

## Review

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# How to get rid of mitochondria: crosstalk and regulation of multiple mitophagy pathways

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**Abstract:** Mitochondria are indispensable cellular organelles providing ATP and numerous other essential metabolites to ensure cell survival. Reactive oxygen species (ROS), which are formed as side reactions during oxidative phosphorylation or by external agents, induce molecular damage in mitochondrial proteins, lipids/membranes and DNA. To cope with this and other sorts of organellar stress, a multi-level quality control system exists to maintain cellular homeostasis. One critical level of mitochondrial quality control is the removal of damaged mitochondria by mitophagy. This process utilizes parts of the general autophagy machinery, e.g. for the formation of autophagosomes but also employs mitophagy-specific factors. Depending on the proteins utilized mitophagy is divided into receptor-mediated and ubiquitin-mediated mitophagy. So far, at least seven receptor proteins are known to be required for mitophagy under different experimental conditions. In contrast to receptor-mediated pathways, the Pink-Parkin-dependent pathway is currently the best characterized ubiquitin-mediated pathway. Recently two additional ubiquitin-mediated pathways with distinctive similarities and differences were unraveled. We will summarize the current state of knowledge about these multiple pathways, explain their mechanism, and describe the regulation and crosstalk between these pathways. Finally, we will review recent evidence for the evolutionary conservation of ubiquitin-mediated mitophagy pathways.

**Keywords:** autophagy; mitochondria; mitochondrial quality control; mitophagy; ubiquitin.

## Introduction: autophagy – insights into an important and highly conserved cellular process

The evolution of autophagy was possibly nearly as important for eukaryotes as the endosymbiosis of  $\alpha$ -proteo-like bacteria leading to a mitochondria-containing eukaryotic ancestor. While bacteria mainly rely on a battery of proteolytic machineries for removal and recycling of unwanted cell material, eukaryotes additionally have evolved the ability to remove such material and even entire organelles within a specialized membrane-enclosed compartment: the lysosome/vacuole. Autophagy (from greek ‘auto’ and ‘phagos’, meaning ‘self’ and ‘eating’) is a highly conserved, tightly regulated and specific process that has been linked to intracellular quality control, cellular homeostasis and cell differentiation or immunity. There are different forms of autophagy described including macroautophagy, microautophagy, and chaperone-assisted autophagy. In this review, we focus on macroautophagy (hereafter only termed autophagy) which involves the engulfment of any material in a double-membrane enclosed autophagosome which subsequently fuses with lysosomes.

The process of selective autophagy, in contrast to bulk autophagy, involves the identification, engulfment, and disposal of a specific cellular compartment or organelle as a result of a defined signal or more general under cellular stress conditions, e.g. starvation. The specific selection of a cargo typically needs a cargo-specific receptor protein, marking it for degradation. On the contrary, the engulfment and disposal utilizes a general machinery, recruiting a double-membrane, the autophagosome, around the cargo. The last step of autophagy is the fusion of the autophagosome with the lysosome, creating an autolysosome, where lysosomal enzymes degrade the entire autophagosomal content to small reusable units (reviewed in Lamb et al., 2013; Anding and Baehrecke, 2017). To distinguish these cargo-specific forms of selective autophagy the following terms have been coined: ER – ER-phagy or reticulophagy, ribosome – ribophagy, peroxisome – pexophagy, pathogens – xenophagy, and mitochondria – mitophagy.

