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Review

Mitophagy

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ABSTRACT

Concurrent mitochondrial elimination and autophagy in many systems has led to the proposal that autophagy is the main mechanism of mitochondrial turnover during development and under pathological conditions. The term mitophagy was coined to describe the selective removal of mitochondria by autophagy but the process itself is still contentious. Three questions are being debated: 1) Is there a specific removal of mitochondria by autophagy or is it non-selective or inadvertent? 2) What are the signals that drive this process? 3) Does removal of mitochondria increase or decrease cell viability? There is a mounting evidence for specific signals in/on mitochondria that drive mitochondrial removal from cells by autophagy. The process itself may be both selective and non-selective. In yeast, surprisingly, mitochondrial elimination occurs more by microautophagy (intracellular pinocytosis by the vacuolar membrane) than macroautophagy (initiated by stand-alone nascent double membrane structures known as autophagosomes). In mammalian cells, macroautophagy seems most prevalent though tools to study microautophagy are not well developed. Whilst lack of mitophagy seems to be deleterious, understanding the interplay between autophagy, mitochondrial performance, and cell pathology is a much-needed area of research.

1. Introduction

The term mitophagy refers to the selective removal of mitochondria by autophagy and was coined by Lemasters [1]. Use of this term followed years of detailed studies on the fate of mitochondria in hepatocytes after activation of the mitochondrial permeability transition (mPT) and loss of mitochondrial membrane potential ($\Delta\Psi m$). The liver, being the body's sensor of nitrogen starvation, forged a connection to autophagy [2]. Presence of mitochondria in autophagosomes in liver is where classically autophagy of mitochondria was first convincingly demonstrated in 1962 [3]. Clear EM evidence is presented of healthy-looking mitochondria, as well as mitochondria in various stages of degradation, engulfed in stand-alone bodies that are not quite lysosomes — the term autophagy/autophagosomes was only coined in 1963 [4]. Ashford and Porter [3] noted that almost every lysosome contained a mitochondrion, albeit together with other organelles. Concluding that mitochondria were being degraded within the lysosomes, it was nevertheless not clear whether decaying mitochondria underwent this process prior to engulfment or whether this was a post-engulfment event, as the authors suggest. Another proposal for the occurrence of mitophagy came from studies of silkmoth muscles undergoing metamorphosis [5] where the authors state: "The observation of a primary phase of autophagy which involves almost exclusively mitochondria argues for a selection of specific cytoplasmic constituents, [suggesting] that certain mitochondria develop functional alterations which would activate autophagy" (see [6] for previous coverage of these topics). In the past few years compelling evidence has begun to emerge indicating that removal of mitochondria from cells can be specific, and the signals that specify mitochondria as targets of the autophagic process have begun to be elucidated, both in yeast and in mammalian cells. Several excellent reviews have appeared on physiological and pathological aspects of this topic [7–12]. Here, the evidence for mitophagy is critically examined with reference to cell function under physiological and pathological conditions.

2. Mitophagy in yeast

Until recently, the best documentation for signals that mediate autophagic removal of mitochondria comes from studies in yeast. Before describing these it is important to appreciate that in yeast, mitochondrial function and morphology, as well as the extent of autophagic activity, depend on growth conditions. Namely, on glucose, yeast obtains energy by anaerobic respiration (fermentation). Glucose represses respiratory enzymes and, as a result, mitochondria are poorly differentiated. On lactate, or ethanol/glycerol, mitochondrial respiration is essential for growth and viability and mitochondrial biogenesis is robust. On galactose, yeast can utilize fermentation and respiration so mitochondrial function/

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biogenesis is not repressed, much like with lactate. Thus, getting rid of mitochondria during growth in glucose may not have any consequences to the cells unless they are starved of nitrogen and autophagy is induced, but getting rid of mitochondria under lactate could lead rapidly to pathology. It follows that there are many conditions under which mitophagy can be examined, and several of these have been utilized, as described below.

The *yme* ("Yeast Mitochondrial Escape") genes were discovered in a search for genes that control escape of mitochondrial DNA (mtDNA) from the mitochondria to the nucleus in the budding yeast *Saccharomyces cerevisiae* (Fig. 1). The question arising is what happens to the mitochondrion body mass during this transfer? In cells growing on glucose, there was an 8–10 fold higher basal rate of escape of mtDNA into the nucleus compared to galactose [13], and almost non-existent transfer was observed in cells growing on ethanol/glycerol. However, in the absence of *yme*1- a mitochondrial protease (an AAA ATPase)- a vastly increased rate of escape of mtDNA was noted in cells on an ethanol/glycerol medium, and this was accompanied by the appearance of "pinched" and fragmented mitochondria adjacent to invaginations at the surface of the vacuole [14]. Vacuole-dependent uptake of mitochondria was confirmed using the alkaline phosphatase (ALP) maturation assay [15] adapted to mitochondria (in which ALP (*PHO*8) targeted to mitochondria in the absence of genomic *PHO*8 acquires activity only through cleavage in the vacuole, thus providing evidence for mitochondrial dissolution). This turnover did not appear to be mediated by macroautophagy, as no intermediate autophagosomes enclosing mitochondria were detected. Surprisingly, mutation of other *yme* genes also enhanced abnormal mtDNA nuclear transfer, but only the *yme*1 strain showed elevated mitophagy. These findings suggest that mitochondrial turnover is triggered by a specific (absence of) protein whose function is dissociated from mtDNA escape from the host mitochondrion. Does this finding indicate mitophagy by the basal autophagic machinery, since nitrogen was plentiful? The authors note that the assay for mtDNA escape depends on the lack of tryptophan, which triggers weak autophagy in glucose-containing medium containing low nitrogen but no amino acids [16]. This effect, however, would apply to all the other *yme* strains, which was not observed. These intriguing studies thus defined the principle of a genetic control over vacuole-dependent mitochondrial elimination but whether autophagy is involved, and what kind of autophagy (micro-, macro-, piecemeal) is open to further investigation. Because the deletion of *yme*1 provokes mitophagy, it is also unclear whether it is the absence of the protein itself that is the signal for mitophagy or whether other proteins do so by acquiring abnormal or novel functions in the absence of the primary protein. It is also possible that mitochondrial biogenesis is disrupted, since a suppressor mutation implicates a subunit of the 26S proteasome in *yme*1-dependent mitophagy [17].

