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Mechanisms, pathophysiological roles and methods for analyzing mitophagy – recent insights

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Abstract

In 2012, we briefly summarized the mechanisms, pathophysiological roles and methods for analyzing mitophagy. As then, the mitophagy field has continued to grow rapidly, and many new molecular mechanisms regulating mitophagy and molecular tools for monitoring mitophagy have been discovered and developed. Therefore, the purpose of this review is to update information regarding these advances in mitophagy while focusing on basic molecular mechanisms of mitophagy in different organisms and its pathophysiological roles. We also discuss the advantage and limitations of current methods to monitor and quantify mitophagy in cultured cells and *in vivo* mouse tissues.

Keywords

autophagy; mitophagy; Mito-QC; mitoTimer; mt-Keima; Parkin

Introduction

The term ‘autophagy’ was coined by De Duve and Wattiaux in 1966 (De Duve and Wattiaux, 1966), and it is a combination of the Greek words ‘auto’ and ‘phagy’ meaning self and eat, respectively. Autophagy was first discovered in the liver (De Duve and Wattiaux, 1966; Yang and Klionsky, 2010). However, research in the autophagy field was once stagnant before the molecular mechanisms for autophagy were revealed due to the complexity of autophagy regulation in mammals. It was not surprising that in 2016, the Nobel Prize in Physiology or Medicine was awarded to Yoshinori Ohsumi for his discoveries regarding roles of autophagy-related (Atg) genes in the autophagy regulation in yeast. Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved and genetically programmed catabolic process that degrades cellular proteins and damaged or excess organelles through the formation of a double-membrane autophagosome. Double-membrane autophagosomes engulf these proteins and organelles in the cytoplasm and then

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fuse with lysosomes to form autolysosomes where the enwrapped contents are degraded via lysosome proteases (Mizushima, 2007; Nakatogawa et al., 2009; Yang and Klionsky, 2010; Parzych and Klionsky, 2014). The basic autophagy machinery and molecular signaling pathways that regulate autophagosome formation and autophagic flux have been extensively reviewed in many recent outstanding review articles, which will not be discussed here.

Autophagy can be both a non-selective and selective process. Non-selective autophagy is a protective process that provides the cell with biomolecule fuels in response to starvation to produce energy. During starvation conditions, autophagy degrades cytoplasmic proteins and organelles and provides amino acids that can be used in gluconeogenesis or to produce ATP by entering the Krebs cycle (Rabinowitz and White, 2010). Degradation of lipids by autophagy produces free fatty acids that can be used to produce energy by β-oxidation in the mitochondria (Singh et al., 2009), although some free fatty acids can also be restored to form lipid droplets via diacylglycerol acyltransferase, which likely reduces lipotoxicity (Nguyen et al., 2017). Selective autophagy removes damaged organelles and protein aggregates using specific receptors, and it can occur in both nutrient-rich and poor conditions (Reggiori et al., 2012a,b). Examples of selective autophagy are reticulophagy or ERphagy for endoplasmic reticulum (ER) degradation (Hamasaki et al., 2005; Yang et al., 2016), ribophagy for degradation of ribosomes (Kraft et al., 2008), xenophagy for degradation of pathogens (Levine, 2005), pexophagy for degradation of peroxisomes (Dunn et al., 2005), lipophagy for degradation of lipids (Singh et al., 2009), aggrephagy for degradation of protein aggregates (Yamamoto and Simonsen, 2011), and mitophagy for the degradation of damaged mitochondria (Lemasters, 2005; Narendra et al., 2008).

Mitochondria were observed in lysosomes as early as 1962 by Ashford and Porter (Ashford and Porter, 1962), however, the term ‘mitophagy’ was created by John Lemasters in 2005 (Lemasters, 2005). Mechanisms of mitophagy induction have been well studied in yeast and in mammalian cells. Recent studies have also begun evaluating mitophagy mechanisms in *Caenorhabditis elegans* (*C. elegans*). In 2012, we briefly summarized the mechanisms, pathophysiological roles and methods for analyzing mitophagy (Ding and Yin 2012). As then, there has been rapid progress in the mitophagy field over the past several years, which has led to a better understanding of the molecular mechanisms involved in mitophagy, the pathophysiological roles of mitophagy in development and disease, and also the appropriate analytical tools and methods for studying and quantifying mitophagy. In this review, we aimed to update the progress of the above aspects in mitophagy.

Mechanisms of mitophagy

Mitophagy in yeast

The first evidence that mitophagy is genetically controlled in yeast was reported by Camougrand’s group. They identified that Uth1p, a protein originally identified from a genetic screening for the regulation of yeast life span (Austriaco, 1996), is required for the autophagic degradation of mitochondria (Kissova et al., 2004). Uth1p is a member of the so-called ‘SUN’ family, which is mainly localized in the mitochondrial outer membrane and is required for the removal of excess mitochondria during starvation (Kissova et al., 2004). In addition to Uth1p, Aup1p, one of a family of protein phosphatase homologs that localizes to

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the mitochondrial intermembrane space, is also required for efficient mitophagy in stationary phase cells (Tal et al., 2007). Interestingly, neither Uth1p nor Aup1p is required for starvation-induced autophagy, suggesting their specificity for mitophagy. Deletion of Mdm38, a mitochondrial inner membrane protein that regulates the mitochondrial K^+/H^+ exchange system, leads to mitochondrial depolarization and fragmentation followed by degradation in the vacuole (Nowikovsky et al., 2007). Blocking fission by the deletion of a pro-fission gene *dnm1* prevented mitochondrial fragmentation and mitophagy (Abeliovich et al., 2013). Thus, it appears that mitochondrial fragmentation facilitates mitophagy, a phenomenon that is also observed in mammalian cells (see below). However, some other studies have also reported that dnm1/Drp1 is not required for mitophagy in yeast (Mendl et al., 2011; Yamashita et al., 2016). It seems that damaged mitochondria themselves could be sufficient to trigger mitophagy because there were no externally added drugs or physiological stresses placed on the mdm38-deficeint cells. Carbonyl cyanide m-chlorophenylhydrazone (CCCP), a common mitochondrial uncoupler, did not induce mitophagy in wild type yeast (Wang et al., 2011), and instead inhibited mitophagy in yeast (Padman et al., 2013). Therefore, although deletion of Mdm38 leads to mitochondrial depolarization followed by mitophagy, whether depolarization is truly required for mitophagy may require further investigation.

Selective degradation of mitochondria by mitophagy in yeast requires the mitochondrial receptor Atg32, which is a 59-kDa outer mitochondrial membrane protein that recruits autophagosomes to mitochondria via its interaction with the autophagosome membrane protein Atg8 and the selective autophagy receptor Atg11 (Kanki et al., 2009a,b; Okamoto et al., 2009a,b; Kondo-Okamoto et al., 2012). Atg32 has an Atg8-family interacting motif (AIM), which is equivalent to an LIR (LC3 interacting region) and allows for its interaction with the ubiquitin-like and phosphatidylethanolamine (PE) conjugated autophagosome membrane protein Atg8 (Okamoto et al., 2009a,b; Kondo-Okamoto et al., 2012). Atg11 is localized to the cytosol and acts as a scaffold protein in yeast, and Atg32 interaction with Atg11 results in recruitment of other autophagy proteins to mitochondria to form an autophagosome (Yorimitsu et al., 2005; Kanki et al., 2009b; Okamoto et al., 2009a,b). Atg32 has a mammalian homolog BCL2-like 13 (BCL2L13/Bcl-rambo) (Murakawa et al., 2015; Otsu et al., 2015), and Atg8 has a mammalian homolog microtubule light chain 3 (LC3) protein. It is currently unknown if Atg11 has a mammalian homolog.

Atg32 is required for mitophagy in yeast, but it is not required for non-selective autophagy. Two independent groups discovered the requirement for Atg32 in yeast mitophagy using genome-wide screens in 2009 (Kanki et al., 2009b; Okamoto et al., 2009a,b). Involvement of Atg32 in yeast mitophagy was confirmed using yeast strains lacking Atg32, which were deficient in mitophagy (Okamoto et al., 2009a,b; Kurihara et al., 2012). Atg32-deficient yeast were unable to degrade excess mitochondria during nitrogen starvation, which resulted in increased levels of defective mitochondria and production of harmful reactive oxygen species (ROS) compared to mitophagy-inducing wild-type yeast cells (Kurihara et al., 2012).

Unlike mammalian mitophagy, there is no evidence of Atg32-induced mitophagy regulation by mitochondrial depolarization or ubiquitin tagging. However, Ser65-phosphorylated ubiquitin, which is an important component of Parkin-induced mammalian mitophagy

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regulation (further discussed below), was found in yeast cells during oxidative stress (Swaney et al., 2015). Atg32-induced mitophagy is regulated by phosphorylation at Ser-114 and Ser-119, which promotes interaction of Atg32 with Atg11 (Aoki et al., 2011) and Atg8 (Farre et al., 2013). Casein kinase 2 (CK2) directly phosphorylates Atg32 and CK2-mutated yeast strains were unable to induce mitophagy, suggesting that CK2 is necessary for mitophagy induction (Kanki et al., 2013). Deletion of high osmolality glycerol (Hog1) or its upstream MAPK Pbs2 also caused inhibition of Atg32 phosphorylation and subsequent mitophagy (Aoki et al., 2011; Mao et al., 2011), but the relationship between Hog1 and Atg32 phosphorylation is not well understood because Hog1 does not directly interact with Atg32 (Aoki et al., 2011). Therefore, another unidentified pathway is likely required for Atg32 phosphorylation via Hog1. Hog1 was suggested to act upstream of CK2 signaling (Kanki et al., 2013). It is possible that Hog1 mediates the interaction of CK2 with Atg32 to initiate its phosphorylation, but this has not been investigated. The PKC MAPK Slt2 may also have a role in mitophagy regulation in yeast by recruiting mitochondria to the pre-autophagosomal structure (PAS) (Mao et al., 2011). However, the exact role for Slt2 in this pathway requires further investigation. Interestingly, cardiolipin was recently shown to have a role in activation of both Hog1 and Slt2 pathways. Yeast cells deficient in cardiolipin had decreased phosphorylation and activation of both Hog1 and PKC MAPK pathways, resulting in decreased mitophagy. Deletion of mitophagy and autophagy genes further exacerbated growth defects in these cells, and activation of Slt2 phosphorylation restored mitophagy and growth. The mechanism for cardiolipin- induced activation of these MAPK pathways for mitophagy induction is still unknown (Shen et al., 2017). Cardiolipin also plays a role in mammalian mitophagy, which is further discussed below.

ROS production may activate mitophagy in yeast because Atg32 expression increased in respiratory growth conditions, but not in fermentable conditions. However, actual ROS levels were not determined in this study (Okamoto et al., 2009a,b). Treatment with the antioxidant NAC also inhibited mitophagy in yeast by fueling the glutathione pool (Deffieu et al., 2009). The mechanism for how modulation of glutathione activates mitophagy is unclear, but it is possible that decreased glutathione levels may alter mitochondrial function and subsequently lead to mitochondrial damage and mitophagy. Even though oxidative stress plays a role in mitophagy activation in yeast, it may not be required to induce mitophagy because respiration-deficient mutants were still able to induce mitophagy during starvation conditions (Eiyama et al., 2013).

In addition to Atg32, Atg33 was also identified during a yeast genome-wide screen to possibly play a role in yeast mitophagy (Kanki et al., 2009a,b), and deletion of Atg33 in yeast decreased mitophagy (Kanki et al., 2009a,b; Welter et al., 2013). However, the exact role of Atg33 in the mitophagy pathway is still unknown.

A non-conventional pathway was recently discovered for mitochondrial protein degradation in aged yeast cells that is independent of Atg32. In this pathway, TOM70 and TOM71 form a mitochondrial-derived compartment (MDC), which is delivered to the vacuole for degradation of its cargo. Interestingly, this pathway selectively removes inner and outer mitochondrial membrane proteins for degradation while leaving the rest of the mitochondrion intact. This pathway does not require Atg5 for formation of the MDC,

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suggesting that the MDC is different from a typical autophagosome. However, Atg5 is required for delivery of the MDC to the vacuole for degradation, so autophagy machinery is still required for mitochondrial protein degradation via this pathway. Failure to form MDCs in TOM70 and TOM71 mutants exacerbated mitochondrial dysfunction, suggesting it is an important secondary pathway to the conventional mitophagy pathway for maintaining mitochondrial homeostasis (Hughes et al., 2016). The possible molecular events and signalings in yeast mitophagy are summarized in Figure 1.

Mitophagy in mammalian cells

Mammalian mitophagy has features different from the yeast mitophagy pathway that may be specific to mammals. There are several mechanisms for mitophagy induction in mammalian cells including both Parkin-dependent and Parkin-independent pathways. The possible mitophagy pathways are summarized in Figure 2 and are further discussed below.

Mitochondrial fission and fusion—Mitochondria are dynamic organelles that constantly undergo fission and fusion, and mitochondrial fission and fusion maintain mitochondrial function and prevent cell death (Westermann, 2010; van der Bliek et al., 2013). Mitochondrial fission allows for equal division of mitochondria during cell division, and it also helps maintain a healthy mitochondria population by segregating damaged mitochondria from healthy mitochondria. Segregated mitochondria are degraded by mitophagy while healthy mitochondria fuse together, resulting in redistribution of mitochondrial proteins and replacement of damaged mitochondrial DNA (Legros et al., 2002; Twig et al., 2008).