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Initially, the general machinery required for autophagy was identified in baker's yeast, but most of the factors and mechanisms are well conserved in mammalian cells. The core complex for the initiation of autophagy, acting downstream of mechanistic target of rapamycin (mTOR), is the Atg1 kinase complex consisting of Atg1 (named ULK1 in mammals), Atg13 and a subcomplex of Atg17-Atg31-Atg29 (only in yeast) that has a scaffold function (Mizushima, 2010). In mammalian cells ULK1 is known to interact with ATG13, RB1CC1 (also known as FIP200) and ATG101 (Hosokawa et al., 2009; Jung et al., 2009). The next step, vesicle nucleation, is mediated by the phosphatidylinositol-3-kinase (PI3K) complex consisting of VPS34, VPS15, Beclin 1, activating molecule in Beclin-1-regulated autophagy (AMBRA1), and ATG14L or VPS38 (Itakura et al., 2008). The expansion of the vesicle is facilitated by two possible pathways: The first pathway employs the covalent conjugation of Atg12 to Atg5 by Atg7 and the E2-like factor Atg10 (Mizushima et al., 1998). This Atg12-Atg5 complex interacts with Atg16-like 1 forming an E3-like active complex that is essential for phagophore expansion (Mizushima et al., 2003). Alternatively, pro-LC3 is converted to microtubule-associated protein 1A/1B-light chain 3 (LC3)-I and further to LC3-II (PE-conjugated LC3) by the protease Atg4, the E1-like protein Atg7 and the E2-like protein Atg3 (Kabeya et al., 2000). The Atg12-Atg5-Atg16-L1-complex can also facilitate LC3-lipidation (Hanada et al., 2007). The lipid conjugation of LC3 mediates its attachment to the phagophore membrane, where it adopts its role in phagophore expansion and cargo recognition. Another important protein involved in cargo recognition is p62 (also called SQSTM1). It can bind to polyubiquitin chains, which are crucial in, e.g. mitophagy, with one side and to LC3-II with its other side, acting as an adapter protein (Matsumoto et al., 2011). Opposed to this, receptor proteins are already located at the cargo and bind directly to LC3-II promoting autophagy of the respective cargo. Receptors for mitophagy are mainly LC3-interacting proteins located at the mitochondrial outer membrane (MOM) and will be discussed in detail later. The last step after completion of the engulfment of the cargo and phagophore closure is fusion of the matured autophagosome with a lysosome. The latter is mediated by Rab7, ESCRTs (endosomal sorting complexes required for transport) and soluble N-ethylmaleimide-sensitive-factor attachment receptors (SNAREs; reviewed in Lamb et al., 2013).

## Mitophagy – a critical endpoint of mitochondrial quality control

The selective removal of mitochondria, in particular of dysfunctional or excess mitochondria, by the autophagy

machinery is termed mitophagy. There are several reasons why a mitochondrion is targeted for removal involving distinct pathways. We will focus on these aspects in the following sections. Furthermore, we will elucidate the current view on the pathways of regulating mitophagy and try to shed light on the relationships and crosstalk between these pathways. First, we will summarize what we know about mitochondrial quality control and the different levels of quality control in general.

## Mitochondrial quality control – many levels ensure the functionality of mitochondria

One of the first quality checkups are ensured by the different protein import complexes – such as the translocase of the outer membrane (TOM), the translocases of the inner membrane TIM23 and TIM22 – importing nuclear encoded proteins into their target submitochondrial compartment (reviewed in Straub et al., 2016; Wiedemann and Pfanner, 2017). A critical contribution to the integrity of the mitochondrial proteome is made by chaperones, which assist in the folding of unfolded or wrongly folded proteins, and proteases, which digest aggregation-prone and dysfunctional proteins (reviewed in Koppen and Langer 2007; Voos et al., 2016). While these mechanisms are capable of dealing with damage on the level of individual proteins, quality control also happens on the level of the whole organelle. On this account, the dynamic nature of mitochondria plays an essential role in maintaining the quality not only of individual mitochondria, but of the whole network.