The evidence that mitophagy is genetically controlled was soon reinforced by work on *UTH1*. Uth1p (UTH, "youth") was first defined in a screen for genes regulating longevity and was independently described by Camougrand and colleagues as being required for Bax activation of cell death in yeast, albeit not for Bax localisation [18,19]. Its connection to autophagy was forged by the discovery that the lethality of rapamycin (which induces autophagy) was decreased in a Δ*UTH1* strain. Kissova et al. [20] went on to demonstrate that Uth1p protein is required for removal of mitochondria into the vacuole during autophagy. Mitophagy inhibition in the Δ*UTH1* strain occurs without diminishing the function of the autophagic machinery, indicating the specificity of this process. In a recent paper [21], the mechanism of mitochondrial removal during starvation in lactate-grown cells was investigated by EM. After vacuolar fragmentation induced by starvation in lactate, within 2–3 h, mitochondrial profiles were found inside vacuoles with and without additional cytoplasm, or closely proximate to the vacuole at what look like specialised sites of protrusion or invagination (similar to [14]). Two categories of encapsulated mitochondria were found, one devoid of cytoplasm and the other containing cytoplasm; profile frequencies were scored to evaluate *UTH1* dependence. Only profiles devoid of cytoplasm, referred to as selective microautophagy, were reduced in the Δ*UTH1* mutant strain whereas the later, more classical microautophagic profiles were not *UTH*1-dependent. Thus, *UTH1* seems to direct one specific mechanism of mitophagy but there is not only one mechanism for mitochondrial removal by autophagy, at least during starvation in lactate. Is Uth1p required for viability? On lactate or glucose media, the *UTH1* deletion rescued cells from rapamycin- or starvation-induced death, whereas death was rescued less robustly, if at all, in the Δ*ATG*5 strain (after returning cells to a glucose medium to relieve stress and dependence on mitochondrial respiration). Since autophagy is activated equally in wt and Δ*UTH*1 strains by rapamycin and starvation, but not in the Δ*ATG*5 strain, this may suggest that it is autophagy, rather than mitophagy, that is required for rescue from rapamycin and starvation and/or that sparing of mitochondria is beneficial to survival. Rapamycin activates autophagy in yeast through inhibition of mTOR, which also has implications for cell growth; thus size of colonies growing in the presence of rapamycin may be smaller because of smaller cells and/or fewer cells. Further confounding issues include a role for *UTH1* in mitochondrial biogenesis and function [19,22]. Thus, the principle of mitophagy being specifically directed and selective is supported by studies of the *UTH1* gene. Not much is known about the function of the UTH1 protein or its orientation in mitochondria, presumably because its overexpression is toxic [22]. It contains a potential Fe–S cluster, aside from having a putative membrane targeting sequence and potential phosphorylation sites. It would be intriguing to investigate what happens when it is expressed in mammalian cells, as no mammalian homologue of *UTH1* has been identified.

Unlike the outcomes reported in the two previous studies in yeast, reduction in mitophagy in Δ*AUP1* mutant yeast [23] is correlated with reduced viability and rapamycin-hypersensitivity, suggesting a pro-survival role for Aup1p-directed mitophagy. In this study (reviewed in [7]), cells were simply followed during stationary-phase maintenance in lactate. In the presence of Aup1p, mitochondria are degraded beginning at ~3 days, evidenced by the loss of the mitochondrial protein aconitase and the appearance of a mito-targeted GFP in the vacuole. That aconitase is degraded in the vacuole is confirmed by a null mutation in the vacuolar protease *pep4*Δ, used similarly in the studies above [14,20]. *AUP1* encodes a mitochondrial phosphatase. Studies with Aup1p-HA show that it is located in the mitochondrial intermembrane space, tethered loosely to one of the membranes, but protected from an externally applied protease unless the outer membrane is breached using hypotonic lysis. Like *UTH1*, *AUP1* is not required for starvation-induced autophagic activity, and is not therefore a crypto-*ATG* gene. Interestingly, Aup1p-GFP ends up in the cytoplasm during mitophagy, while at least two mitochondrial proteins end up in the vacuole. However, Aup1p-HA expression was reduced similarly to aconitase but whether its remnants were cytoplasmic is not reported. To understand how Aup1p ends up in the cytoplasm, Abeliovich's [7] simplest proposition is that Aup1p is segregated in bits of mitochondria that remain viable after it signals, while the damaged compartment is cut away by fission and targeted for degradation. How the protein ends up in the cytoplasm is not clear; perhaps newly synthesised Aup1-GFP cannot be loaded into the mitochondria because of a loading defect, due to changes in the properties of mitochondria (loss of membrane potential, or transport proteins). These studies indicate that mitophagy is specific, in that Aup1p marks mitochondria for degradation in stationary phase cells, a physiologically relevant circumstance. How specific the process of mitochondrial elimination is in relation to that of other organelles is not yet clear. It will be illuminating to see