Mitochondrial fusion in mammals is mediated by the dynamin-related GTPases mitofusin 1 (Mfn1), Mfn2 and optic atrophy 1 (Opa1). Mfn1/2 are located on the outer mitochondrial membrane and are responsible for fusion of the outer mitochondrial membranes of adjacent mitochondria, and Opa1 is located on the mitochondrial inner membrane and is responsible for the fusion of adjacent inner mitochondrial membranes (Youle and van der Bliek, 2012). Mfn1 and Mfn2 likely have redundant functions during mitochondrial fusion. However, although mitochondrial fusion occurred when lacking either Mfn1 or Mfn2 alone, mitochondrial fusion rates were lower (Chen et al., 2005), suggesting that Mfn1 and Mfn2 are both still required for efficient mitochondrial fusion. However, Mfn1 may be more important for mitochondrial fusion than Mfn2. For example, mitochondrial fusion was shown to require cooperation between Opa1 and Mfn1, but not Mfn2 (Cipolat et al., 2004). In addition, Mfn1-deficient MEF cells had greater mitochondrial fragmentation than Mfn2-deficient MEFs (Chen et al., 2003, 2005). Individual roles for Mfn1 and Mfn2 during the fusion process require further investigation.

Activity of fusion proteins is regulated by cleavage and several post-translational modifications. Both Mfn1 and Mfn2 are post-translationally regulated by ubiquitination, which leads to their degradation in the proteasome. Degradation of Mfn1 and Mfn2 promotes mitochondrial fission, which is an important step in induction of mitophagy in mammalian cells (Gegg et al., 2010; Tanaka et al., 2010; Youle and Narendra, 2011). Opa1 is regulated by alternative splicing and proteolytic cleavage. Mammalian Opa1 has 8 mRNA

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isoforms generated by alternative splicing (Delettre et al., 2001), which are further cleaved into long and short forms of Opa1 (S- and L-Opa1). Opa1 is known to be cleaved by the large intermembrane space AAA-protease Yme1L under normal conditions (Griparic et al., 2007; Song et al., 2007) or by Oma1 when mitochondria lose membrane potential or have low levels of ATP (Ehses et al., 2009; Head et al., 2009). Inducible cleavage of Opa1 by Oma1 during mitochondrial dysfunction prevents fusion of mitochondria, leading to mitochondrial fragmentation and subsequent removal of damaged mitochondrial fragments as a quality control mechanism (Head et al., 2009).

Similar to mitochondrial fusion, mitochondrial fission requires a dynamin-related GTPase, which is Drp1 in mammals. Drp1 is a cytosolic protein that is recruited to the mitochondrial outer membrane during stress where it initiates mitochondrial fission. Once recruited to the mitochondria, Drp1 forms spirals and undergoes a conformational change using GTP hydrolysis, which causes constriction and initiation of mitochondrial fission (Ingerman et al., 2005; van der Bliek and Payne, 2010; Mears et al., 2011). In addition, mitochondria and ER are associated and in physical contact. Drp1 is localized to this mitochondria-ER contact site, and mitochondria are thought to constrict once they make contact with the ER (Friedman et al., 2011). This mitochondria constriction may be initiated by the ER-associated inverted formin 2 (INF2), which causes actin polymerization at the ER-mitochondria contact site (Korobova et al., 2013). Drp1-mediated constriction is thought to follow the initial constriction mediated by IFN2 for completion of mitochondrial fission (Korobova et al., 2013).

Similar to mitochondrial fusion proteins, the mitochondria fission protein Drp1 is also regulated by posttranslational modifications including ubiquitination, SUMOylation, phosphorylation, O-GlcNAcylation and S-nitrosylation (Elgass et al., 2013; Otera et al., 2013). The mechanism of Drp1 recruitment to mitochondria to induce fission in mammalian cells is not entirely understood, but it may require fission protein 1 (Fis1), mitochondrial fission factor (Mff), Ganglioside induced differentiation associated protein 1 (Gdap1) and the mitochondrial dynamics (Mid) proteins Mid49 and Mid51 (Mozdy et al., 2000; Smirnova et al., 2001; Niemann et al., 2005; Westermann, 2010; Palmer et al., 2011).

Mitochondrial fragmentation/fission is important for mitophagy. Inhibition of mitochondrial fission was shown to inhibit mitophagy (Twig et al., 2008), and elongated mitochondria were spared from autophagosome sequestration (Gomes and Scorrano, 2011; Rambold et al., 2011). Mitochondrial fission is thought to separate damaged mitochondria from healthy mitochondria. After photo-labeling, mitochondria underwent continuous cycles of fission and fusion, and fission events resulted in two sets of daughter mitochondria with either increased or decreased membrane potential. Daughter mitochondria with higher membrane potential proceeded to fusion while depolarized daughter mitochondria were degraded by mitophagy (Twig et al., 2008). Furthermore, smaller mitochondria fragments are thought to be more readily taken up by autophagosomes than larger mitochondria (Twig and Shirihai, 2011). Therefore, mitochondrial fission is a necessary step for mitophagy induction. However, mitochondrial fission alone is not enough to induce mitophagy. It seems that mitochondria must be both depolarized and fragmented for mitophagy induction to occur (Narendra et al., 2008; Twig et al., 2008; Gomes and Scorrano, 2011).

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The Pink1-Parkin signaling pathway—The Pink1-Parkin mitophagy pathway is the most studied mitophagy pathway in mammalian cells. Parkin is an E3-ubiquitin ligase encoded by the *Park2* gene (Kitada et al., 1998), and it is well known for its protective role in the brain because a variety of *Park2* loss-of-function gene mutations have been associated with development of autosomal recessive Parkinson's disease (Matsumine et al., 1997; Houlden and Singleton, 2012; Wauer and Komander, 2013; Seirafi et al., 2015). In addition to brain, Parkin is also expressed in thymus, muscle, heart, kidney, spleen, and liver (Ding and Yin 2012).

Parkin was originally discovered to regulate mitophagy by Richard Youle's group in 2008 (Narendra et al., 2008). In their study, CCCP-treatment induced Parkin translocation from the cytosol to depolarized mitochondria in Parkin-expressing HEK293 cells and in HeLa cells with YFP-Parkin overexpression, and mitochondrial-localized Parkin co-localized with the autophagosome marker LC3 after CCCP treatment. In addition, mitochondrial mass was decreased in Parkin-overexpressing HeLa cells after CCCP treatment but unchanged in non-Parkin expressing HeLa cells, suggesting that Parkin recruitment to depolarized mitochondria caused their degradation.

Phosphatase and tensin homolog (PTEN)-induced kinase 1 (Pink1) is required for recruiting Parkin to depolarized mitochondria and activating its E3 ligase activity (mechanism discussed below). Pink1 overexpression initiates translocation of Parkin to mitochondria (Kawajiri et al., 2010), and ectopic expression of Pink1 on peroxisomes recruits Parkin and causes their degradation by autophagy (Lazarou et al., 2013). Pink1 is suggested to act upstream of Parkin because overexpression of Parkin in Pink1-deficient *Drosophila* partially rescued the Pink1 mutant phenotype, but overexpression of Pink1 did not rescue Parkin-deficient *Drosophila* (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). Pink1 is rapidly cleaved and degraded in healthy mitochondria. When mitochondria are healthy, Pink1 is imported by the transporter outer membrane (TOM) complex and into the inner mitochondrial membrane through the transporter inner membrane (TIM) complex where it is first processed by the matrix processing peptidase (Greene et al., 2012) and then cleaved by the inner mitochondrial membrane protease Parl between amino acids A103 and F104 (Jin et al., 2010; Deas et al., 2011; Meissner et al., 2011). Truncated Pink1 is released into the cytosol where it is degraded by the proteasome through the N-end rule pathway (Yamano and Youle, 2013). Cleaved Pink1 fragments in the cytosol have also been suggested to inhibit Parkin translocation to mitochondria (Fedorowicz et al., 2014). Studies in *Drosophila* suggest that Pink1 degradation in healthy mitochondria is regulated by the Lon protease in the mitochondrial matrix, but this process has not been confirmed in mammalian cells (Thomas et al., 2014). A theory for regulation of Pink1 degradation in healthy mitochondria in mammalian cells involves the SPY (Slp2-Parl-Yme1) complex, which is made up of the membrane scaffold protein stromatin-like protein 2 (Slp2) and the inner mitochondrial membrane proteases Parl and Yme1L. Spl2 interaction with Parl enables Parl to cleave and process Pink1. Slp2 inhibits the stress-activated peptidase OMA1, which can bind to Slp2 and cleaves the phosphatase phosphoglycerate mutase family member 5 (PGAM5) in depolarized mitochondria (Wai et al., 2016). PGAM5 is an inner mitochondrial membrane

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protein that has been shown to protect Pink1 from degradation by Parl through an 'inside-out Pink1 translocation model' (Lu et al., 2014).

Once stabilized on the mitochondrial membrane, Pink1 phosphorylates itself on Serine 228 and Serine 402 to mediate Parkin recruitment to depolarized mitochondria (Okatsu et al., 2012). In addition, Pink1 phosphorylates Ser65 within Parkin's UBL domain and Ser65 on ubiquitin, resulting in recruitment and tethering of Parkin to mitochondria, increased Parkin activity and greater Parkin-induced outer mitochondrial membrane protein ubiquitination. Once activated, Parkin adds ubiquitin chains onto mitochondrial outer membrane proteins, and these chains are further phosphorylated by Pink1, resulting in additional recruitment and activation of Parkin (Ordureau et al., 2014; Shiba-Fukushima et al., 2014). Therefore, Pink1 and Parkin work together to promote the selective turnover of damaged mitochondria, and Pink1 induces a feed-forward loop for outer mitochondrial membrane protein ubiquitination and mitophagy induction.

Parkin ubiquitinates a variety of outer mitochondrial membrane proteins through lysine48 and lysine63 ubiquitin linkages (Chan et al., 2011) including the mitochondrial fusion proteins Mfn1 and Mfn2, the mitochondrial trafficking protein Miro1, the translocase of mitochondrial membrane 20 (TOM20), and the voltage-dependent anion channel (Vdac). Mfn1/2 ubiquitination causes their proteasomal degradation, resulting in mitochondrial fragmentation (Gegg et al., 2010; Geisler et al., 2010b; Chan et al., 2011). Ubiquitination of Miro1 implements mitochondrial arrest to separate damaged mitochondria from healthy mitochondria to facilitate the envelope of damaged mitochondria by autophagosomes (Wang et al., 2011).

The exact mechanism for how autophagosomes are recruited to damaged mitochondria is not completely understood, but it may involve recruitment of various mitophagy receptor proteins to ubiquitinated mitochondrial proteins. These mitophagy receptor proteins share similar structures and generally all have domains to bind with ubiquitinated proteins (via UBA) and the autophagy protein LC3 (via LIR). These proteins include p62/SQSTM1, Neighbor of BRCA Gene 1 (NBR1), NDP52 and optineurin. It is likely that these receptor proteins may compensate for each other and thus loss of one receptor protein may not necessarily lead to severe mitophagy defect. In addition, the importance of each of these receptor proteins in mitophagy may also be context and cell type dependent. For instance, p62 seems to be important for selective mitophagy in 293 cells, mouse macrophages and in pre-implantation embryos for paternal elimination of mitochondria (Ding et al., 2010b; Geisler et al., 2010b; Rojansky et al., 2016; Zhong et al., 2016), but it is dispensable for mitophagy in MEFs and HeLa cells and may play a role in the clustering of damaged mitochondria (Narendra et al., 2010; Okatsu et al., 2010). However, recent evidence suggests that NDP52 and Optineurin may be the main adaptor proteins for mitophagy (Heo et al., 2015; Lazarou et al., 2015). Interestingly, recruitment of NDP52 and optineurin to mitochondria by Pink1 can be independent of Parkin (Lazarou et al., 2015). When mitochondria are depolarized, Pink1 activates Tank binding kinase 1 (TBK1), which then phosphorylates adaptor proteins optineurin, NDP52, and p62. Phosphorylated optineurin and p62 exhibit better binding to LC3 and recruitment to mitochondria (Heo et al., 2015). In

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addition, TBK1 phosphorylation of optineurin enhances its binding to phosphorylated Ser65 ubiquitin chains (Richter et al., 2016).

Parkin-induced mitophagy is both positively and negatively regulated by deubiquitination. The deubiquitinase USP8 positively regulates Parkin-induced mitophagy by removing K6-linked ubiquitin chains from Parkin, which is required for Parkin recruitment to damaged mitochondria (Durcan et al., 2014). Ubiquitin-specific peptidase 30 (USP30) and USP15 both negatively regulate Parkin-induced mitophagy by removing ubiquitin from outer mitochondrial membrane proteins previously ubiquitinated by Parkin. Knockdown of USP30 and USP15 in cells and flies rescued defective mitophagy (Nakamura and Hirose, 2008; Bingol et al., 2014; Cornelissen et al., 2014). In addition, Parkin ubiquitinates itself to induce its own degradation (Zhang et al., 2000).

Intriguingly, the inner mitochondrial membrane protein prohibitin 2 (PHB2) was also recently shown to play a role in Parkin-mediated mitophagy by interacting with LC3 via its LIR domain. PHB2 binds to LC3 following Parkin-mediated ubiquitination and proteasomal degradation of outer mitochondrial membrane proteins, which causes the outer mitochondrial membrane to rupture and likely exposes PHB2. Park2-expressing HeLa cells with siRNA knockdown of PHB2 had decreased mitochondrial degradation after treatment with antimycin A and oligomycin (Wei et al., 2017), suggesting it is an important regulator of mitophagy.

In addition to the Parkin-induced mitophagy pathway described above, a new Parkin-regulated pathway has been identified for degrading depolarized mitochondria that is independent of the autophagy pathway. Hammerling et al. showed that a single-membrane Rab5-positive endosome with help from the endosomal sorting complexes required for transport (ESCRT) machinery engulfs damaged mitochondria and delivers them to the lysosome for degradation (Hammerling et al., 2017). Outer mitochondrial membrane proteins from depolarized mitochondria are ubiquitinated by Parkin, which recruits ESCRT complexes on the early endosome. The ESCRT complex induces invagination and scission of the endosome membrane, allowing for engulfment of damaged mitochondria. Hammerling et al. suggested that endosomes can eliminate damaged mitochondria more rapidly than autophagosome-mediated degradation because they are continuously synthesized, unlike autophagosomes (Hammerling et al., 2017). Therefore, even though Parkin-mediated degradation of mitochondria is the best understood pathway for ridding of damaged mitochondria, there is still much to learn about Parkin-regulated mitophagy and other roles of Parkin in maintaining mitochondrial health independent of mitophagy.