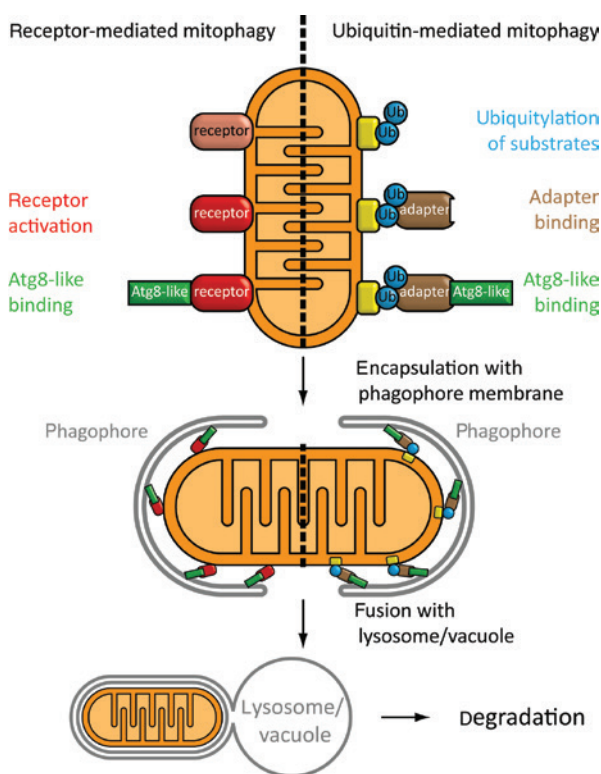
Mitochondria form a huge and dynamic network within a healthy cell and individual mitochondria constantly undergo fission and fusion. During fusion the contents of all subcompartments of mitochondria mix. This can be seen as beneficial, as scarcity of or a plethora of certain molecules in one mitochondrion is leveled during the fusion process and moreover damaged molecules get diluted. On the other hand, it could also lead to an infection-like spreading of damaged, potentially dominant-negative, molecules, impairing the overall quality (Figge et al., 2012). The latter may occur when a damaged mitochondrion can still reconnect to the network. Therefore a key function of the fission-fusion-dynamics is the spatial separation of dysfunctional organelles and adapting the relative rates of fusion and fission is predicted to be a critical factor in mitochondrial quality control (Figge et al., 2012). On the molecular level this is mediated by several GTPases, Mitofusin (Mfn) 1 and Mfn2 for the fusion of the MOM and optic atrophy protein 1 (OPA1) for the fusion of the mitochondrial inner membrane (MIM). OPA1 is alternatively spliced into eight isoforms and is

subsequently processed by several proteases including the m-AAA protease paraplegin, the i-AAA protease Yme1L, and OMA1 into long and short forms, l-OPA1 and s-OPA1, respectively. l-OPA1 is responsible for the MIM fusion process, hence cleavage to short isoforms impairs mitochondrial fusion (Ishihara et al., 2006; Griparic et al., 2007; Ehses et al., 2009; Head et al., 2009). The fission process involves the recruitment of the GTPase dynamin-related protein 1 (Drp1) to mitochondrial receptor proteins Fis1, Mff, MID49 and MID51 (Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010; Palmer et al., 2011; Loson et al., 2013). The action of Drp1 is regulated via phosphorylation mediated by Cdk1/Cyclin B or Protein kinase A and via dephosphorylation by Calcineurin (Cribbs and Strack, 2007; Taguchi et al., 2007). The quality of the mitochondrial network is in particular safeguarded by the fact that only healthy mitochondria are able to reconnect to the network, leading to the isolation of dysfunctional organelles which is a prerequisite for their selective elimination by mitophagy (Herlan et al., 2004; Lyamzaev et al., 2004; Duvezin-Caubet et al., 2006; Twig et al., 2008).

A third process, acting at a different level, is the formation of mitochondria-derived vesicles (MDVs). The proposed function of MDVs is the vesicular transport of mitochondrial cargo to peroxisomes and to late endosomes/lysosomes. While the importance for peroxisome-targeted transport is unclear, lysosomal targeting is thought to be a novel mitochondrial quality control pathway (Soubannier et al., 2012a). MDVs generated *in vitro* upon induction of artificial stress were rich in oxidized proteins and even contained different cargo depending on the stress target (Soubannier et al., 2012b). Interestingly, the *in vivo* formation of MDVs was dependent on the ubiquitin-mediated mitophagy proteins PTEN-induced putative kinase 1 (Pink1) and Parkin, indicating that MDVs are part of the mitochondrial quality machinery (McLelland et al., 2014). Although the exact relationship of MDV formation to other pathways of mitochondrial quality control are still to be elucidated, it appears that MDVs do nicely complement molecular quality control of misfolded and damaged proteins by chaperones/proteases and the organellar quality control by mitophagy (reviewed in Sugiura et al., 2014).

While the former two processes are a first line of defense against mitochondrial damage, they presumably also play a role during, or happen in parallel to, the complete removal of damaged mitochondria by mitophagy. Especially mitochondrial dynamics is tightly entangled with mitophagy, as dysfunctional mitochondria need to be spatially separated from the healthy network to isolate the damage and to ensure efficient engulfment by autophagosomal membranes. Importantly, there are two

distinct principal mechanisms (Figure 1) how mitochondria are marked for their removal: (1) receptor-mediated mitophagy, where a membrane/cargo-bound receptor is activated which then interacts directly with autophagosomal marker proteins (LC3/Atg8-like); and (2) ubiquitin-mediated mitophagy, which in mammals is commonly facilitated by the Pink1/Parkin pathway. The molecular signals for removal of a cargo are, in one case, the activation of the receptor and, in the other, the massive ubiquitylation of mitochondrial proteins. They are subsequently bound by bifunctional adapter proteins, containing ubiquitin-binding motifs as well as LC3-interacting regions (LIR) facilitating the binding to Atg8-like proteins such as LC3 and gamma-aminobutyric acid receptor-associated proteins (GABARAPs). Although these pathways have been analyzed separately in the past, there is emerging data on a possible crosstalk between these pathways.