EM images of the cells, alongside immunogold labeling of Aup1p-GFP during the autophagy process. Mutagenesis studies are also awaited to define whether the mitophagic signaling by Aup1p depends on its phosphatase activity and its mitochondrial localisation.

The notion of selective mitophagy in yeast is further strengthened by the study of Nowikovsky et al. [24]. Mdm38p/Mkh1p is a mitochondrial inner membrane protein that is an essential component of the mitochondrial K^+/H^+ exchange system. A conditional deletion of *MDM38* causes mitochondria to swell, lose $\Delta\Psi$m, fragment, and reduce cell growth on non-fermentable substrates (petite phenotype). On galactose medium, Mdm38p depletion led to fragmentation of mitochondria, which ended up proximate to a vacuole showing many surface indentations, as described above [14,21,25] followed by microautophagy. Again, no evidence for autophagosomes, or macroautophagy, was detected. That mitochondria ended up being digested in the vacuole was assayed by using a pH-sensitive biosensor consisting of a mitochondrially-targeted GFP-RFP tandem protein. In the vacuole, GFP fluorescence fades in the acidic environment whereas the RFP fluorescence is stable. Amazingly, this type of mitophagy was prevented by nigericin (an inhibitor of the K^+/H^+ exchanger, a well-characterised transporter), indicating that K^+/H^+ exchange activity is primarily responsible for mediating the effects. Although these data correlate with loss of $\Delta\Psi$m as a signal for mitophagy, when the pro-fission gene Dynamin 1 (*DNM1*) was also deleted (yielding a Δ*MDM38*Δ*DMN1* strain), mitochondria still had reduced $\Delta\Psi$m, but did not now undergo autophagy, implicating fission in the mitophagic process. Mitochondria thus retained in the cytoplasm were not fragmented but had no cristae, and were still swollen. Nowikovsky et al. [24] mention that whilst deletion of the fission gene *DNM1* inhibited Δ*MDM38*-mediated fission and mitophagy, overexpression of the pro-fusion proteins Fzo1p or Mgm1p did not inhibit Δ*MDM38*-mediated mitophagy but whether these genes were able to restore a mitochondrial network was not discussed. Interestingly, it is reported that despite the mitophagy due to the lack of Mdm38p, log phase cells growing in galactose survived normally but in late stationary phase, these cells were more prone to die. Whether this death is related to autophagy and/or to K^+/H^+ transporter malfunction is not reported.

Hence, out of the four genes that regulate mitophagy mentioned thus far, two induce mitophagy with opposing effects on survival, and two lead to suppression of autophagy, each under its own peculiar set of conditions. The one feature these mechanisms have in common are the profiles of microautophagy through which mitophagy apparently occurs. More work will reveal how these pathways converge.

The suggestion that following Aup1p signaling, a damaged portion of the mitochondria is targeted for removal, fits work trying to elucidate whether mitochondrial membrane potential deficiency or another intra-mitochondrial signaling mechanism is a marker for mitophagy in yeast. Priault et al. [25] aimed to collapse $\Delta\Psi$m to examine whether this is sufficient to cause autophagy and/or mitophagy. Yeast grown on fermentable glucose was switched to O_2-depleted medium at 37 °C in a strain Δ*FMC1*ts which leads to accumulation of aggregates of dysfunctional F0F1 ATPase and collapse of $\Delta\Psi$m, because glycolytic ATP cannot be used for $\Delta\Psi$m maintenance. EM studies found that macro- and microautophagy were triggered under these conditions, but not in WT or in oxygenated Δ*FMC1* cells. Autophagy was activated under this treatment and was *ATG*5-dependent, measured using the cytoplasmic ALP assay, but the activity was not as strongly induced as that induced by rapamycin. Perhaps specific mitophagy does not require a huge amount of autophagy. Selective mitophagy was evidenced by the finding that two integral membrane proteins of the outer (porin) and inner (F1α or Atp1p) membranes were degraded but not those of other organelles (late Golgi membrane, cytosolic, ER membrane, nuclear pore). Reduced $\Delta\Psi$m was also induced by several independent means without anaerobiosis, each showing increased rates of ALP maturation that was reversed, where tested, by disabling autophagy through Δ*ATG5*. Importantly, ATP increased in cells under each of the conditions that lowered $\Delta\Psi$m and activated autophagy, although no measurement was carried out in the Δ*ATG*5 strain to demonstrate that autophagy is the source of the ATP. What was the impact on viability? This was only reported for the Δ*FMC1*/anaerobic condition, where death commitment point was advanced in the mutant cells. Since ATP increased while viability decreased, the authors propose that the lack of mitochondrial biogenesis is source of autophagy, implying a feedback between mitochondrial energy/functional status, turnover, and autophagy. How this feedback might be regulated is an open question.