Even though autophagosome recruitment and engulfment of depolarized mitochondria likely involve the previously discussed adaptor proteins, the mechanisms for engulfment of mitochondria by autophagosomes are not completely understood. Activating molecule in Beclin 1-regulated autophagy (Ambra1) was suggested to play a role in this final step of mitophagy. Parkin interacts with Ambra1 during mitochondrial depolarization, and Ambra1 is recruited to perinuclear clusters during Parkin-mediated mitophagy to activate class III PI3K for autophagosome formation surrounding these mitochondrial clusters for their clearance (Van Humbeeck et al., 2011). Two GAP proteins, TBC1D15 and TBC1D17, form

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heterodimers and bind to a damaged mitochondrion via the outer mitochondrial membrane protein Fis1 and also bind to the autophagosome as it grows and engulfs the mitochondrion (Yamano et al., 2014). Successful completion of autophagy/mitophagy also needs a sufficient number of lysosomes to fuse with autophagosomes to meet the needs of autophagic/mitophagic flux. Youle's group recently showed that transcription factor EB (TFEB), an important regulator of autophagosome and lysosome biogenesis (Settembre et al., 2013), was activated in a Pink1- and Parkin-dependent manner (Narendra et al., 2008). Interestingly Parkin-mediated TFEB nuclear translocation requires Atg9A and Atg5 but is negatively regulated by Rag guanosine triphosphatase-mediated mTORC1 activation (Nezich et al., 2015). Together, current evidence indicates that Pink1 and Parkin regulate mitophagy at multiple layers including mitochondrial dynamics and trafficking, autophagosome formation, tagging of damaged mitochondria for their recognition by autophagosomes and endosomes/lysosomes as well as lyosomal biogenesis.

Parkin-independent mitophagy signaling pathways—Even though Parkin has been shown to be important for the mitophagy pathway, mitophagy can also be induced via Parkin-independent pathways. Parkin-independent mitophagy may be mediated by other E3 ubiquitin ligases such as Smurf1, March5 or Mul1. In addition, Parkin-independent mitophagy may occur via Bcl2/adenovirus E1B 19 kDa interacting protein 3 (Bnip3), Fun 14 domain containing 1 (Fundc1), Nix, Bcl2L13, cardiolipin or Ambra1. Furthermore, mitochondrial-derived vesicles (MDVs) and mitochondrial spheroids may also serve as alternative avenues for mitochondrial quality control and provide novel protective mechanisms against mitochondrial stress.

Nix and Bnip3—Bnip3 was first identified in a yeast two-hybrid screen that interacted with adenovirus E1B 19 kDa (Boyd et al., 1994). Bnip3 contains a Bcl-2 homology 3 (BH3) domain, and it acts as a pro-apoptotic mitochondrial protein (Yasuda et al., 1998). Nix/Bnip3L shares 53%–56% amino acid sequence identity with Bnip3 (Chen et al., 1999). Both Nix and Bnip3 are inserted into the outer mitochondrial membrane through their C-terminal transmembrane domains, and their N termini are exposed to the cytoplasm. Bnip3 and Nix both have an LIR and interact with the autophagosome membrane protein LC3 (Novak et al., 2010; Novak and Dikic, 2011; Hanna et al., 2012). Ser34/35 phosphorylation adjacent to Nix's LIR region enhanced the binding of Nix with LC3 (Rogov et al., 2017). Nix may also induce mitophagy by promoting ROS production because Nix-deficient MEFs had less ROS production (Ding et al., 2010b), and ROS production has been shown to activate autophagy (Scherz-Shouval and Elazar, 2011). The mechanisms for ROS-induced mitophagy are not clear, but our studies suggest that ROS may inhibit mTOR signaling, leading to subsequent induction of autophagy (Ding et al., 2010b). Other studies suggest that ROS inactivates Atg4 and reduces its de-conjugation activity, which consequently increases LC3 association with the autophagosome membrane (Scherz-Shouval et al., 2007).

Bnip3 and Nix are expressed in liver, skeletal muscle, heart, kidney and brain, but they are not ubiquitously expressed under normal conditions (Galvez et al., 2006). They are both highly induced during hypoxic conditions by Hif-1 alpha (Hif-1α) to remove damaged mitochondria and prevent accumulation of ROS (Sowter et al., 2001; Zhang et al., 2008).

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Nix and Bnip3 seem to have complementary roles in the mitophagy response during hypoxia because depletion of either Nix or Bnip3 alone did not affect mitophagy, but simultaneous depletion of both inhibited the mitophagic response to hypoxic conditions. In addition, overexpression of both Nix and Bnip3 induced mitophagy under normal oxygen concentrations (Bellot et al., 2009). In addition to Hif-1α, Bnip3 and Nix expression are also regulated by the Foxo3 transcription factor during starvation or alcohol exposure (Mammucari et al., 2007; Ni et al., 2013b). Starvation increases Foxo3 activity, and it binds to Bnip3 and Nix promoter regions to increase their expression in muscle cells (Mammucari et al., 2007). However, the role of Foxo3-induction of Nix and Bnip3 in mitophagy requires further study.

Nix is also involved in mitophagy induction in cells that are undergoing high rates of oxidative phosphorylation, which leads to increased ROS production, mitochondrial dysfunction and cell death. When cells are undergoing high rates of oxidative phosphorylation, the GTPase Rheb is recruited to the outer mitochondria membrane where it interacts with Nix and LC3 to induce mitophagy (Melser et al., 2013). Bnip3 can also activate mitophagy by interacting with Rheb (Li et al., 2007). Nix also activates mitophagy during red blood cell maturation, which is discussed in a later section.

Both Nix and Bnip3 may also play roles in Parkin-dependent mitophagy induction. Nix may play a role as a substrate of Parkin in Parkin-dependent mitophagy (Gao et al., 2015). Bnip3 may interact with Pink1 to prevent its proteolytic processing and support its accumulation on the mitochondrial membrane for Parkin recruitment to damaged mitochondria (Zhang et al., 2016).

Fundc1—In addition to Nix and Bnip3, Fundc1 has also been shown to have a role in hypoxia-induced mitophagy (Liu et al., 2012). It is currently unknown if Fundc1 has a more important role in hypoxia-mediated mitophagy than Nix or Bnip3, but Fundc1 has a greater binding affinity for LC3 than Nix (Novak et al., 2010). Fundc1 is an outer mitochondrial membrane protein. Similar to Nix and Bnip3, Fundc1 interacts with LC3 through its LIR motif, which is essential for Fundc1-mediated mitophagy because mutation of this site inhibits mitophagy (Liu et al., 2012).

Even though Nix and Bnip3 also mediate hypoxia-induced mitophagy, Fundc1 activates mitophagy via a different mechanism. While mRNA expression of Nix and Bnip3 are increased by Hif-1α or FoxO3, Fundc1 levels are decreased during hypoxia (Guo et al., 2001; Liu et al., 2012). Unlike Nix and Bnip3, Fundc1-mediated mitophagy is mainly regulated by phosphorylation and ubiquitination instead of transcriptional regulation (Liu et al., 2012; Chen et al., 2014). Fundc1 is phosphorylated on Tyr18 by the Src kinase (Liu et al., 2012; Chen et al., 2017) and also on Ser13 by CK2 (Chen et al., 2014) within the LIR motif under oxygenated conditions. During hypoxia, CK2 and Src kinase are inhibited, and PGAM5 dephosphorylates Fundc1 on Ser13, which increases binding affinity of Fundc1 for LC3 (Chen et al., 2014). Bcl2-like 1 (Bcl2l1), but not Bcl2, interacts with and inhibits PGAM5, which prevents Fundc1 dephosphorylation and subsequent mitophagy (Wu et al., 2014).

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In addition to regulation by phosphorylation, Fundc1 is also ubiquitinated and degraded by the proteasome during hypoxia (Chen et al., 2017). Interestingly, Fundc1 is not ubiquitinated by Parkin. Hypoxia did not induce Parkin translocation to mitochondria, and overexpression of Parkin in HeLa cells did not affect Fundc1 ubiquitination levels or its degradation when exposed to hypoxic conditions (Chen et al., 2017). Fundc1 is instead ubiquitinated by the mitochondrial E3 ligase March5 at lysine 119 in hypoxic conditions. HeLa cells with ectopic March5 expression had increased levels of Fundc1 ubiquitination and degradation in hypoxic conditions, which were decreased with Fundc1 knockdown (Chen et al., 2017). Chen et al. proposed a model where hypoxia initially promotes interaction of Fundc1 with March5 followed by Fundc1 ubiquitination and degradation, which protects mitochondria from degradation by mitophagy. Severe hypoxia instead causes dephosphorylation of Fundc1, which enhances its reaction with LC3 and induces mitophagy, as previously discussed (Chen et al., 2017).

Interestingly, Fundc1 was shown to play an important role in mitophagy in platelets. Hypoxia in platelets increased mitochondrial degradation by mitophagy, which was inhibited in Fundc1 KO platelets (Zhang et al., 2017). In ischemia reperfusion (I/R) mouse models, mitophagy occurred in wildtype mouse platelets during late-stage I/R when oxygen levels were low, which decreased activation of platelets to prevent further cardiac injury. Fundc1 platelet-specific knockout (KO) mice had decreased mitophagy and an abundance of damaged mitochondria, which correlated with continuous platelet activity and greater cardiac injury (Zhang et al., 2017).

In addition to acting as a mitophagy receptor, Fundc1 was also recently shown to modulate mitochondrial fission and fusion by interacting with Drp1 and Opa1, respectively (Chen et al., 2016). Fundc1 interacts with Drp1 and recruits it to the mitochondria to promote mitochondrial fragmentation. In addition, Fundc1 knockdown in HeLa cells increased mitochondrial fusion. Fundc1 modulates mitochondrial fusion by interacting with Opa1 on lysine 70, and this interaction must be lost for mitophagy to occur. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) treatment in HeLa cells enhanced the interaction of Fundc1 with Drp1 and decreased its interaction with Opa1 to promote mitochondrial fission and subsequent mitophagy. Fundc1 phosphorylation status affects its interaction with Drp1 and Opa1, suggesting that Fundc1 phosphorylation regulates mitochondrial fission and fusion in addition to mitophagy (Chen et al., 2016).

Smurf1—Smurf1 is an E3 ubiquitin ligase similar to Parkin that was found to have a role in CCCP-induced mitophagy mediated by Parkin. Smurf1 was identified in a high-content genome wide small interfering RNA screen. Unlike Parkin, the ubiquitin ligase function of Smurf1 does not play an important role in mitophagy induction. Instead, the C2 domain of Smurf1 is required for engulfment of damaged mitochondria by autophagosomes. In addition, Smurf1 deficient mice have accumulated mitochondria that are damaged in their heart, brain and liver (Orvedahl et al., 2011).

Mul1—Mul1 is an E3 ligase that acts in parallel to the Parkin-induced mitophagy pathway. Mul1 was recently shown to ubiquitinate outer mitochondrial membrane Mfn proteins in *Drosophila* and mammalian cell lines during mitochondrial depolarization. In addition, Mul1

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overexpression in *Drosophila* reversed Parkin/Pink1 mutant phenotypes, and Pink1 and Mul1 or Parkin and Mul1 double mutant flies had worsened phenotypes than either mutant alone. Furthermore, Mul1 knockdown in Parkin KO primary cortical neurons increased mitochondrial depolarization, and Parkin KO mice with Mul1 knocked down had neuronal mitochondrial depolarization and neuron degradation (Yun et al., 2014). Mul1 was shown to act independently of Parkin because neither Mul1 overexpression nor knockdown in Parkin-expressing HeLa cells affected Parkin translocation to depolarized mitochondria (Yun et al., 2014). In addition, Mul1-induced mitophagy was still activated in the absence of Parkin with selenite treatment in Park2-deficient MEFs (Li et al., 2015). Furthermore, knockdown of Mul1 in Parkin deficient cells decreased mitochondrial ubiquitination and mitophagy, but knockdown of Mul1 alone did not affect mitochondrial ubiquitination levels or mitophagy induced by CCCP or Oxphos conditions (Rojansky et al., 2016). These data suggest that Parkin and Mul1 have redundant functions for mitophagy activation. The mechanism of activation for Mul1-induced mitophagy is still not clear. Li et al. demonstrated that Mul1 was important for selenite-induced mitophagy in HeLa cells, but not for mitophagy induced by FCCP or hypoxia. In addition, they suggested that Atg5 and ULK1 are both required for mitophagy induction by Mul1, and that Mul1 ubiquitinates ULK1 for degradation during mitophagy activation (Li et al., 2015). The role of Mul1 in Parkin-independent mitophagy still requires further investigation.

Mammalian Atg32 (Bcl2-L-13)—Bcl2-L-13 is a Bcl-2 family member with four BH motifs (Kataoka et al., 2001), which was recently identified as the mammalian homolog of yeast Atg32 (Murakawa et al., 2015). Bcl2-L-13 is a single transmembrane domain outer mitochondrial membrane protein with 2 WXXI motifs, allowing it to bind to the autophagosome membrane protein LC3 (yeast homolog Atg8). Bcl2-L-13 induced mitochondrial fission and mitophagy in HEK293 cells after CCCP treatment independent of Parkin, and Bcl2-L-13 induces mitophagy in yeast deficient in Atg32, confirming it as an Atg32 homolog (Murakawa et al., 2015). Bcl2-L-13 induction may also be regulated by phosphorylation, similar to its yeast homolog Atg32. The mechanisms for how Bcl2-L-13 induces mitophagy are not completely understood, but it was suggested that it may bind to an unknown homolog of Atg11 or act as an adaptor protein similar to Atg11 in mammalian cells to induce mitophagy.