**Figure 1:** Two mechanisms of mitophagy in mammalian cells. Left, receptor-mediated mitophagy – a MOM-localized receptor is activated by phosphorylation, increasing its binding affinity for Atg8-like proteins (LC3s or GABARAPs) and recruiting them to mitochondria. Right, MOM-proteins become highly ubiquitylated, recruit bifunctional adapter proteins (e.g. p62), which in turn recruit Atg8-like proteins. Bottom, binding of Atg8-like proteins initiates encapsulation of mitochondria into autophagosomes via the expansion of the phagophore membrane; fusion with the lysosome leads to the formation of autolysosomes ensuring degradation of the selected cargo. MOM, mitochondrial outer membrane.

These two principal pathways will be delineated one by one in the following sections.

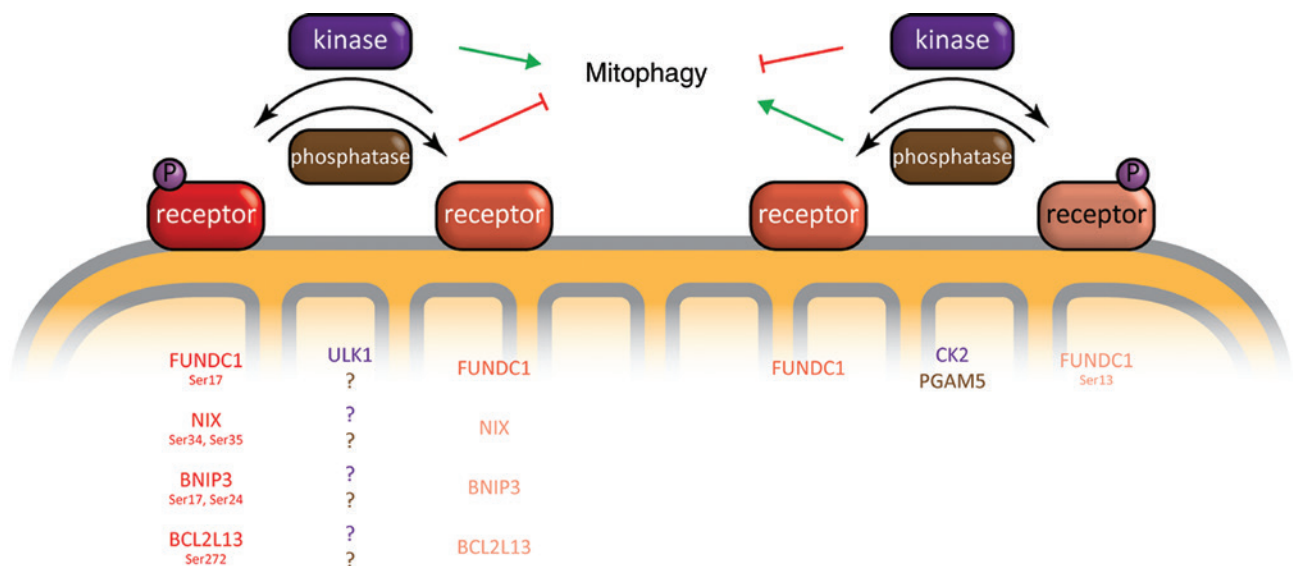
and the mammalian proteins that are known to be regulated by this are shown in Figure 2.