While it was shown that mitochondrial deficits trigger autophagy under non-starvation conditions [26], Zhang et al. [27] explored the role of autophagy in mitochondrial maintenance. Using Δ*ATG*1/Δ*ATG6*/Δ*ATG8*/Δ*ATG12* single mutant strains that would disable all forms of autophagy, and yeast grown in glucose to stationary phase, nulls in each of the autophagy genes preserved mitochondrial proteins significantly, suggesting that autophagy was removing mitochondria. Additionally, autophagy-deficient *ATG* strains exhibited defects in mitochondria-dependent growth with an increased cell population at the G1 phase of the cell cycle. All the mutant strains also exhibited growth defects and lower oxygen consumption rates in non-fermentable medium, which supports the idea of compromised mitochondrial functions. Consequently, the mutant cells displayed lower $\Delta\Psi$m, reduced mitochondrial electron transport chain activities, higher levels of reactive oxygen species (ROS), and higher mitochondrial mutation rates. Together, these results provide evidence that autophagy plays a significant role in mitochondrial maintenance and that an autophagy defect compromises mitochondrial and cell functions. This supports a feedback relationship between autophagy and mitochondria, which would need to be untangled to examine cause and effect. As the authors suggest, it is possible that each of the *ATG* genes has additional roles outside of autophagy. One testable possibility is that the role of autophagy has less to do with mitochondrial elimination per se but rather serves to elevate ATP [28], thereby promoting survival and conditions where mitophagy can flourish alongside mitochondrial biogenesis.

We have seen that several different “types” of autophagy may be responsible for removal of mitochondria to the vacuole. Kanki and Klionsky [29] investigated whether mitophagy is selective using a battery of *ATG* gene knockouts that, in various combinations, reveal whether mitophagy uses the same machinery as the cytoplasmic to vacuole targeting (Cvt) pathway, pexophagy, or a non-selective pathway. To assay mitophagy, a novel assay was developed based on the finding that free GFP can be retrieved and detected on immunoblots after it is cleaved in the vacuole off two mitochondrial GFP-fusion proteins. Though the previous study used Aup1p-GFP to localise it to the cytoplasm during mitophagy, this is the first report of GFP being retrieved intact from degradation in the vacuole. For this study log-phase cells were switched from a lactate medium to a glucose medium lacking nitrogen as the switch to glucose alone did not provoke any mitophagy in the time frame of this study. Moreover, only a small amount of mitophagy was observed when cells were switched to a lactate medium lacking nitrogen (as in the study on *UTH1*), although autophagy was as strongly provoked during starvation in lactate as in glucose. Hence, starvation in a fermentative substrate is required for the activity they have measured, thus demonstrating again that mitophagy is not simply a result of non-selective autophagy. Under these conditions, deletion of genes (*ATG11*, *ATG20*, *ATG24*) that did not inhibit

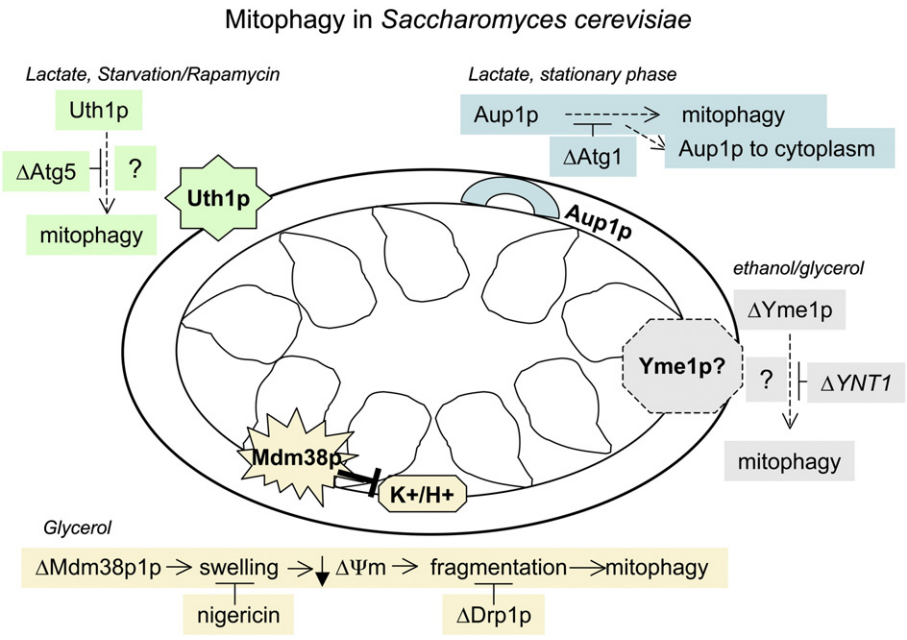

Fig. 1. Genes and processes that mediate mitophagy in *S. cerevisiae*. Mitochondria are depicted with cristae contacting the inner membrane at tightly restricted contact sites, based on serial reconstruction of electron tomography. Figure gives location of each protein (except Yme1p, whose location is unknown). A brief overview of mechanisms that lead to mitophagy is given. See text for details.