Cardiolipin—Cardiolipin is a phospholipid dimer that is synthesized in the inner mitochondrial membrane (Ren et al., 2014). When mitochondria are depolarized, cardiolipin translocates to the outer mitochondrial membrane by phospholipid scramblase-3 (Chu et al., 2013) and/or the intermembrane space protein NDPK-D (Kagan et al., 2016). Cardiolipin induces mitophagy in SH-SY5Y cells and primary cortical neurons after rotenone treatment. Rotenone treatment caused LC3 colocalization with mitochondria, which was inhibited when cardiolipin translocation to the outer mitochondrial membrane was blocked (Chu et al., 2013). Interestingly, cardiolipin also functions to induce apoptosis. If cardiolipin translocates to the outer mitochondrial membrane and it is in its peroxidized form, it initiates apoptosis. If it is not peroxidized on the outer mitochondrial membrane, it induces mitophagy (Ren et al., 2014).

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Cardiolipin and Parkin may have some overlap in mitophagy induction or they may induce mitophagy under different levels of mitochondrial depolarization. CCCP-treated cells caused Pink1 and Parkin-induced mitophagy, but rotenone treatment caused cardiolipin translocation to the outer mitochondrial membrane without recruitment of Parkin. This may be due to less mitochondrial depolarization induced by rotenone compared to CCCP treatment (Chu et al., 2013). Cardiolipin may also function as a compensatory mitophagy pathway in the absence of functional Parkin. The role of cardiolipin for inducing mitophagy requires further evaluation.

Ambra1—In addition to its previously discussed role in Parkin-mediated mitophagy (Van Humbeeck et al., 2011), Ambra1 can also activate mitophagy independent of Parkin. Overexpression of a mitochondrial form of Ambra1 induced mitophagy in Parkin-deficient HeLa cells and in HEK293 cells with Parkin knocked down (Strappazzon et al., 2015). Ambra1 also induced mitophagy after FCCP treatment in Parkin-deficient cells. In addition, Ambra1 has an LIR enabling it to interact with LC3, and Ambra1 still interacts with LC3 in the absence of Parkin. Mutations in the LIR region of Ambra1 inhibit mitophagy (Strappazzon et al., 2015). Ambra1 likely has a role in both Parkin-dependent and independent mitophagy, but the exact roles of Ambra1 in mitophagy or the mechanisms by which it activates mitophagy are not well understood.

Atg-independent mitophagy—While Nix is critical for autophagic removal of mitochondria during erythrocyte maturation as discussed below, recent evidence revealed a novel ULK1-dependent but Atg5-independent macroautophagy for the clearance of mitochondria from fetal definitive reticulocytes. Honda et al. showed that mitochondria are retained in reticulocytes in ULK1-deficient and ULK1/Atg5 double deficient mice but not in ULK1 wild type and Atg5-deficient mice (Honda et al., 2014). Moreover, during erythroid differentiation, early evidence suggested that 15-lipoxygenase increased the permeability of organelle membranes and could be involved in mitochondria degradation (van Leyen et al., 1998). Therefore it seems that both Atg-dependent and independent mechanisms can be involved in the regulation of mitochondrial homeostasis during erythroid differentiation. Moreover, it is also possible that mitochondria can be removed directly by lysosomes via microautophagy independent of Atg-mediated macroautophagy, although it still remains to be further investigated.

Mitochondrial-derived vesicles—Parkin and Pink1 stimulate the formation of MDVs, which bud off mitochondria for delivery of oxidized mitochondrial proteins to lysosomes. MDVs are single-membraned and contain outer mitochondrial membrane proteins such as VDAC and TOM20 (Soubannier et al., 2012; McLelland et al., 2014). The Parkin-induced mitophagy pathway is stimulated by mitochondrial depolarization while MDV formation occurs during oxidative stress generated within the mitochondria. Another key difference between mitophagy and MDVs is that MDVs bud off of damaged mitochondria and are degraded in the lysosome without any prior sequestration in autophagosomes (McLelland et al., 2014; Roberts et al., 2016). Formation of MDVs may actually help conserve mitochondrial function and prevent mitophagy by selectively degrading damaged mitochondrial contents (Soubannier et al., 2012; McLelland et al., 2014).

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Mechanisms for MDV formation and delivery to lysosomes are not completely understood, but it was suggested that the GTPases Rab9 and Rab7 are required for vesicle budding and formation on mitochondria as well as for membrane fusion between MDVs and endosomes, respectively (Vanlandingham and Ceresa, 2009; Matheoud et al., 2016). The SNARE protein syntaxin-17 was recently suggested to target the mitochondrial outer membrane and mediate trafficking of MDVs to the late endosome/lysosome. Once delivered, syntaxin-17 remains on mature MDVs and forms a ternary SNARE complex with VAMP7 and SNAP29, which induces fusion of the MDV with the late endosome/lysosome. MDV fusion with the lysosome also requires the homotypic fusion and vacuole protein string tethering complex (McLelland et al., 2016), MDVs also deliver their cargo to peroxisomes, but the reason for delivery to peroxisomes is currently unknown (Neuspiel et al., 2008; Mohanty and McBride, 2013).

Various roles for MDVs have recently surfaced. MDVs may be involved in Parkinson's disease pathogenesis through MDV-mediated degradation of Drp1 (Wang et al., 2016a,b). Interestingly, MDV pathway can also be used for mitochondrial antigen presentation and loss of Pink1 and Parkin increased levels of mitochondrial antigens presented on MHC Class I molecules in both dendritic cells and macrophages, suggesting that Parkin and Pink1 inhibit mitochondrial antigen presentation (Matheoud et al., 2016). MDVs were also recently shown to help maintain mitochondrial homeostasis in the heart when mice were treated with doxorubicin to induce acute cardiotoxicity. MDVs were present in normal physiological conditions in H9c2 myoblasts and were upregulated under stress when treated with antimycin-A (Cadete et al., 2016). Together, formation of MDVs may represent another mechanism for mitochondrial quality control and mitochondrial antigen presentation with involvement in many pathophysiological conditions.

Mitochondrial spheroids—We recently discovered a novel autophagosome-like structure that derived from damaged mitochondria, which we termed as 'mitochondrial spheroids' (Ding et al., 2012a,b). Mitochondrial spheroids are mitochondria with a ring or cup-like morphology that have a similar appearance to autophagosomes, but their lumen is connected to the cytosol by a small opening. Spheroids can enwrap cellular contents such as ER, lipid droplets, or other mitochondria. They are positive for lysosome proteins and may be able to degrade their contents, but their degradation capacity is currently unknown. Mitochondrial spheroids are formed during oxidative stress, and their formation depends on the Mfn1 and Mfn2 fusion proteins (Ding et al., 2012a,b; Yin and Ding, 2013). Parkin inhibits mitochondrial spheroid formation by initiating proteasomal degradation of Mfn1 and Mfn2. MEF cells, which lack detectable Parkin, formed mitochondrial spheroids after CCCP treatment, and Parkin overexpression in these cells prevented mitochondrial formation due to degradation of Mfn1 and Mfn2 (Ding et al., 2012a,b).

Interestingly, neuronal expression of mutant huntingtin in *Drosophila* induced formation of mitochondrial spheroids in the photoreceptor neurons, which were blocked by overexpression of Pink1 (Khalil et al., 2015). Mitochondrial spheroids were also found in mouse livers exposed to acetaminophen or high fat as well as alcohol diet (Ding et al., 2012a,b; Manley et al., 2014; Ni et al., 2015; Williams et al., 2015a,b). These observations clearly support the physiological relevance of formation of mitochondrial spheroids.

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However, the function of mitochondrial spheroids and whether or not they serve as a protective mechanism requires further investigation.

Mitophagy in *Caenorhabditis elegans*

Similar to mammalian cells, mitophagy is activated in *C. elegans* to maintain mitochondrial homeostasis. It is also activated in *C. elegans* as a protective response during mitochondrial stress and contributes to longevity (Palikaras et al., 2015a,b; Schiavi et al., 2015). In addition, iron chelators activate mitophagy in *C. elegans*, which is thought to recycle iron as a protective mechanism (Kirienko et al., 2015). Mitophagy also helps eliminate paternal mitochondria in *C. elegans* (Sato and Sato, 2011; Wang et al., 2016b; Wei et al., 2017). Similar to mammalian cells, mitophagy in *C. elegans* is mediated by homologs of mammalian Parkin (*C. elegans* pdr-1) (Springer et al., 2005), Pink1 (*C. elegans* pink-1) (Samann et al., 2009), BNIP3 (*C. elegans* dct-1) (Palikaras et al., 2015a,b), and the autophagy adaptor protein sequestosome 1/p62 (*C. elegans* sqst-1) (Tian et al., 2010; Schiavi et al., 2015). Pdr-1 is ubiquitously expressed in *C. elegans*. Similar to Parkin in mammalian cells, pdr-1 is an E3 ubiquitin ligase, and basal ubiquitination levels were decreased after pdr-1 deletion (Springer et al., 2005; Ved et al., 2005). Pink-1 function also appears to be conserved between mammalian cells and *C. elegans* (Samann et al., 2009), suggesting that the Parkin and Pink1-induced mitophagy pathway is conserved in *C. elegans*.

Schiavi et al. showed that mitophagy is activated in *C. elegans*, and mitophagy activation requires pdr-1, dct-1, sqst-1 and pink-1 after frataxin depletion, which causes iron starvation and a hypoxia-like stress response (Schiavi et al., 2015). Frataxin is a nuclear-encoded mitochondrial protein responsible for regulation of iron-sulfur-cluster-containing proteins necessary for mitochondrial respiratory chain function (Martelli and Puccio, 2014). The frataxin homolog in *C. elegans* is frh-1 (Vazquez-Manrique et al., 2006), and slight depletion of frh-1 in *C. elegans* is associated with longevity (Ventura and Rea, 2007; Schiavi et al., 2015). Silencing of pdr-1, dct-1, sqst-1 or pink-1 reduced longevity in *C. elegans* during frataxin suppression (Schiavi et al., 2015). Longevity was also reduced after knockdown of dct-1, pdr-1 or pink-1 during heat stress, starvation, and treatment with the mitochondrial uncouplers CCCP or paraquat (Samann et al., 2009; Palikaras et al., 2015a,b). In addition, decreased animal survival was correlated with reduced mitochondrial ATP levels, increased mitochondrial ROS, and an overall decrease in the number of functional mitochondria (Palikaras et al., 2015a,b). Palikaras et al. showed co-localization of dct-1 with Lgg-1 (homolog of yeast Atg8 and mammalian LC3) on the outer mitochondrial membrane, suggesting that dct-1 mediates mitophagy in *C. elegans* (Palikaras et al., 2015a,b). Furthermore, Bess et al., 2012 showed that mitophagy removes mitochondrial DNA lesions after UVC radiation, and deletion of pink-1 in *C. elegans* inhibited the elimination of damaged mitochondrial DNA lesions.

Mitophagy is also important for degrading paternal mitochondria during development in *C. elegans*. When paternal mitochondria enter the oocyte during fertilization, they become permeabilized and depolarized and are surrounded and engulfed by autophagosomes. In zygotes with defective autophagy, paternal mitochondria are maintained, which prevents inheritance of maternal mitochondria (Sato and Sato, 2011; Wang et al., 2016b). Recently it

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was discovered that phb-2, the ortholog of mammalian PHB2, is required for degradation of maternal mitochondria by mitophagy. Phb-2 knockdown decreased mitochondrial degradation (Wei et al., 2017). Overall, the mammalian mitophagy pathway appears to be conserved in *C. elegans* for maintenance of mitochondrial health, but the various roles of this pathway in *C. elegans* need further exploration.

Pathophysiological role of mitophagy

Mitochondria are essential for regulating cellular energy metabolism and cell death. Therefore, it is not a surprise that defects in mitochondrial function and homeostasis have been linked to many pathophysiological conditions and diseases, which are discussed below.

Development

Mitophagy is necessary for several developmental processes. For example, mitophagy is required for red blood cell maturation. Red blood cells must eliminate their mitochondria during maturation in order to be able to better carry and provide oxygen. Nix was shown to be required for this process because mitochondrial engulfment by autophagosomes was blocked in Nix-deficient mice, which led to reduction of red blood cell life-span and development of anemia (Schweers et al., 2007; Sandoval et al., 2008). This mechanism of mitophagy induction was shown to require mitochondrial depolarization (Sandoval et al., 2008). Treatment with the mitochondrial uncoupler CCCP induced depolarization and restored mitochondria sequestration into autophagosomes in Nix-deficient erythroid cells (Sandoval et al., 2008). In addition, Nixdeficient MEFs were resistant to CCCP-induced mitochondrial depolarization and general autophagy induction (Ding et al., 2010b), confirming the requirement of mitochondrial depolarization for Nix-induced mitophagy.

Mitophagy also occurs during embryonic development. Mitochondria are well known to be inherited from the maternal parent in most eukaryotes. Paternal mitochondria and mitochondrial DNA are selectively degraded from the embryo by mitophagy (Ankel-Simons and Cummins, 1996), In addition to *C. elegans*, it was recently discovered that sperm mitochondria are removed by mitophagy in mice. LC3 and p62 were seen surrounding fertilizing sperm inside of mouse embryos (Al Rawi et al., 2011) suggesting that their mitochondria may be eliminated by mitophagy. Paternal mitochondria in mice were shown to depolarize after entering the oocyte, and Parkin and Mul1 were suggested to have redundant functions for degrading them via mitophagy. Embryos with loss of Parkin or Mul1 alone had a slight decrease in paternal mitochondria elimination, but simultaneous loss of Parkin and Mul1 caused severe decreases in paternal mitochondria elimination so that 60% of embryos retained their paternal mitochondria. In addition, embryos with loss of p62, Tbc1d15, or Pink1 also had decreased degradation of paternal mitochondria, suggesting they are important for paternal mitochondrial degradation in addition to Parkin (Rojansky et al., 2016).