## Receptor-mediated mitophagy

Although this review focuses mainly on the molecular mechanisms of mitophagy in mammalian systems, the first receptor identified was Atg32 in baker's yeast (Kanki et al., 2009; Okamoto et al., 2009). It has no known ortholog in mammalian cells and there is apparently no mitophagy pathway which is independent of Atg32 in yeast (reviewed in Müller et al., 2015b). It can be considered as an archetype of mitochondrial mitophagy receptors consisting of a transmembrane domain (TMD), anchoring it to the MOM, and an Atg8-interacting motif (AIM), also termed LIR motif, facilitating the interaction with downstream autophagy factors. Although there are additional post-translational modifications of Atg32 known (Levchenko et al., 2016), it shares the prototypical way of receptor activation by phosphorylation of serine residues (S114 and S119) with its mammalian counterparts (Aoki et al., 2011; Kanki et al., 2013). A general model for receptor activation

### NIX (BNIP3L) and BNIP3

One of the first receptors discovered in mammalian cells was Nip3-like protein X (NIX), also termed BNIP3L. Initially being described as a proapoptotic or cell death factor (Chen et al., 1999), its essential role for the elimination of mitochondria from reticulocytes during erythrocyte differentiation became clear (Schweers et al., 2007; Sandoval et al., 2008). It is a MOM protein containing a LIR motif by which it recruits, e.g. GABARAP-L1 to damaged mitochondria (Novak et al., 2010). While most of the data link NIX to mitochondrial elimination in reticulocytes, it was connected to Pink1/Parkin-mediated mitophagy (Ding et al., 2010) and recently shown to be a substrate of Parkin (Gao et al., 2015). Furthermore, a recent study indicated a role for NIX during neuronal differentiation (Esteban-Martinez et al., 2017). The authors observed a hypoxia-induced, NIX-mediated mitophagy subsequently inducing a metabolic switch in developing retinal ganglion cells. The activation mechanism of NIX



**Figure 2:** Known mechanism of mitophagy receptor regulation.

Left, an unmodified receptor gets phosphorylated by a kinase, activating downstream mitophagy by, e.g. increasing Atg8-like affinity; this can be counteracted by phosphatases. Right, an unmodified receptor can be phosphorylated by a kinase, deactivating downstream mitophagy by, e.g. lowering Atg8-like affinity; this can be counteracted by phosphatases. Bottom, for NIX (BNIP3L), BNIP3, and BCL2L13 only the activating phosphorylation mechanism and the modified residues are known but the kinases and phosphatases have not been identified yet; for FUNDC1 both the activating and deactivating phosphorylation mechanisms, the modified residues, and participating enzymes are known. No activation mechanism is known so far for the receptors AMBRA1 and SMURF1. MIM-receptor prohibitin 2 probably lacks an activation mechanism as its activity is dependent on MOM-rupture. FKBP8 contains no residues N- and C-terminal of its LIR motif that can be phosphorylated and thus is likely to be activated via a different, unknown mechanism.



was very recently shown to be dependent on phosphorylation at residues Ser34 and Ser35. The study showed an enhanced interaction of phosphomimetic NIX-derived peptides with LC3 and presented the structure of an artificial interaction complex (Rogov et al., 2017). Though the initiation signal for the activation is not known so far, it has been shown that the small GTPase Rheb is recruited to mitochondria under conditions of enhanced oxidative phosphorylation, promoting the interaction of NIX with LC3-II (Melser et al., 2013).

Reminiscent to NIX, the LIR-containing MOM protein BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) is a proapoptotic protein that is also involved in hypoxia-induced mitophagy (Quinsay et al., 2010). In cardiomyocytes, it induces mitochondrial translocation of the fission factor Drp1 leading to fragmentation and subsequent induction of Pink1/Parkin-mediated mitophagy (explained in a latter section in detail) through translocation of Parkin to mitochondria. Furthermore, BNIP3-induced mitophagy is reduced in Parkin-deficient cells (Lee et al., 2011), and BNIP3 can stabilize Pink1, the sensor kinase of Pink1/Parkin-mediated mitophagy (Zhang et al., 2016), linking the receptor-mediated with the Pink1/Parkin-mediated pathway. BNIP3 is regulated by phosphorylation by a yet unknown kinase at Ser17 and Ser24, residues flanking the LIR, which enhances its binding to the Atg8 protein LC3B and the SNARE GATE-16 (Zhu et al., 2013).