macroautophagy, inhibited mitophagy (as well as pexophagy and Cvt), whereas deletion of genes (*ATG17*, *ATG29*, *ATG31*) that spared Cvt, blocked mitophagy (as well as pexophagy and macroautophagy). These data show that mitophagy is a selective process, but the subtype of autophagy involved is not known. Interestingly, the same assay was run to investigate whether mitophagy occurred after treatments that reduce ΔΨm. However, when cells were treated with oligomycin or after Mdm38p knock down very little free GFP was generated relative to the total amount of fusion protein expressed, in contrast to the conditions described above [24,25]. This finding suggests that the majority of mitochondrial degradation observed during starvation using a fermentative substrate (where the amount of free GFP generated was a high proportion of total GFP-fusion protein) was not mediated by a reduction in ΔΨm (although the authors' interpretation is that reduced ΔΨm does coincide with mitophagy).

2.1. Conclusion

1) Is there a specific removal of mitochondria by autophagy or is it non-selective or inadvertent? In yeast, mitophagy can be specific and selective (as defined by a subset of *ATG* genes being involved), though this depends on growth and assay conditions. It seems that mitophagy is mediated primarily by microautophagy rather than through autophagosomes (though this mitophagy is blocked by mutating the general genes *ATG1*, *ATG8*, *ATG9*). 2) What are the signals that drive this process? There are at least four genes (summarised in Fig. 1) that specify mitophagy depending again on conditions; whether they lie on a common generic pathway, and where they converge, is not known. A reduction in ΔΨm can provoke mitophagy, but this is not essential. 3) Does removal of mitochondria lead to increased or decreased cell viability? Because the removal of mitochondria cannot be separated from autophagy, which by itself impacts on cell survival, there is no good answer to this question at present. The reasons why Δ*UTH1* decreases rapamycin-induced inhibition of growth/viability while Δ*AUP1* enhances its toxicity could be simply due to differences in assay conditions (stress during acute starvation compared to physiological conditions), or to some fundamental process to do with the roles of each protein.

3. Mitophagy in mammalian cells

While loss of ΔΨm may not be a necessary signal for mitophagy in yeast, in mammalian cells loss of ΔΨm appears to be a common feature of mitophagy (Fig. 2). Lemasters and colleagues have used dyes and confocal techniques to image mPT, ΔΨm and mitochondrial engulfment by GFP-LC3-positive autophagosomes during starvation [30,31]. When hepatocytes were starved and treated with glucagon, thereby activating autophagy, mitochondria underwent cyclosporine-inhibitable mPT [32] prior to engulfment, suggesting that damaged mitochondria were targets for autophagosomal removal. Quantitatively, however, it did not appear that more than a handful of mitochondria were removed. When mitochondria were photo-damaged deliberately, GFP-LC3-positive structures appeared in the damage area, suggesting that a signal is produced in this area. One possible source is ROS that activates the protease Atg4B, the enzyme that cleaves the c-terminus of LC3-I, an essential precursor to conversion of LC3I to LC3II [11,33]; since the damaged mitochondria are no longer labeled upon loss of ΔΨm, however, one cannot see them within the GFP-LC3-positive autophagosomes. However, the authors have also used dyes that are not removed from mitochondria upon loss of ΔΨm to demonstrate colocalisation of mitochondria in acidic compartments [31], suggesting that mitochondria reach the auto/lysosomes. These data do not indicate whether this form of mitophagy is a selective process.

The theme that a damaged mitochondrion signals the autophagic machinery to remove it – perhaps via ROS production [11], or, in the case of apoptosis, via Bif-1/endophilinB's triple function of binding to Bax at the mitochondria, fragmenting mitochondria, and activating autophagy [34–36] – has support from other data, but not all concur. The fission/fusion machinery of mitochondria has been linked to autophagy in several recent papers but whether mitophagy occurs as a result is still open to question. Fusion and fission of mitochondria are dependent on several genes (reviewed

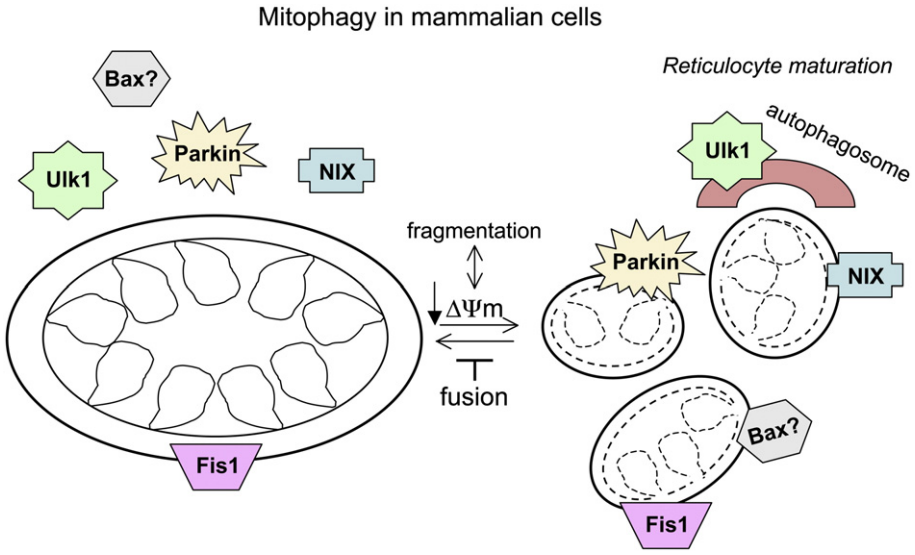

Fig. 2. Genes and processes that mediate mitophagy in mammalian cells. Figure gives location of each protein before and after fragmentation/loss of ΔΨm, the main driving forces for mitophagy in these systems. Bax is included based on the fact that it drives mitophagy in cells in which apoptosis has been activated but caspases are inhibited. See text for details.