Mitophagy also plays a role in adipogenesis. During early adipogenesis, mitochondria numbers increase dramatically prior to differentiation to provide the high levels of energy and substrates needed for differentiation and lipogenesis (Wilson-Fritch et al., 2003; Goldman et al., 2011). However, mature adipocytes contain significantly less mitochondria,

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likely due to mitophagy. Autophagy is important in differentiation of adipocyte progenitor cells to white adipocytes. Blocking Atg5 or Atg7 in MEF cells caused failure of progenitor adipocytes to differentiate into white adipocytes (Baerga et al., 2009; Zhang et al., 2009). In addition, conditional adipose Atg7 KO mice had reduced white adipose tissue containing significantly more mitochondria compared to wild-type mice (Baerga et al., 2009; Zhang et al., 2009) suggesting that mitophagy plays an important role in adipocyte differentiation to white adipocytes. However, the exact role for mitophagy and mechanisms involved in adipogenesis require further investigation.

Mitophagy and mitochondrial biogenesis were recently shown to have a role in myogenesis during skeletal muscle development. Differentiation of immature myoblasts into mature myotubes requires a metabolic shift from glycolysis to oxidative phosphorylation, which involves mitophagy. C2C12 myoblasts treated with siRNA against Atg5 or p62 maintained a fibroblast morphology instead of developing into a myotube. Mitophagy is upregulated soon after differentiation begins, and mitophagy during myoblast differentiation requires p62. As differentiation continues, mitochondrial biogenesis is also required to provide mitochondria capable of performing oxidative phosphorylation (Sin et al., 2016).

Mitophagy is also important for metabolic differentiation of cardiomyocytes. Fetal perinatal cardiac myocytes utilize carbohydrate metabolism as an energy source, and adult myocytes require fatty acid metabolism as an energy source. Using Mfn2 mutant mice that inhibited mitophagy by preventing Parkin from binding to Mfn2, Gong et al. found that mitophagy was required for transition between fetal carbohydrate metabolism and adult fatty acid metabolism in myocytes. Mfn2 mutant mice had persistence of fetal carbohydrate metabolizing mitochondria instead of transitioning to fatty acid metabolizing mitochondria, suggesting that Parkin interaction with Mfn2 initiates mitophagy to degrade fetal mitochondria in myocytes to allow their replacement with fatty acid metabolizing mitochondria (Gong et al., 2015). Therefore, the Parkin mitophagy pathway and Parkin interaction with Mfn2 are important for proper development of cardiac myocytes.

Aging

Mitochondrial dysfunction and mitochondrial DNA mutations are well known to be associated with aging (Sun et al., 2016). Mitophagy declines in mice with aging (Sun et al., 2015), and Parkin-induced mitophagy has been suggested to prolong lifespan in *Drosophila* (Greene et al., 2003; Rana et al., 2013) and *C. elegans* (Palikaras et al., 2015a,b). In *Drosophila*, Parkin loss decreased whereas Parkin overexpression increased longevity (Greene et al., 2003). Increased lifespan in mice with Parkin overexpression was due to increased ubiquitination and subsequent reduction in protein aggregates. Flies with Parkin overexpression also had increased mitochondrial activity (Rana et al., 2013). Parkin and mitophagy also have a role in *C. elegans* for maintaining life. Longevity was decreased in *C. elegans* with knockdown of dct-1, pdr-1, or pink-1 during heat stress, starvation, and treatment with the mitochondrial uncouplers CCCP or paraquat, as previously discussed (Samann et al., 2009; Palikaras et al., 2015a,b). In addition, the natural mitophagy inducer urolithin A increased life span in *C. elegans* by preventing accumulation of dysfunctional mitochondria (Ryu et al., 2016). Even though mitophagy clearly has a role in maintaining

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longevity, the exact reasoning for this is not well understood. It is likely due to the ability of mitophagy to remove damaged mitochondria, which helps maintain mitochondrial homeostasis and function to prevent ROS accumulation and mitochondrial DNA damage (Sun et al., 2016).

Cancer

Autophagy is well known to play a role in cancer because the tumor suppressor genes *Lkt, Ampk* and *Pten* are positive regulators of autophagy (Cully et al., 2006; Liang et al., 2007; Degtyarev et al., 2008; Hezel and Bardeesy, 2008; Kimmelman, 2011) while the oncogenes class I PI3K, Akt, and anti-apoptotic Bcl-2 family proteins suppress autophagy (Maiuri et al., 2009). The role of autophagy in cancer is dynamic because autophagy can either promote or suppress tumorigenesis depending on the stage of tumorigenesis and the tumor's microenvironment (White, 2015).

Impaired autophagy causes increased DNA damage, mitochondrial damage, and increased ROS, which all are associated with tumorigenesis (White, 2015). Therefore, autophagy can help suppress early-stage tumorigenesis. Mice with *Beclin 1* depletion develop hepatocellular carcinoma, lung adenocarcinoma, mammary hyperplasia, and lymphoma (Qu et al., 2003; Yue et al., 2003). Mice with liver-specific depletion of Atg5 or Atg7 accumulate damaged mitochondria, which results in development of liver injury, steatohepatitis, and eventual adenocarcinoma (Inami et al., 2011; Takamura et al., 2011; Ni et al., 2014).

In addition to general autophagy proteins acting as tumor suppressors, the mitophagy proteins Parkin and Bnip3 also act as tumor suppressors, suggesting that degradation of damaged mitochondria via mitophagy is an important mechanism for cancer prevention. Parkin is one of the most frequently deleted genes in cancer. The *Park2* gene is mutated or has copy number loss leading to decreased Parkin expression in many different types of cancer including breast, ovarian, thyroid, pancreatic, cervical, bladder, colorectal, gastric, glioblastoma, kidney, melanoma, liver and lung adenocarcinoma (Cesari et al., 2003; Denison et al., 2003; Wang et al., 2004; Fujiwara et al., 2008; Tay et al., 2010; Veeriah et al., 2010a,b; Yeo et al., 2012; Toma et al., 2013; Xu et al., 2014; Hu et al., 2016; Lee et al., 2016). Restoration of Parkin expression in breast cancer cell lines (Tay et al., 2010) and glioma cells (Veeriah et al., 2010a,b) slowed cellular proliferation, confirming Parkin's role as a tumor suppressor. Parkini-nduced mitophagy likely plays an anti-tumorigenic role because damaged mitochondria are well-known to be involved in cancer progression (Gogvadze et al., 2008). However, Parkin also has other anti-tumorigenic functions independent of mitophagy.

Parkin plays a role in cell cycle regulation or its transcriptional regulation by p53. Parkin also ubiquitinates and mediates proteasomal degradation of Cyclin E and Cyclin D1 *in vitro*, and Parkin loss upregulates expression of these proteins leading to progression through the cell cycle and increased proliferation (Veeriah et al., 2010a,b; Gong et al., 2014).

The mitophagy protein Bnip3 was also recently shown to have a tumor suppressive role in a mouse model of breast cancer. Loss of Bnip3 in these mice caused breast cancer metastasis, which was suggested to be due to defective mitophagy and accumulation of dysfunctional

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mitochondria and ROS production. Increased ROS production led to increased expression of Hif-1α and its target genes and increased glycolysis, which promoted the Warburg effect and subsequent cancer progression (Chourasia et al., 2015). Loss of Bnip3 expression was also associated with breast cancer metastasis to lymph nodes, lung, liver, and bone (Manka et al., 2005; Koop et al., 2009) and chemoresistance in pancreatic cancer patients (Akada et al., 2005). Bnip3 is down-regulated in pancreatic cancer by hypermethylation of its promoter region (Okami et al., 2004; Erkan et al., 2005). Bnip3 promoter hypermethylation and decreased Bnip3 expression were also found in some colorectal and gastric cancers (Murai et al., 2005). Restoration of Bnip3 expression made pancreatic tumor cells more susceptible to hypoxia-induced cell death (Okami et al., 2004), confirming its role as a tumor suppressor.

Even though general autophagy and mitophagy have been shown to prevent tumorigenesis, autophagy can actually help existing tumors survive in pancreatic, lung, melanoma and brain cancers (Guo et al., 2013; White, 2015). In addition, autophagy may cause tumor cells to be resistant to chemotherapy (Guo et al., 2013). The ability of autophagy to protect existing tumors is not surprising because autophagy helps cells survive in stressful and nutrient deprived environments. Whether mitophagy activation can also aid in tumor cell survival is unknown.

Neurodegenerative diseases

Parkin and mitophagy play protective roles against Parkinson's disease. Loss of Parkin can cause autosomal recessive (early onset) Parkinson's disease. Approximately 10% of early onset Parkinson's disease cases are caused by mutations in the *Park2* gene (Houlden and Singleton, 2012), and gene mutations can occur throughout the *Park2* gene and lead to a decrease in Parkin function or its increased proteasomal degradation (Wauer and Komander, 2013; Seirafi et al., 2015; Zhang et al., 2015). In addition to genetic mutations, Parkin can also be inactivated post-translationally via oxidation, nitrosylation, or phosphorylation (Walden and Martinez-Torres, 2012; Zhang et al., 2015). Pink1 mutations were also identified and associated with early-onset Parkinson's disease (Valente et al., 2001, 2002; Geisler et al., 2010a). Defects in Pink1 and Parkin lead to an accumulation of damaged and dysfunctional mitochondria, which is likely due to a lack of mitophagy. The accumulation of dysfunctional mitochondria causes loss of dopaminergic neurons. Therefore, defects in mitophagy are at least partially responsible for development of early-onset Parkinson's disease (Pickrell and Youle, 2015). Interestingly, mitochondrial motility and mitophagy were also recently shown to be impaired in sporadic Parkinson's disease due to impaired Parkin-mediated degradation of miro (Hsieh et al., 2016). MDVs were also recently shown to have a role in Parkinson's disease via degradation of Drp1, as previously discussed (Wang et al., 2016b).

Parkin and mitophagy may also play protective roles in other neurodegenerative diseases such as Alzheimer's disease (AD) (Hong et al., 2014; Corsetti et al., 2015), amyotrophic lateral sclerosis (ALS) (Wong and Holzbaur, 2014; Moore and Holzbaur, 2016), and Huntington's disease (HD) (Khalil et al., 2015). Overexpression of Parkin decreased β-amyloid load, gliosis, and inflammation in an AD mouse model. In addition, Parkin

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overexpression protected against behavioral abnormalities in these mice (Hong et al., 2014). Interestingly, Parkin was also shown to play a pro-death role in AD. N-terminal truncation of tau occurs in AD and results in excessive removal of mitochondria by the Parkin-Pink1 mitophagy pathway, and inhibition of mitophagy partially protected against neuronal cell death (Amadoro et al., 2014; Corsetti et al., 2015). Parkin and mitophagy likely protect against development of ALS. As previously mentioned, optineurin is a selective autophagy receptor in the Parkin-induced mitophagy pathway. Optineurin is commonly mutated in ALS (Maruyama et al., 2010), and ALS-associated mutations in optineurin prevent its association with mitochondria, resulting in decreased mitophagy (Wong and Holzbaur, 2014). TBK1, which phosphorylates Optineurin to increase its interaction with LC3 as previously discussed, was also recently shown to be mutated in ALS (Freischmidt et al., 2015). Finally, Parkin-induced mitophagy was suggested to be protective in HD. Khalil and colleagues showed that Parkin-mediated mitophagy was defective in a mouse model of HD. Parkin was able to ubiquitinate mitochondrial proteins, but autophagosomes were unable to be recruited to mitochondria requiring degradation. Overexpression of Pink1 In striatal cells from these mice partially restored mitophagy (Khalil et al., 2015). The exact mechanisms for Parkin and mitophagy involvement in Parkinson's, AD, ALS, and HD still require further investigation.

Innate immunity and host defense

Autophagy plays an important role in innate immunity by defending against microbes and viruses including *Mycobacterium tuberculosis, Salmonella, Listeria, Shigella, Psuedomonas aeruginosa*, HIV-1, and Sindbis viruses (Deretic, 2012a,b; Kirienko et al., 2015). Autophagy can be regulated by the DAMP molecules HMGB1 and IL-1β in addition to toll-like receptors, RIG-I-like receptors, and Nod-like receptors including NLRC4, NLRP3, and NLRP4 (Deretic, 2012a,b). The innate immune signaling molecules TBK-1 and IKKα/β are also associated with autophagy (Weidberg and Elazar, 2011; Deretic, 2012a,b). Interestingly, TFEB was recently shown to have a protective role during *S. aureus* infection. During infection, TFEB became activated and translocated to the nucleus in macrophages where it activated transcription of proinflammatory cytokines and chemokines (Visvikis et al., 2014). Whether TFEB is involved in the immune response against other types of infection is unknown.

Mitophagy also has a role in innate immunity and host defense. Suppression of mitophagy leads to an accumulation of damaged and ROS-producing mitochondria, which activates the NLRP3 inflammasome. Over-activation of the inflammasome can cause tissue damage and organ failure (Zhou et al., 2011). Sestrin 2 protected against NLRP3-induced injury by inducing mitophagy. Mice deficient in sestrin 2 had decreased mitophagy, increased activation of NLRP3, and subsequent increased mortality rates (Kim et al., 2016). Mitophagy is also protective against bacterial infection-mediated sepsis via regulation of NLRP3 inflammasome activation. Pink1 and Parkin KO mice had increased organ failure and mortality rates during sepsis, and use of a NLPR3 inhibitor protected mice against septic death (Kang et al., 2016). LC3B and Beclin-1 depletion also promote an accumulation of damaged mitochondria and cytosolic translocation of mtDNA in macrophages, which promotes secretion of IL-1β and IL-18 after LPS or ATP stimulation (Nakahira et al., 2011). These studies suggest that mitophagy can regulate NLRP3-dependent inflammation and host

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defense by preserving mitochondrial integrity. Even though mitophagy is mostly thought of as a protective mechanism, it actually enhances Hepatitis C Virus infection by allowing virus-infected cells to escape cell death (Kim et al., 2014).