## FUNDC1

FUN14 domain containing 1 (FUNDC1) is another MOM receptor, known to be involved in hypoxia-induced mitophagy. Although a recent publication questioned the overall importance of FUNDC1, LC3, and p62 in hypoxia-induced mitophagy (Hirota et al., 2015), FUNDC1 was shown to physically interact with LC3 (Liu et al., 2012). Additionally, it interacts with Drp1 and OPA1, balancing fusion and fission under hypoxic stress (Chen et al., 2016; Wu et al., 2016). FUNDC1 is regulated by two distinct pathways: one involves the kinase CK2 and the phosphatase phosphoglycerate mutase 5 (PGAM5), the other is mediated by the kinase ULK1, a central kinase regulating induction of autophagy. CK2 phosphorylates FUNDC1 at Ser13, inhibiting downstream mitophagy, while the minor fraction of PGAM5 localized to the outer membrane was suggested to dephosphorylate the same residue, thereby enhancing LC3 interaction (Chen et al., 2014). PGAM5 is itself regulated by Bcl2-L1/Bcl-XL, but not Bcl2. Bcl2-L1/Bcl-XL binding to PGAM5 inhibits its phosphatase activity, preventing FUNDC1-mediated mitophagy (Wu et al.,

2014a). ULK1, as the other major regulator, was shown to be recruited to fragmented mitochondria under hypoxia and to phosphorylate Ser17 of FUNDC1, enhancing the binding to LC3 (Wu et al., 2014b). Although these phosphorylation sites are just a few residues apart, their impact on the interaction of the protein to LC3 is remarkable and the structural basis of this interaction was intensively studied (Lv et al., 2017).

## BCL2L13

Bcl2 like protein 13 (BCL2L13), a member of the apoptosis-regulating Bcl2 protein family, was identified as a possible functional homolog of yeast Atg32. It is a MOM protein consisting of an N-terminal cytosolic domain which also carries the LIR motif and a C-terminal TMD. BCL2L13 binds to LC3 inducing mitochondrial fragmentation and mitophagy (Murakawa et al., 2015). An overexpression of BCL2L13 induced mitochondrial fission in wild type and in Drp1 knockdown cells, though to a lesser extent. However, the fission was apparently not mediated by Drp1 itself and changes in the phosphorylation pattern of Drp1 appeared to compensate BCL2L13-induced changes of mitochondrial morphology. Mitophagy was induced upon overexpression of BCL2L13 in human embryonal kidney (HEK) cells and even in HeLa cells after dissipation of the membrane potential by carbonyl cyanide m-chlorophenyl hydrazone (CCCP). The latter implies a Parkin-independent mechanism as HeLa cells lack Parkin. Furthermore, knockdown of BCL2L13 attenuated mitophagy after CCCP treatment suggesting a substantial role in parallel to the Pink1/Parkin-mediated pathway. Though a detailed mechanistic study is still missing, first insights into the regulation were gained with the analysis of a S272A mutant (a residue in close proximity to the LIR), indicating that similarly to FUNDC1, phosphorylation at this position could enhance mitophagy by facilitating the interaction with LC3. Lastly, the authors complemented a yeast strain lacking Atg32 by heterologous expression of BCL2L13 rescuing the defect in mitophagy (Murakawa et al., 2015).

## AMBRA1 and SMURF1

Even though the four above-mentioned receptors share certain structural and functional similarities among each other, e.g. the LIR-motif and the regulation by phosphorylation/dephosphorylation, there are two more factors which are putative mitophagy receptors: AMBRA1 and SMAD ubiquitination regulatory factor 1 (SMURF1).

SMURF1 was identified as a selective autophagy factor in a genome-wide siRNA screening (Orvedahl et al., 2011). It is a HECT-domain ubiquitin ligase but it apparently has a dual role in the proteasomal degradation pathway with its ligase activity and in autophagy with its C2 domain. SMURF1 KO MEFs show strongly impaired CCCP-induced mitophagy. While this suggests a role in Pink1/Parkin mitophagy this has not been studied further. More recently, SMURF1 was rather linked to selective autophagy of intracellular bacteria, termed xenophagy (Franco et al., 2017).

The case of AMBRA1 is even more complex. AMBRA1 is known to be involved in autophagy as a positive regulator of Beclin1, as an interaction partner of Bcl-2, and it was proposed to be required for late stages of mitophagy (Van Humbeeck et al., 2011). In a more recent study, the presence of a functional LIR motif and LC3-interaction as well as the enhancement of Pink1/Parkin mitophagy was demonstrated (Strappazzon et al., 2015). Furthermore, an artificially mitochondria-anchored AMBRA1 induced massive mitophagy in different cells even without carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone treatment and in the absence of Pink1 or Parkin. Moreover, the authors observed a high degree of ubiquitination of mitochondria and the expendability of p62 during AMBRA1-induced mitophagy (Strappazzon et al., 2015). As this approach utilized an artificial localization of AMBRA1, it is not clear whether endogenous AMBRA1 under physiological conditions indeed acts as a mitophagy receptor that is recruited to mitochondria by a specific signal, acts like an adapter protein comparable to p62, or primarily functions as previously described during nucleation of autophagosomes at a distinct location (Di Bartolomeo et al., 2010).