in [37]). There are two pro-fusion genes, mitofusin (*mfn*) 1 and 2, on the outer mitochondrial membrane feeding in to the regulation of Optic atrophy 1 (*OPA*1) inside the mitochondria. The fission machinery is comprised of dynamin related protein 1 (*Drp*1) which is recruited to the mitochondria to bind to Fission 1(*Fis*1), a resident mitochondrial outer membrane protein. Anyone observing mitochondria in living cells will have noted the dynamic nature of mitochondrial fission and fusion, as well as the high sensitivity of these events to various signals, not least amongst them the finding that mitochondria fragment in conjunction with Bax-mediated apoptosis [38,39].

Parone et al. [40] studied mitochondria in HeLa cells after knocking down *DRP*1, and found that mitochondria lost respiratory function, ATP concentration dropped, cell cycle was reduced, and mtDNA was lost. Although LC3II was elevated, possibly as a result of increased ROS [33], there were few incidences of colocalisation of mitochondria and autophagic vacuoles demarcated by GFP-LC3. This may be due to the absence of lysosomal inhibitors to stabilise the autophagosomes (see [41] below) but taken at face value, the data indicate that mitophagy in Drp-knockdown cells was suppressed. These findings are in agreement with Arnoult et al. [42] that mitochondria disappear through excess fission driven by Drp1 over-expression.

Twig et al. [41,43] tested the role of fusion and fission in mitophagy by following several individual mitochondria through rounds of fission and fusion. They found that mitochondria that had undergone a round of fusion followed by fission divided into two subpopulations: those that re-fuse and those that never re-fuse. The re-fusing mitochondria regained ΔΨm before fusion but the non-fusing ones had reduced ΔΨm and some ended up in GFP-LC3-positive autophagosomes. Tracking these events individually using dyes demonstrated preferential segregation of the depolarized mitochondria into LC3-positive structures, using a brief exposure to the lysosomal inhibitors PepstatinA and E64D at the end of the tracking period to trap the mitochondria in the autophagosomes for observation. Sequestration of the mitochondria by autophagosomes occurred long enough after depolarization to dissociate the act of depolarization per se from mitophagy. Because depolarization occurred well before autophagy, and was maintained, the authors sought an irreversible event that prevented re-fusion, focusing on *OPA*1. When OPA1 was over-expressed, a reduced fraction of mitochondria were found in autophagosomes, implying loss of OPA1 as the signal. Likewise, knockdown of Fis1 or Drp1 reduced autophagy. Here, mitophagy occurs through macroautophagy and the process is clearly selective in terms of which mitochondria become engulfed, but whether the autophagosomes contain only mitochondria without surrounding cytoplasm is not reported. In the absence of fission, defective mitochondria accumulated, and insulin secretion in response to glucose was reduced. Whether the same changes occur in the absence of autophagy (rather than fission) was not reported. Interestingly, it is noted that autophagy-deficient *Atg5*$^{-/-}$ MEF have less mitochondrial respiration than *Atg5*$^{+/+}$ MEF (not unlike the conclusion in [27]) but whether this is a general feature of autophagy-deficient cells is not clear.

Another link between mitochondrial fission/damage and mitophagy comes from recent work from Youle's lab [44], involving Parkin. Parkin is an ubiquitin ligase encoded by *Park*2, loss of function mutations in which are associated with a familial juvenile form of Parkinson's disease [45]. A link between Parkin, Pink1 (a Parkinson's disease-associated protein kinase that is resident in mitochondria [46,47]) and mitochondrial fission/fusion has been noted [48–51]. In *Drosophila*, Parkin knockout was associated with mitochondrial swelling which occurred prior to degeneration of neurons and muscles (reviewed in [52]). Narendra et al. [44] found that when they reduced ΔΨm with uncouplers (CCCP) (which feeds back on mitochondrial fission due to recruitment of Drp1 through its dephosphorylation by calcineurin [53]) mitochondria fragmented and Parkin relocalised from the cytoplasm to the mitochondria. Within 48 h, mitochondrial markers Tom20, cytochrome *c*, and TRAP1 (which binds Pink1) were lost. By EM, no mitochondria remained whilst numerous lysosomes appeared in the cytoplasm. The specificity of mitochondrial elimination was remarkable since even peroxisomes were left intact. Evidence for autophagic removal of mitochondria is that mitochondria appeared within GFP-LC3-positive structures, and the percentage of cells with no detectable mitochondria was reduced after 24 h from ~30% in wt cells to ~3% in wt cells treated with the lysosomal inhibitor bafilomycin A1 or in *Atg5*$^{-/-}$ MEFs. Interestingly, the percent of nonapoptotic live cells was undiminished in a glucose-containing medium but in a galactose medium, cells without mitochondria died within 3 days. The autophagic mechanisms employed are not yet reported, nor is it known whether endogenous Parkin regulates mitochondrial morphology and function in the same manner. It is also of interest to see what other types of stimuli lead to Parkin recruitment, and whether Drp1 and calcineurin are involved in the fragmentation process [54].