Mitophagy is important for proper T-cell differentiation. During differentiation, mitochondria are degraded in thymocytes by mitophagy. Mature T-cells with deficient mitophagy have more mitochondria and increased ROS, leading to impaired peripheral survival (Pua et al., 2009). Mitophagy is also important for maturation of invariant natural killer T (iNKT) cells. During differentiation of iNKT cells in the thymus, autophagy is activated to rid of excess mitochondria. Atg7 T-cell conditional KO mice had decreased iNKT cell development and lacked mature iNKT cells in peripheral organs due to increased levels of mitochondrial ROS and subsequent apoptosis (Salio et al., 2014). Mitophagy was also shown to be important for degrading dysfunctional ROS-producing mitochondria in natural killer (NK) cells in a mouse model of cytomegalovirus infection. Mitophagy enabled NK cells to effectively transition from effector cells to memory cells. Mitophagy in NK cells in this model was mediated by Bnip3 and Nix (O'Sullivan et al., 2015).

Tissue injury

Autophagy and mitophagy have been shown to be protective in several models of tissue injury including the brain, liver, heart, and kidney. Parkin and mitophagy play protective roles in the brain to prevent development of neurodegenerative diseases, as previously discussed. Studies from our lab have demonstrated that Parkin and mitophagy also provide protection in the liver against acetaminophen (APAP) and alcohol-induced liver injuries (Ni et al., 2012, 2013a,b; Williams et al., 2015a,b). Pharmacological induction of autophagy by rapamycin protected against APAP-induced liver injury, likely by removing damaged mitochondria via mitophagy and reducing ROS production (Ni et al., 2012). In addition, autophagy activation in the liver was protective during APAP overdose by removing acetaminophen adducts (Ni et al., 2016). Parkin-induced mitophagy may play a role in protection against APAP because Parkin translocated to mitochondria in mice after APAP treatment, and Parkin knockdown in mice exacerbated APAP-induced liver injury (Williams et al., 2015a). In addition, lysosomal cholesterol accumulation caused by acid sphingomyelinase deficiency was recently shown to exacerbate APAP-induced liver injury by inhibiting mitophagy. Acid sphingomyelinase deficiency inhibited mitophagy by blocking fusion of autophagosome-containing mitochondria with lysosomes after APAP treatment (Baulies et al., 2015). Our work also suggests that mitochondrial spheroid formation may also play a protective role in APAP overdose (Ding et al., 2012a,b; Ni et al., 2013a), but this remains to be determined.

Induction of autophagy and mitophagy also protect against alcohol-induced liver injury and steatosis (Ding et al., 2010a, 2011; Lin et al., 2013; Lu and Cederbaum, 2015). Parkin may also have a role in mediating mitophagy during alcoholic liver disease because we found that Parkin KO mice had increased liver injury, mitochondrial damage, oxidative stress, and steatosis in addition to decreased mitochondrial respiration, β-oxidation, cytochrome c oxidase activity and mitophagy compared to wildtype mice after alcohol feeding (Williams et al., 2015b). Alcohol also caused an increased number of autophagosomes containing

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mitochondria and Parkin colocalization with mitochondria and autophagosomes in rat hepatocytes after alcohol treatment (Eid et al., 2016).

Parkin and mitophagy may also have protective roles in the heart. Parkin translocated to mitochondria in cardiomyocytes during myocardial infarction. In addition, Parkin KO mice had decreased mitophagy and an accumulation of dysfunctional mitochondria after myocardial infarction, which reduced survival rates (Kubli et al., 2013). Parkin-mediated mitophagy also protected against ischemia-induced cardiac injury (Huang et al., 2011). Moreover, the mitophagy inhibitor TP53-induced glycolysis and apoptosis regulator exacerbated ischemia-induced cardiomyocyte cell death and cardiac injury (Hoshino et al., 2012). Cytosolic p53 inhibits Parkin mitochondrial translocation and subsequent mitophagy, leading to mitochondrial dysfunction and heart failure in mice after doxorubicin treatment (Hoshino et al., 2013). Pink1 protein levels were also reduced in end-stage human heart failure (Billia et al., 2011). In addition, Pink1 KO mice had high levels of oxidative stress, mitochondrial dysfunction, cardiomyocyte death and cardiac injury (Billia et al., 2011) and were more susceptible to I/R injury (Siddall et al., 2013).

Mitophagy also plays an important role in maintaining mitochondrial homeostasis in kidney to prevent injury and disease. Mitochondrial dysfunction is a hallmark of acute kidney injury, and damaged mitochondria require removal by mitophagy in order to prevent pathogenesis due to accumulation of ROS and release of DAMPs (Darisipudi and Knauf, 2016; Duann et al., 2016). Cisplatin is well known to induce acute kidney injury. Zhao et al. (2017) found that knockdown of Pink1/Parkin in human renal proximal tubular cells exacerbated cisplatin-induced kidney injury, and overexpression of Pink1/Parkin maintained mitochondrial function and protected against injury by promoting mitophagy. Mitophagy also has a role in protection against injury in diabetic kidney disease. Mitophagy is downregulated in diabetic kidney disease, which exacerbates tubule injury and disease pathogenesis. Treatment with the mitochondrial targeted antioxidant mitoQ reversed tubular damage caused by diabetic kidney disease by inducing mitophagy (Zhan et al., 2015; Xiao et al., 2017). These data suggest that mitophagy plays a role in maintaining mitochondrial homeostasis and health in the kidney, but specific mechanisms for mitophagy induction in the kidney require further exploration.

Analysis of mitophagy

There are several mechanisms for studying mitophagy in yeast and mammalian cells, which have led to remarkable progress in the mitophagy field by providing a better understanding of mitophagy mechanisms and the role of mitophagy in many diseases. However, reliable quantitative assays for studying mitophagy in mammalian cells have been lacking, and most of the current knowledge of the mitophagy pathway came from *in vitro* experiments. Excitingly, novel *in vivo* methods have recently been developed for measuring mitophagy (Williams et al., 2017), which will help further our understanding of the mitophagy pathway. Mitophagy is a dynamic process that begins with engulfment by an autophagosome and ends with degradation in a lysosome. Therefore, a lysosome degradation inhibitor, such as chloroquine, should be used to accurately monitor mitophagy flux for each of these assays. It is important to use a variety of assays when monitoring mitophagy in both *in vitro* and *in*

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vivo models. A thorough review of current modulators of mitophagy was recently published (Georgakopoulos et al., 2017). Examples of mitophagy modulators include protonophores CCCP and FCCP, potassium ionophores valinomycin and salinomycin, respiratory chain inhibitors Antimycin A and Oligomycin A, Parkinsonian toxins paraquat and rotenone, iron chelators, and Pink1 enhancers among others (Georgakopoulos et al., 2017). Current assays for studying and quantifying mitophagy are discussed below.

Electron microscopy

Electron microscopy (EM) is one of the best tools for studying mitophagy because it provides visualization of double-membrane autophagosomes that contain cytoplasmic material and/or organelles at various stages of degradation in the lysosome. It also allows for accurate identification of structures and gives an idea of what is happening to mitochondria in relevance to other structures within the cell. Early stages of mitophagy can be identified via EM because mitochondrial structures such as cristae can be seen inside of double-membrane autophagosomes (Figure 3). Late-stage autolysosomes can also be identified in EM by their single membrane, and mitochondria in various stages of degradation can be seen.

Cautionary notes—Pros: EM provides visual evidence of early and late stages of mitophagy. Cons: Due to limited cell numbers/sections, it is difficult to use EM to quantify mitophagy activation. Data from EM studies may be highly variable, so it is important to analyze several cells from different sections, if possible. Unbiased sample and microscope section selections are important, and the analyst must be familiar with autophagosome, lysosome, and organelle structures for proper identification. Expertise is also required for sample preparation to avoid sample artifacts.

Fluorescence microscopy for mitochondria-autophagosome or mitochondria-lysosome colocalization

Two approaches can be used for determining colocalization of mitochondria with either autophagosomes or lysosomes depending on if the cell being imaged is live or fixed. For live-cell imaging microscopy, MitoTracker dye should be used. MitoTracker dye (ThermoFisher Scientific, USA) is taken up electrophoretically by mitochondria and is dependent upon mitochondrial membrane potential, making it useful for live cell imaging. It covalently binds to mitochondrial proteins and remains in mitochondria even if they become depolarized (Elmore et al., 2001). Colocalization of mitochondria with autophagosomes can be examined using a GFP-LC3 (for autophagosome labeling) and MitoTracker Red co-labeling approach, which will produce yellow puncta when mitochondria are colocalized with autophagosomes (Figure 4, arrows). For fixed cells, mitochondria can be visualized using immunostaining for mitochondrial proteins. A co-labeling approach using antibodies against mitochondrial proteins such as Tom20, VDAC, or COX IV and GFP-LC3 labeled autophagosomes may be used to look for mitophagy activation. Immunostaining may also be used for monitoring colocalization of mitochondria with lysosomes. Fixed cells can be stained using antibodies specific for mitochondrial proteins and an antibody against lysosomal-associated membrane protein 1 (LAMP-1) or LAMP-2. For live cell imaging, MitoTracker stained mitochondria and LysoTracker (ThermoFisher Scientific, USA) stained

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lysosomes can also be visualized. Cells stained with MitoTracker and LysoTracker can also be fixed followed by confocal microscopy analysis. To assess mitophagic flux using above assays, one should also combine the lysosomal inhibitors such as chloroquine or Bafilomycin A1 to block lysosomal degradation of mitochondria.

Cautionary notes—Pros: These assays can visualize a large number of cells and can also monitor the entire dynamic process of mitophagy in a live cell setting. Cons: This assay demonstrates colocalization of LC3 positive autophagosomes with mitochondria, but it cannot be used to determine mitochondrial degradation. In addition, quantifying colocalization of mitochondria with autophagosomes or lysosomes is subjective, so consistent criteria need to be used across experiments. It should also be noted that not all GFP-LC3 positive puncta are autophagosomes. Some positive GFP-LC3 positive puncta may be GFP-LC3 aggregates and produce misleading results. Furthermore, LysoTracker is not specific for lysosomes because it stains all acidic compartments, and lysosomal pH change can affect the Lysotracker staining patterns.

Mitochondrial mass

The last step of the degradation process of mitophagy can be monitored by determining mitochondrial mass, which can be measured in several ways. Mitochondrial mass can be measured using MitoTracker staining and flow cytometry where fluorescence intensity is decreased during loss of mitochondrial mass (Mauro-Lizcano et al., 2015). Immunohistochemistry or Western blot using antibodies for mitochondrial proteins can also be used to assess mitochondrial mass. Mitochondrial matrix and inner membrane proteins should be used for monitoring loss of mitochondrial mass due to mitophagy because outer mitochondrial membrane proteins can be degraded by the proteasome (Tanaka et al., 2010; Chan et al., 2011; Yoshii et al., 2011). Finally, a citrate synthase assay may be used to measure mitochondrial mass (Watts et al., 2004; Hargreaves et al., 2007). Citrate synthase is a citric acid cycle mitochondrial matrix enzyme that can be used to measure mitochondrial content because it is not affected by mitochondrial dysfunction (Watts et al., 2004; Hargreaves et al., 2007; Gegg et al., 2010). In Parkin-overexpressing SH-SY5Y cells, CCCP treatment induced mitophagy and resulted in decreased mitochondrial mass, which correlated with decreased citrate synthase activity (Gegg et al., 2010).

Cautionary notes—Pros: These assays are more objective and quantitative than previous assays. Cons: MitoTracker staining depends on mitochondrial membrane potential and may not stain damaged mitochondria. Measuring mitochondrial protein degradation by Western blot can suggest mitophagy activation, but high levels of mitophagy may be necessary for successful detection of mitochondrial degradation. In addition, data interpretation may be challenging because mitochondrial proteins can be degraded at different rates, and mitochondrial membrane proteins can be degraded by the proteasome in addition to autophagy (Tanaka et al., 2010; Chan et al., 2011; Yoshii et al., 2011). Therefore, several mitochondrial proteins including inner membrane and matrix proteins should be used if monitoring mitochondrial mass by immunohistochemistry or Western blot. Results from citrate synthase activity should be interpreted with caution because they may be non-specific. It is currently not clear if other factors affect citrate synthase activity.

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OM45-GFP processing assay

The Om45-GFP processing assay is useful for detecting mitophagy in yeast (Kanki et al., 2009a). Om45-GFP is a mitochondrial outer membrane protein tagged with GFP. During yeast mitophagy, Om45 along with mitochondria are delivered to the vacuole for degradation. Om45 is degraded while GFP is relatively stable and accumulates in the vacuole. The free GFP can be measured by Western blotting.

Cautionary notes—Pros: This assay has high specificity and is more objective and quantitative. Cons: GFP stability in lysosomes may vary in different cell types depending on the acidity and activity levels of their lysosomes. It seems that this assay is more reliable in yeast systems but not in mammalian cells due to different pH values in vacuoles vs lysosomes (more acidic).

Antibodies against phosphorylated ubiquitin (p-S65-Ub)

Phosphorylated ubiquitin (p-Serine65-Ub) is formed during Parkin-Pink1 mediated mitophagy as previously discussed, and antibodies to p-Serine65-Ub may be used to detect mitophagy activation by this pathway (Fiesel et al., 2015; Klionsky et al., 2016). Under basal conditions, p-Serine65-UB has low detection levels, but it is induced during mitochondrial stress and is further increased in the presence of Parkin in cells and primary neurons (Fiesel et al., 2015). The p-Serine65-Ub antibody can be used as a marker for mitophagy activation when combined with other assays.