## FKBP8

FK 506 binding protein 8 (FKBP8) is a very recently characterized mitophagy receptor protein (Bhujabal et al., 2017), known before as a non-canonical FKBP and peptidyl-prolyl-cis-trans-isomerase (PPIase). It was also previously described as an important anti-apoptotic protein. FKBP8 was shown to interact with LC3, depending on its LIR motif, under mitophagy inducing conditions, but also upon overexpression alone. Its overexpression furthermore induced changes in mitochondrial morphology and mitophagy independent of Parkin. In contrast to most other known receptors, it seems not to be regulated by a (de-)phosphorylation mechanism, as it does not possess any phosphorylatable residues near the LIR motif (Bhujabal et al., 2017).

## Prohibitin 2

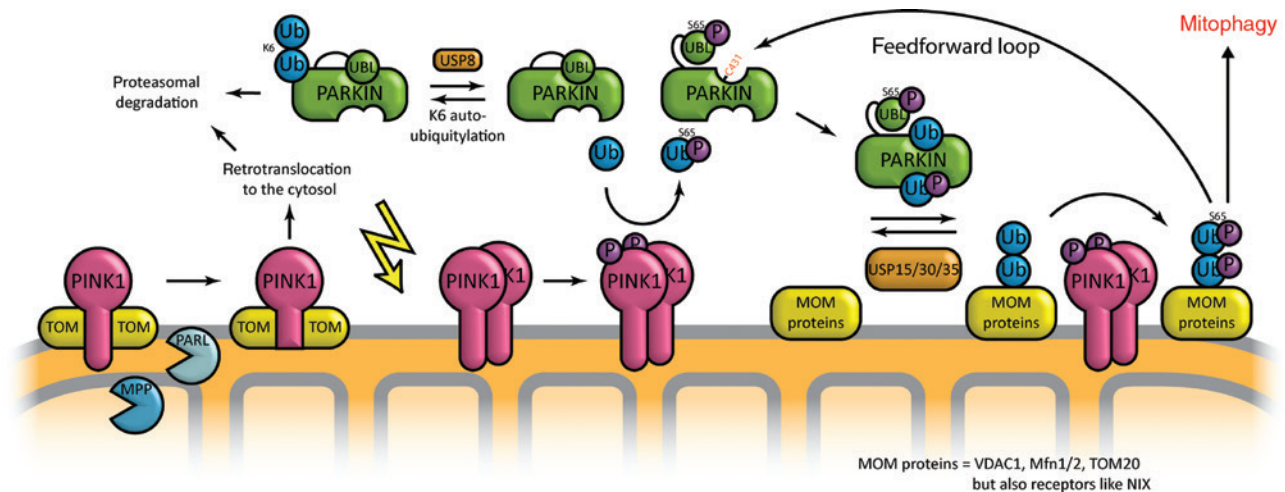
One of the youngest members of the family of putative mitophagy receptors, Prohibitin 2/PHB2, was found in a screening for LC3-interacting proteins under mitophagy inducing conditions (Wei et al., 2017). At first sight this appears unexpected as Prohibitin 2 is located in the MIM as a part of the ring-shaped, heterodimeric Prohibitin 1/2 mega-complex which has important roles in mitochondrial biogenesis including degradation of mitochondrial membrane proteins, controlling mitochondrial protein folding, and cristae morphogenesis (reviewed in Artal-Sanz and Tavernarakis, 2009; Bavelloni et al., 2015). Prohibitin 2 was shown to bind LC3-II, but not GABARAP with its LIR motif in a Pink1/Parkin-dependent manner and knockdown of the protein inhibited mitochondrial degradation (Wei et al., 2017). The Prohibitin 2-mediated mitophagy is dependent on proteasomal degradation and rupture of the MOM. The authors conclude that Prohibitin 2 acts as a MIM mitophagy receptor which becomes accessible to LC3 after MOM rupture during Pink1/Parkin mitophagy. The binding to LC3 then promotes the efficient removal of dysfunctional mitochondria (Wei et al., 2017). It is remarkable to note that in this way Prohibitin 2 acts as a true mitophagy receptor located in the MIM, but at the same time Prohibitin 2-mediated mitophagy remains fully dependent on the Pink1/Parkin pathway, suggesting an interplay of these two pathways.