Investigating whether mitophagy is induced by Parkin in yeast may also be of interest. Taken together, this paper demonstrates that a specific mammalian protein can signal mitophagy. The implications of Parkin mutations found in Parkinson's disease on mitochondrial mitophagy can now be examined in model systems (*Drosophila*, *Caenorhabditis elegans*) where little is known about mitophagy at present.

Although the data suggest that mitochondrial fission is requisite for mitophagy, Gomes et al. [54] found that overexpression of a form of Fis1 (Fisα1) mutated in its ability to cause fission was at least as efficacious as wt Fis1 in promoting mitochondrial autophagy. They conclude that loss of ΔΨm is the common feature enabling the two Fis1 forms to induce mitophagy. They also noted lack of complete autophagy of all damaged mitochondria, a common feature of many of the studies in mammalian systems. There are cases where the entire cohort of mitochondria seems to disappear given time, however. We have noted that mitochondria are fragmented and have lost ΔΨm when they disappear from neurons induced to undergo apoptosis but prevented from dying using caspase inhibitors [26,55,56]. Removal of large numbers of mitochondria including mitochondrial DNA was partially prevented with the Bafilomycin A1 (which prevents lysosomal proteolytic activity by causing lumen alkalinisation), indicating an autophagy-mediated event.

Lens, erythrocytes, and sperm are ideal systems to study mitochondrial elimination since these organs get rid of mitochondria as part of their developmental process [57,58]. Matsui et al. [57] examined lens and erythrocytes in *ATG5*$^{-/-}$ mice and could not find evidence for a defect in the elimination of mitochondria from these two structures. However, recent work has implicated NIX, a non-canonical BH3 member of the Bcl2 family of proteins [59–62], and ULK1 [63], a kinase homologue of *ATG1* that is expressed on autophagosomal membranes [64,65] and is implicated in autophagy in mammalian cells [66].

Kundu et al. [63] show that Ulk1, a serine threonine kinase, is a critical regulator of mitochondrial and ribosomal clearance during the final stages of erythroid maturation. Thus, many more reticulocytes with normal/numerous mitochondria that retained normal membrane potential accumulated in *ULK1* knockout mice. In maturing reticulocytes in vitro, *ULK1*$^{-/-}$ cells were less efficient than wt cells in clearing the mitochondria (based on prestaining with a mitochondrial dye) but addition of the uncoupler CCCP led to clearing with similar efficiency to clearing mitochondria in untreated wt reticulocytes. The process was not selective for mitochondria, however, as ribosomes were also cleared, although CCCP-induced clearance of ribosomes was not reported upon. As with all the other studies that show specificity, expression of Ulk1p was not essential for induction of macroautophagy in response to nutrient deprivation or for survival of newborn mice. It should be noted that eventually, *ULK1*$^{-/-}$ cells cleared their organelles. The authors implicate lipid peroxidation [67] and NIX [61,62] in this process. Together, these data suggest that the *ATG*1 homologue ULK1 is a component of the selective autophagy machinery that leads to the elimination of organelles in erythroid cells. Unlike *ATG1*, it is not an essential mechanistic component of mitophagy in erythrocyte maturation.

NIX is a homologue of BNIP3, an inducible BH3 family member that is regulated by hypoxia [68]. Schweers et al. [62] demonstrated that mitochondrial clearance is defective in *NIX*$^{-/-}$ reticulocytes. The function of Nix is specific because no other Bcl2-family protein alone or in combination could replace it (but see [61] below). The participation of mPT in the signaling was eliminated because drugs that inhibit mPT did not prevent mitochondrial removal in wt reticulocytes. Also, *NIX*$^{-/-}$ mitochondria were robustly filled with a ΔΨm-dependent mitochondrial dye. By EM, mitochondria in *NIX*$^{-/-}$ reticulocytes appeared normal but mostly clustered around the outside of autophagosomal membranes (reminiscent, perhaps, of profiles of mitochondria touching the yeast vacuole). In wt reticulocytes, many autophagosomes contained mitochondrial remnants, indicating autophago/lysosomal maturation. Remarkably, however, most autophagosomes loaded with mitochondria seemed to be exocytosed. Hence, NIX is required for the selective elimination of mitochondria through autophagy during reticulocytes maturation but the process of this elimination is not canonical autophagy.