Cautionary notes—Pros: The p-Serine65-Ub should be a specific marker of Parkin-Pink1 induced mitophagy. Cons: The p-Serine65-Ub antibody is novel and specificity for mitophagy activation needs to be further evaluated.

mt-Keima

Mitophagy can be quantified *in vitro* or *in vivo* by fluorescence imaging using a mitochondria-matrix targeted mKeima (mt-Keima) fluorescent protein, which accumulates in lysosomes and changes color depending on if it is in a neutral (green color) or acidic (red color) pH environment. Mitochondria undergoing mitophagy will exhibit a red signal when labeled with mt-Keima after delivery to lysosomes. In addition, the excitation peak of mt-Keima shifts from 440 nm to 586 nm when mitochondria are delivered to lysosomes, indicating mitophagy activation. Mt-Keima can also be combined with GFP-Parkin for assessing mitophagy activation. While fluorescence intensity for mt-Keima increases during mitophagy activation, Parkin fluorescence decreases due to its degradation in the lysosome (Katayama et al., 2011; Sun et al., 2015).

A transgenic mouse expressing mt-Keima was recently developed by Sun et al. to monitor mitophagy *in vivo*. Interestingly, levels of mitophagy differed greatly among different mouse tissues with high levels of basal mitophagy in the heart and low levels in the thymus, and mitophagy levels were affected by changes in genetics and environmental conditions. Furthermore, a decline in mitophagy was seen with aging in these mice (Sun et al., 2015). Therefore, these mice may be used to monitor mitophagy levels in various disease states or treatment conditions.

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Cautionary notes—Pros: mt-Keima can monitor mitophagy with great specificity *in vitro* and *in vivo* without affecting mitochondrial function. For *in vitro* measurement, mt-Keima can be used to measure mitophagy in a live cell setting using a large population of cells (Sun et al., 2015). Cons: For *in vitro* measurement, mt-Keima samples cannot be fixed without disrupting the pH gradient. Excitation spectrums for Keima may also overlap resulting in an orange color when expressed in the lysosome instead of red. In addition, mt-Keima results may be affected by long exposure times during imaging. For *in vivo* analysis, the precise location of analysis in mouse tissue may be difficult to determine unless a separate fluorescent reporter is used or there are easily identifiable morphological landmarks present. There is also a possibility that *ex vivo* imaging of tissue from mt-Keima mice may contain artifacts as Keima is pH dependent (Sun et al., 2015).

MitoTimer

MitoTimer is a Timer fluorescent protein targeted to the mitochondrial matrix that changes its emitted fluorescence from green to red with protein maturation, and it can be used to monitor mitophagy both *in vitro* and *in vivo* during mitochondrial aging or stress (Ferree et al., 2013; Hernandez et al., 2013; Laker et al., 2014; Stotland and Gottlieb, 2016). MitoTimer was created by fusing the Timer protein with the mitochondrial targeting sequence of the COX8A subunit (Terskikh et al., 2000; Ferree et al., 2013; Hernandez et al., 2013). Alterations in green MitoTimer expression levels suggest changes in mitochondrial import, biogenesis, or synthesis. Alterations in red expression levels without changes in green expression levels indicate mitochondrial degradation (Trudeau et al., 2014). We used MitoTimer to monitor mitochondrial biogenesis in primary cultured mouse hepatocytes (Williams et al., 2017). As can be seen, MitoTimer signals display green-only mitochondria (newly synthesized mitochondria), red-only mitochondria (old/aging mitochondria) and yellow mitochondria (intermediate stage of mitochondria) (Figure 5). The majority of mitochondria are yellow, likely indicating the dynamic fusion process of matured/old mitochondria (red) with newly synthesized mitochondria (green) in hepatocytes.

MitoTimer analysis may be performed by both flow cytometry and fluorescence microscopy (Ferree et al., 2013; Hernandez et al., 2013). Analysis by fluorescence microscopy allows for fixation of samples and simultaneous analysis of all samples in an experimental group (Hernandez et al., 2013). However, bleaching of MitoTimer must be avoided during analysis, and imaging settings should be consistent among all samples within an experimental group (Ferree et al., 2013; Trudeau et al., 2014). During flow cytometry analysis, excitation at 488 nm and 543 nm and emission at 497–531 nm and 583–695 nm should be used for green and red-fluorescing mitochondria, respectively (Trudeau et al., 2014). It is recommended to use a DOX-inducible vector to synchronize MitoTimer expression in cells. In addition, MitoTimer expression should be monitored for at least a week to confirm stability of red and green expression levels, and its effects on mitochondrial structure and function should be evaluated before beginning experiments (Trudeau et al., 2014).

Cautionary notes—Pros: MitoTimer may be used both *in vitro* and *in vivo* to monitor mitophagy. MitoTimer may also be used *in vitro* and *in vivo* to monitor mitochondrial biogenesis. Cons: MitoTimer protein typically matures from green to red expression within

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48 h and remains in the mitochondria after maturation for several days. Mitochondrial half-lives vary among different tissues. Therefore, it may be difficult to distinguish new red-fluorescing mitochondria from mitochondria that retained red expression in mitochondria with longer half-lives (Terskikh et al., 2000; Hernandez et al., 2013; Gottlieb and Stotland, 2015). In addition, changes in MitoTimer fluorescence are not specific for mitochondrial damage, degradation, or biogenesis. MitoTimer must be combined with other methods to accurately determine causes of changes in fluorescence (Ferree et al., 2013; Gottlieb and Stotland, 2015). Furthermore, MitoTimer expression may be affected by temperature, oxygen levels, and light exposure (Terskikh et al., 2000; Hernandez et al., 2013; Gottlieb and Stotland, 2015). Finally, even though MitoTimer has been used in *in vivo* studies, it has so far been limited to *Drosophila* and mouse skeletal and heart muscle. Development of wholebody transgenic MitoTimer mice or conditional MitoTimer expression in other mouse tissues would greatly benefit the mitophagy field.

Mito-mRFP/mCherry-EGFP fluorescent reporter assay: mito-QC

Mito-QC is a pH-sensitive mitochondrial fluorescent probe made up of a tandem mCherry-GFP tag fused to the mitochondrial targeting sequence of the outer mitochondrial membrane protein FIS1. The mito-QC mouse allows for monitoring of mitophagy *in vivo*. Under steady-state conditions, mito-QC displays red and green fluorescence. When mitophagy is induced, mito-QC displays the mCherry red signal, which is stabilized when mitochondria are delivered to the lysosome. The green GFP signal is quenched upon delivery of mitochondria to the lysosome (McWilliams et al., 2016).

The mito-QC transgenic mouse has ubiquitous expression of mito-QC and allows for monitoring of mitophagy *in vivo* with high specificity. Mito-QC does not appear to affect mitochondrial structure or function. Mito-QC also allows for monitoring of mitochondria populations within different areas of a particular tissue because it enables visualization of entire mitochondrial networks making it a very useful new tool for studying mitophagy and mitochondria *in vivo* (McWilliams et al., 2016).

Cautionary notes—Pros: Tissues from mito-QC mice can be fixed before analysis if the fixative is maintained at 7.0 pH. In addition, there is no overlap in emission spectra using the mito-QC mouse. Cons: Expression levels of mito-QC vary among different tissues with lower expression in liver. In addition, the precise location of analysis in mouse tissue may be difficult to determine unless a separate fluorescent reporter is used or there are easily identifiable morphological landmarks present. Fluorophore selection for additional immunolabeling is also limited because mito-QC uses red and green fluorescence (McWilliams et al., 2016). Finally, as FIS1 is on the outer mitochondrial membrane, it is unclear whether some mito-QC could be degraded by the ubiquitin proteasome system instead of autophagy. To avoid this possibility, Chan's group developed a similar probe (Cox8-EGFP-mCherry) that targets the mitochondrial inner membrane protein Cox8 (Rojansky et al., 2016).

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Future perspectives and concluding remarks

Mitophagy plays critical roles in maintaining cellular homeostasis and functions. Mitophagy is important for the development and remodeling of many tissues. Impaired mitophagy contributes to the pathogenesis of many human diseases. With the progress of our understanding regarding the molecular mechanisms that regulate mitophagy and the development of new reliable quantitative assays to monitor mitophagy, we are now able to reveal and better understand basic biochemical mechanisms regulating mitophagy and its pathophysiological roles *in vivo*. Despite these recent significant advances in our knowledge of selective mitophagy, many questions remain unanswered. While ubiquitination of mitochondrial proteins is important for mitophagy, it remains unclear whether a special group of ubiquitinated mitochondrial proteins are required for recruitment of autophagy receptor proteins to mitochondria. How different cell types utilize different receptor proteins for mitophagy also needs to be further investigated. Moreover, in the Pink1-Parkin-independent mitophagy pathways, additional alternative ubiquitin E3 ligases remain to be identified, although some of them have been recently discovered such as Mul1. In addition, more details need to be studied regarding the coordination between the ubiquitin proteasome system and autophagy for removal of mitochondria. Answering these questions will improve our knowledge on the general mechanisms of mitochondrial homeostasis and may lead to new therapeutic approaches for treatment of diseases with dysfunctional mitochondria.

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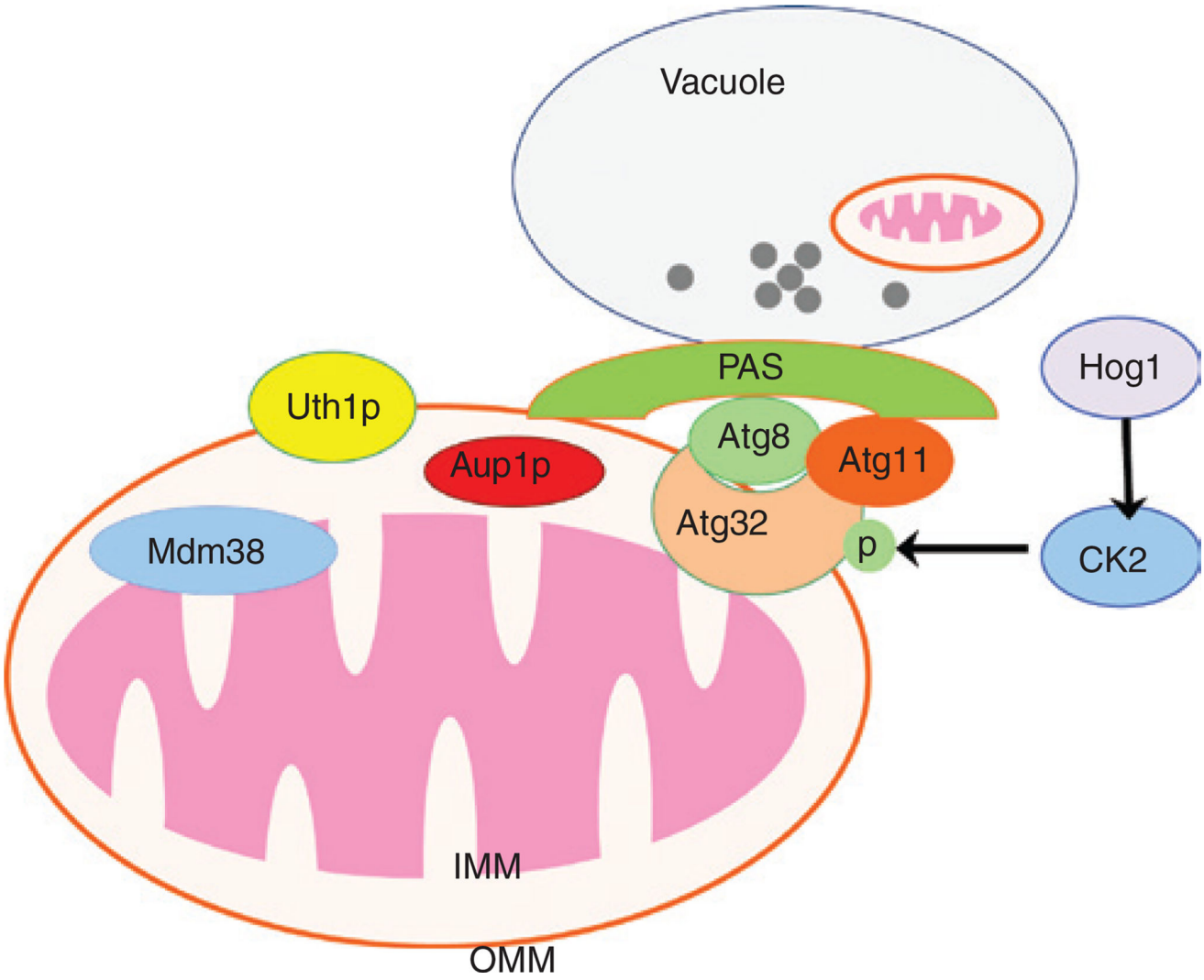

Figure 1. A Proposed model for mitophagy in yeast cells

In yeast cells, the outer mitochondrial membrane protein Uth1p and the intermembrane space protein Aup1p are required for mitophagy whereas deletion of the mitochondrial inner membrane protein Mdm38 leads to mitophagy. The mitophagy receptor protein Atg32 is an outer mitochondrial membrane protein, which interacts with Atg11 and Atg8 positive phagophore assembly site (PAS) that allows autophagosomes to specifically envelop Atg32 positive mitochondria. CK2 directly phosphorylates Atg32 to promote mitophagy, which is mediated by Hog1 that acts upstream of CK2. Autophagosomes enveloped with mitochondria then fuse with the vacuole where the mitochondria are degraded. IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane.

