where retinal ganglion cell axonal mitochondria are shuttled to neighboring astrocytes for degradation [221]. This neuro-glial communication was also observed after cerebral ischemia, where astrocytes released functional mitochondria to damaged neurons to ensure their survival [222]. Certainly, mitophagy in glial cells and neuro-glial interactions could have a critical contribution to neurodegenerative diseases that up-to-date has been largely overlooked.

If mitophagy is validated as a viable therapeutic pathway, a big question remains as to what exactly to target. Several natural compounds have been reported to induce mitophagy and achieve anti-aging and cytoprotective effects (i.e., urolithin A, spermidine or nicotinamide mononucleotides) [68,223–225]. In fact, the first-in-human clinical trial for Urolithin A has been recently published showing a transcriptional increase in some mitochondrial function, biogenesis and degradation markers from quadriceps muscles biopsies [226]. However, it is not clear if the anti-aging effects associated with these compounds are only due to mitophagy. The chronic administration of these metabolites can result in a more boarder impact on cell function or other aspects of mitochondria biology, including mitochondrial biogenesis. PINK1 and Parkin would be the obvious candidates to develop selective activators, but they are not essential for all forms of mitophagy, so may not be relevant for diseases outside of PD. In addition, small molecule activators for PINK1 and Parkin would be of no use in patients that lack expression of these proteins, or at least have limited use in PD patients harboring inactivating mutations in these genes. An alternative approach to directly targeting PINK1 and Parkin, and focusing on mitochondrial ubiquitylation more broadly, involves inhibiting USP30. Blocking USP30 would facilitate a faster and easier ubiquitylation of mitochondria to signal for mitophagy, which could compensate for a defective or reduced mitophagy activity. While USP30 has been primarily studied as a counterpart of PINK1/Parkin-mitophagy, its activity has been shown to also regulate basal mitophagy and pexophagy *in vitro* [150,227]. However, it is unclear how USP30 regulates mitophagy in a physiological or disease context, so more work is needed in this area. It may also be possible to enhance mitophagy by directly activating the autophagy initiation machinery, for example, by pharmacological activation of ULK1, VPS34, AMPK or inhibition of mTORC1. Autophagy activation has been proposed as a potential therapeutic opportunity to enhance the clearance of protein aggregates in neurodegenerative disease or in cancer therapies [228]. However, aiming to activate the autophagy machinery to clear dysfunctional mitochondria will likely not be specific. Therefore, there are no clear therapeutic candidates to specifically modulate mitophagy yet. While most therapeutic efforts are focused on enhancing mitophagy, it cannot be excluded that upregulation of mitophagy could also have pathological implications. Indeed, evidence exists of deleterious upregulation of mitophagy such as in intracellular pathogen infection [170] or cancer cell survival during mitotic arrest [122].

Conclusions

Significant advances have been achieved over the years to understand how mitophagy is regulated on a cellular basis. However, we are far from understanding how mitophagy signaling is regulated *in vivo* and its physiological contribution to diseases and organism development. Furthermore, the current data in the field suggest that mitophagy is more complex than anticipated. Mitophagy is not restricted to one or two signaling pathways; instead, it has an important contextual regulation with multiple signaling mechanisms, depending on the tissue, stress, metabolic state or development phase. In addition, mitophagy dynamism has to be framed under the light of mitochondrial network function and dynamics, where the orchestrated interplay of mitophagy with other mitochondrial quality control pathways remains unclear. Here, we aimed to review our current knowledge in order to answer basic questions about mitophagy: “What is it?”,“What is it used for?” “Why is important?”,“How is regulated?” and “Can it be therapeutically useful?” By doing so, we have also highlighted important gaps in our understating of mitophagy that indeed raise outstanding questions to resolve in the years to come. Now, more than ever before, we have the *in vitro* and *in vivo* tools to gain deeper insight on our understanding of mitophagy and its contribution to physiology and disease.

Acknowledgments

We would like to thank the Ganley Laboratory for critical reading of this manuscript. This work was funded by a grant from the Medical Research Council, UK (I.G.G.; MC_UU_00018/2).

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Declarations of interest: None.

Received 1 May 2019;
Received in revised form 28 June 2019;
Accepted 30 June 2019
Available online xxxx

Keywords:
mitophagy;
mitochondria;
Parkinson's disease;
autophagy;
metabolism

Abbreviations used:
OXPHOS oxidative phosphorylation; ROS reactive oxygen species; MDV mitochondrial-derived vesicle; ONL outer nuclear layer; PD Parkinson's disease; OMM outer mitochondria membrane; LIR LC3-interacting region; OMMAD outer mitochondrial membrane-associated degradation; DUB deubiquitylase enzyme; CL cardiolipin; IMM inner mitochondria membrane; PAS phagophore assembly site; GEF guanine nucleotide exchange factor; GAP GTP-hydrolysis activating protein; MitAP MHC-I mitochondrial antigen presentation.

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Please cite this article as: L. Montava-Garriga and I. G. Ganley, Outstanding Questions in Mitophagy: What We Do and Do Not Know, Journal of Molecular Biology, https://doi.org/10.1016/j.jmb.2019.06.032