Sandoval et al. [61] studied the same phenomenon and performed some perturbation studies additionally. Unlike the case with ULK1, ribosomes were cleared normally in *NIX*$^{-/-}$ reticulocytes but mitochondria failed to enter the autophagosomes. Remarkably, as in the case of ULK1, autophagy of mitochondria was restored in *NIX*$^{-/-}$ reticulocytes simply by uncoupling (and presumably depolarizing and/or fragmenting mitochondria) with FCCP or depolarizing (and perhaps fragmenting) mitochondria with the potent BH3 mimetic compounds ABT-737 (perhaps implying Beclin release from Bcl2 and induction of autophagy [69]). However, Bim, a BH3-only protein whose activities are mimicked by ABT-737, did not trigger mitophagy. Interestingly, K562 cells that can be developed into the erythrocytes, also depended on NIX to clear mitochondria during maturation and this occurred in an *ATG7*-dependent manner (see [66] Supplementary Fig. 9), implying that NIX involvement in mitophagy is more widespread. *NIX*−/− reticulocytes retaining mitochondria were more prone to active apoptosis and phagocytosis by macrophages, leading to anaemia, suggesting that lack of removal of mitochondria (or NIX) is deleterious to the cells. A remaining conundrum is the finding that *ATG5*$^{-/-}$ reticulocytes from neonate animals clear mitochondria normally but knockdown of *ATG7* reduced clearance in K562 cells. Perhaps it is all a question of timing, as in the case of ULK1, since neither NIX nor ATG7 knockdown completely inhibited mitochondrial loss up to 6 days. Taken together, NIX appears to be used physiologically to initiate mitophagy through its reduction in ΔΨm, since lack of NIX can be overcome by irreversibly depolarizing the mitochondria. This does not exclude the possibility that it may still fulfil other functions in mitophagy.

Interestingly, there is no mention of exocytosis of mitochondria-laden autophagosomes by Sandoval et al. [61], as observed by Schweers et al. [62]. While autophagy can eliminate mitochondria, it may not be the only way of getting rid of damaged mitochondria. For example, Lyamzaev et al. [70] inhibited respiration and uncoupled oxidative phosphorylation (using the uncoupler DNP or FCCP and the inhibitors antimycin A or myxothiazol) for 48 h and found cells devoid of mitochondria. They could not observe colocalised mitochondria within monodansylcadaverine-positive vesicles by imaging (but did not report on the consequences of inhibiting lysosomal proteases or knocking down autophagy genes). Nevertheless, by imaging an irreversible mitochondrial dye, they find large membrane-bound clusters of mitochondrial remnants ("mitoptotic bodies") that are extruded from cells. Lyamzaev et al. name this process mitoptosis. Whether these mitoptotic bodies are autophagosomes requires further examination.

Finally, in addition to genes, signaling by kinases has also been reported to mediate mitophagy in mammalian cells. 1-Methyl-4-Phenylpyridinium (MPP+) is a mitochondrial toxin that mediates the toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which causes Parkinson's disease symptoms in vivo. When Zhu et al. [71] added MPP+ to neuroblastoma cells in culture this induced numerous autophagic vacuoles. This activity was accompanied by loss of two mitochondria proteins (pyruvate dehydrogenase E2 and a p110 protein), loss of which was blocked by inhibitors of MEK/ERK signaling pathway, with ERK2 being localised in mitochondria. The inhibitors also blocked LC3II production, vacuole number, and reversed about 50% of mitochondrial loss, detected by immunochemistry. Decreases in ΔΨm and ultrastructural damage were not prevented, implying that the ERK signaling acts downstream of mitochondrial injury in stimulating autophagy. Autophagy was also activated in primary dopaminergic

neurons, in keeping with other work that associates oxidative damage of mitochondria in neurons with autophagy and cell death [72]. A further trigger of mitophagy was reported in human cells in response to coenzyme Q deficiency [73]. Identifying a kinase that mediates mitophagy may open the way to elucidating downstream targets in the mitophagic pathway.

3.1. Conclusion

1) Is there specific removal of mitochondria by autophagy or is it non-selective or inadvertent? As in yeast, in mammalian cells, mitophagy can be selective and specific. It seems that mitophagy is mediated primarily through autophagosomes, but perhaps this finding is because of the tools being used, namely GFP-LC3. It is clear that mitochondrial elimination is mostly dependent on autophagic genes but whether digestion of mitochondrial contents always occurs inside lysosomes is unclear. 2) What are the signals that drive this process? As summarised in Fig. 2, NIX and ULK1 (in vivo) and Parkin are three proteins that can signal specific mitophagy in mammalian cells. When these are deficient, a reduction in $\Delta\Psi m$ can re-install mitophagy. Consistent with this conclusion, the Fisα1 mutant induces autophagy and some mitophagy by de-energising the mitochondria and not through mitochondrial fission. Whether energised mitochondria can be autophagocytosed independently is still open to question. 3) Does removal of mitochondria lead to increased or decreased cell viability? In reticulocytes, removal of mitochondria by autophagy produces erythrocytes. Although erythrocytes have to survive on glycolysis, and have a limited life span, their relative longevity of 120 days attests to the fact that mitophagy is not in itself deleterious, despite the fact that mitochondria lose $\Delta\Psi m$ prior to their removal. Rather, diseases may develop as a result of deficient mitophagy, as usually this leaves behind damaged mitochondria. The notion of pinching off defective parts of mitochondria and deleting them by autophagy whilst maintaining functional parts (and perhaps using the energy provided by autophagy as a stop gap to prevent cell death) is an attractive proposition. An interesting question in relation to diseases is whether catching cells during partial mitophagy – by arresting autophagy – is early enough to repair the remaining mitochondria so that they can be returned to normal function.

Altogether, a strong case for mitophagy as a selective process can be made although the detailed mechanisms are not completely resolved. A more thorough genetic approach awaits studies in *Drosophila* and *C. elegans*, where autophagy is well studied but genetic control over mitophagy is yet to be reported.

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