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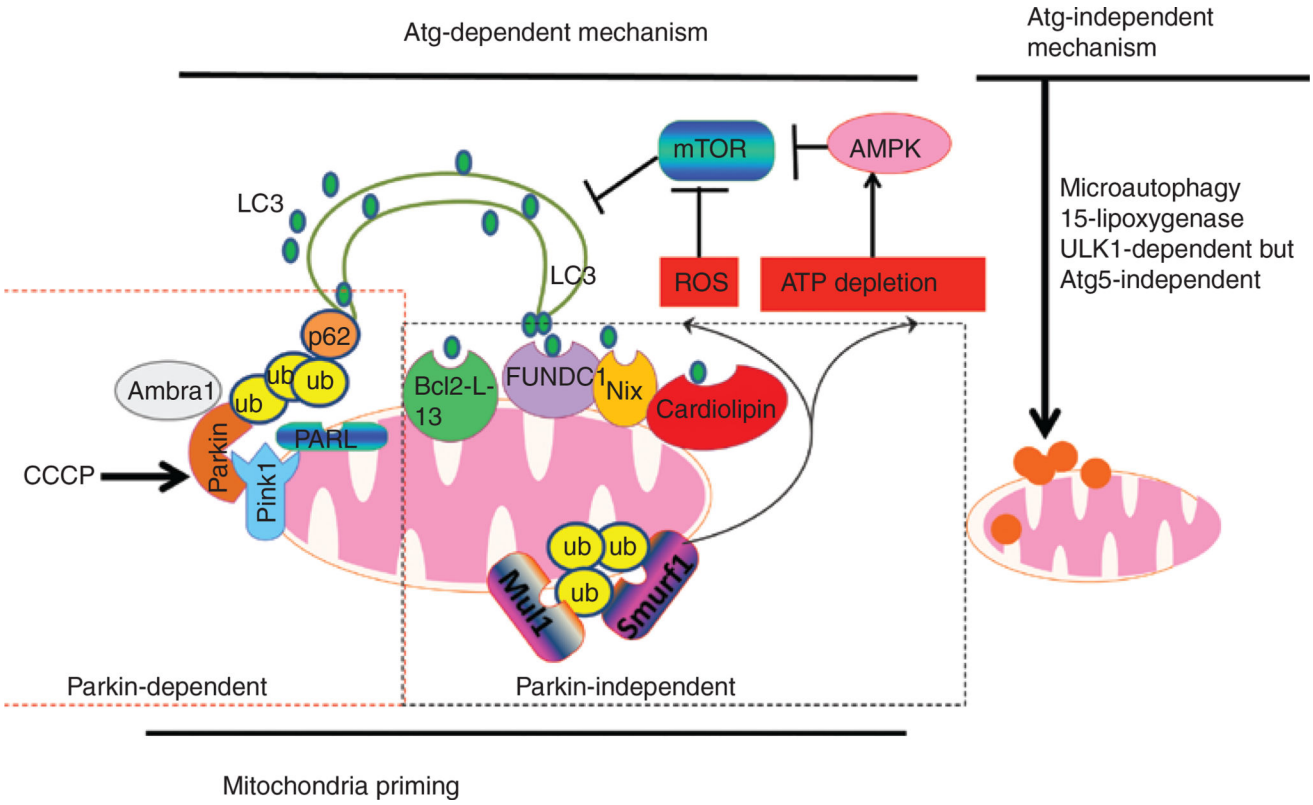

Figure 2. Proposed models for mitophagy in mammalian cells

We propose a two-step mitophagy model in mammalian cells: the induction of canonic Atg-dependent macroautophagy and mitochondrial priming. The induction of canonic autophagy requires Atg proteins and mTOR suppression, which is mediated by mitochondrial damage-induced ROS production and ATP-depletion-mediated AMPK activation. Priming of mitochondria is mediated by multiple mechanisms that could be Parkin-dependent or Parkin-independent. In the presence of Parkin, one common mechanism is that mitochondrial depolarization (e.g. following CCCP treatment) results in impaired PARL-mediated Pink1 cleavage, leading to Pink1 stabilization and Parkin recruitment to the mitochondria. Mitochondria-located Parkin promotes ubiquitination of outer membrane proteins, which may either be degraded via the proteasome or serve as binding partners for autophagy receptor proteins such as p62. p62 may in turn act as an adaptor molecule through direct interaction with LC3 to recruit autophagosomal membranes to the mitochondria. Parkin can also interact with Ambra1, which in turn activates the PI3K complex around mitochondria to facilitate selective mitophagy. For the Parkin-independent mechanism, damaged mitochondria (particularly under hypoxia conditions) may increase Fundc1 and Nix expression, which may in turn recruit autophagosomes to mitochondria by direct interaction with LC3 through their LIR domains. Upon mitochondrial depolarization, Smurf1 and Mul1, two E3 ubiquitin ligases, also target mitochondria to promote mitophagy, most likely via the ubiquitination of mitochondrial proteins. Atg-independent mitophagy is less understood, but Ulk1-dependent and Atg5-independent macroautophagy or 15-lipoxygenase have been shown to promote mitochondrial degradation. Direct lysosomal invagination or interaction with damaged mitochondria (microautophagy) could also play a role.

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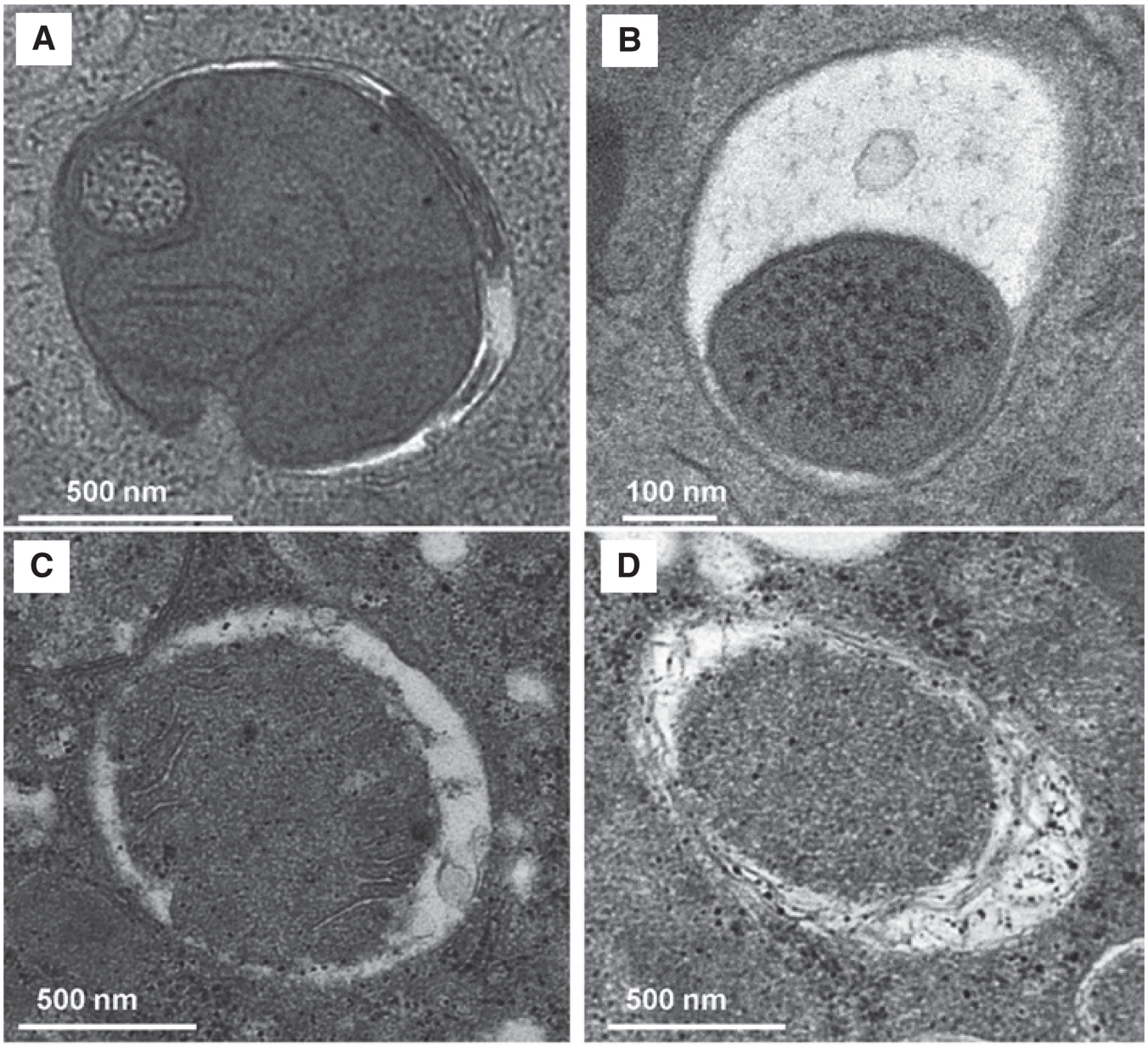

Figure 3. Electron microscopic (EM) depiction of mitophagy

Wild type C57BL/6 mice were treated with acetaminophen (i.p., 500 mg/kg) for 6 h (panels A, B) or ethanol (gavage, 4.5 g/kg) for 6 h (panels C, D). Thin liver sections were processed for EM studies. Panels (A, C) show an early double-membraned autophagosome enveloping mitochondria; panels (B, D) show a late autolysosome containing degrading mitochondria.

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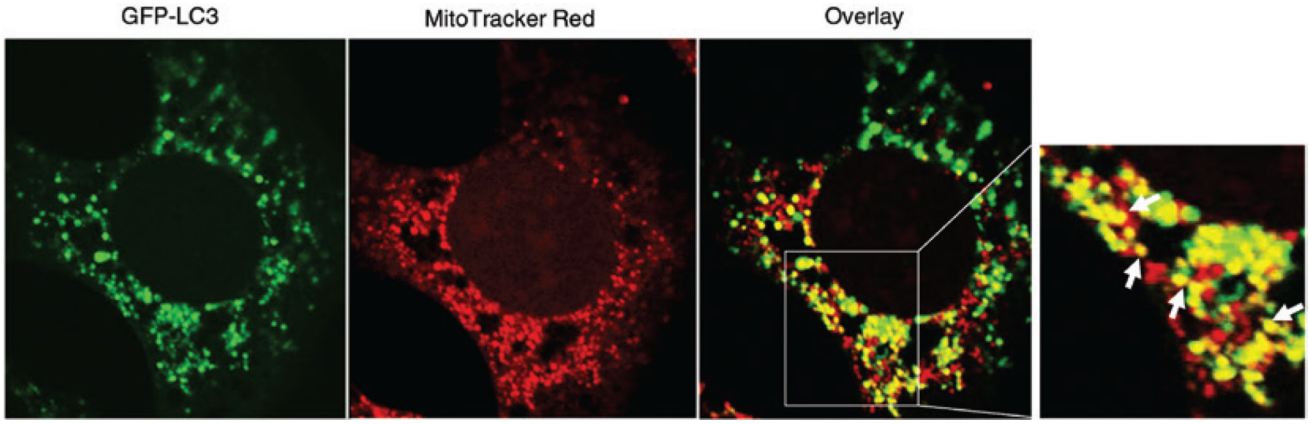

Figure 4. Confocal microscopy for the co-localization of autophagosomes with mitochondria

GFP-LC3-expressing MEFs were first loaded with MitoTracker Red (50 nm, ThermoFisher Scientific, USA) for 30 min and then treated with CCCP (30 μm) plus the lysosomal protease inhibitors E64D (10 μm) and Pepstatin A (10 μm) for 6 h. The cells were fixed with 4% paraformaldehyde followed by confocal microscopy. Arrows: yellow dots represent co-localized GFP-LC3 positive autophagosomes with red fluorescence labeled mitochondria.

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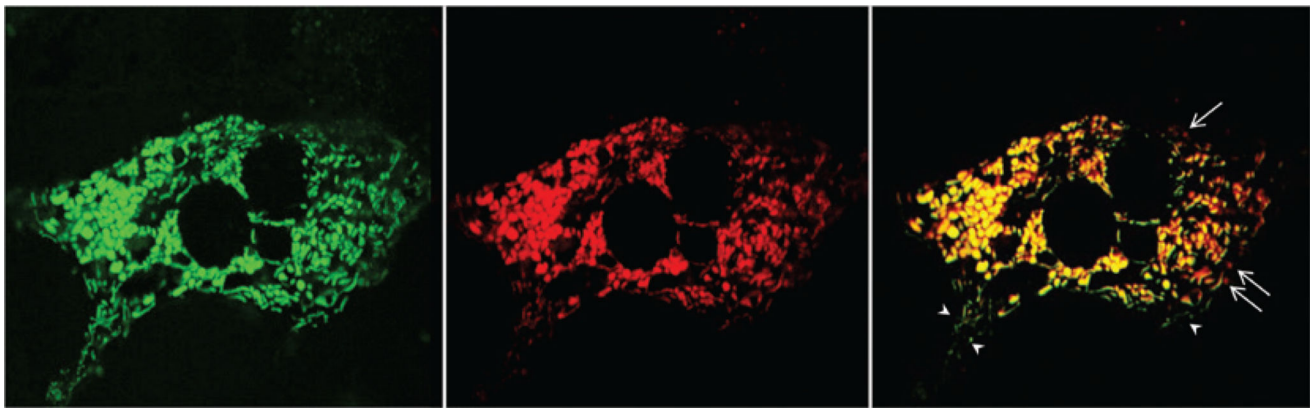

Figure 5. Confocal microscopy for monitoring mitochondria biogenesis in primary hepatocytes infected with AAV-Mito-Timer

Primary mouse hepatocytes were infected with AAV-TBG-MitoTimer (1000 GC/cell) for 72 h. Hepatocytes were fixed with 4% paraformaldehyde in PBS followed by confocal microscopy. Arrows: red-only old mitochondria; arrow heads: green-only newly synthesized mitochondria.