## Pink1/Parkin-mediated mitophagy

The receptor-mediated mitophagy pathway has very important and often very specific functions, e.g. the role of NIX during maturation of reticulocytes or the role of BNIP3 under hypoxic conditions. Considering the maintenance of mitochondrial quality, the Pink1/Parkin-pathway is considered to play a major role for the removal of damaged mitochondria (Figure 3). Nonetheless, this pathway does not stand alone and there are many overlaps and an interplay with other pathways such as bulk autophagy, the ubiquitin-proteasome system (UPS), and also apoptosis.

## Pink1 – a major sensor of mitochondrial quality

The protein Pink1 has been linked to mitochondrial diseases for quite some time, but the underlying molecular



**Figure 3:** Pink1/Parkin mitophagy.

In healthy mitochondria Pink1 gets imported by the TOM complex into the IMS and the MIM (not depicted) and is rapidly degraded by MPP, PARL and the proteasome. When mitochondria are dysfunctional (flash) Pink1 accumulates in the MOM, dimerizes and autophosphorylates to become fully active. Pink1 phosphorylates both ubiquitin and Parkin. The latter acquires its fully active form upon binding of phosphoubiquitin. Parkin polyubiquitylates MOM proteins which get phosphorylated by Pink1. This helps to recruit and activate even more Parkin creating a strong feed-forward loop. Highly phosphoubiquitylated MOM proteins recruit adapters for the progression of mitophagy. USP15, USP30 and USP35 can remove ubiquitin moieties and thereby counteract the progression of mitophagy. By K6 autoubiquitylation Parkin promotes its own proteasomal degradation. This is counteracted by the deubiquitinase USP8, stabilizing Parkin.

mechanisms were only elucidated in detail in the last years. Pink1 comprises an N-terminal MTS, a transmembrane segment and a kinase domain. Under regular growth conditions, Pink1 is imported by the TOM and TIM23 complexes into the MIM. The matrix processing peptidase (MPP) cleaves the MTS (Greene et al., 2012) and the presenilin-associated rhomboid like (PARL) protease cleaves off the N-terminal part between Ala103 and Phe104 (Jin et al., 2010; Deas et al., 2011; Meissner et al., 2011). The  $\Delta 103$ -Pink1 is known to be rapidly degraded in line with the N-end rule pathway (Yamano and Youle, 2013). Pink1 turnover is dependent on the proper targeting of Pink1 into the MIM and on these proteases. Reduced mitochondrial membrane potential is thought to result in the incomplete transport of Pink1 which consequently accumulates in the MOM (Lazarou et al., 2012). Accumulated Pink1 undergoes dimerization and intermolecular autophosphorylation at Ser228 and Ser402 thereby activating itself, leading to downstream phosphorylation events including Parkin recruitment to mitochondria (Okatsu et al., 2012, 2013). There is also a Parkin-binding role attributed to the 53kDa truncated fragment of Pink1 (Fedorowicz et al., 2014). It is known that this fragment can be stabilized via K63-linked ubiquitylation by TRAF6 (Lim et al., 2015) which might contribute to the recruitment of Parkin to mitochondria. Stabilized Pink1 at the MOM further promotes mitophagy by phosphorylating Parkin and ubiquitin as discussed in more detail below.

## Parkin – amplifier of the key mitophagy signal

As Pink1 is a critical sensor for changes in mitochondrial quality, its accumulation is an important initiation step for mitophagy. However, Pink1 accumulation alone is not sufficient for the progression of mitophagy. While this signal is sufficient to induce mitophagy, the full activation of mitophagy needs a signal amplification, which is mediated by Parkin, an E3-ubiquitin ligase. It belongs to the RING-betweenRING-RING (RBR) domain family of E3 ligases, transferring ubiquitin from an E2 ligase on an intermediate catalytic Cys residue on to a substrate protein (Iguchi et al., 2013; Zheng and Hunter, 2013). Under non-mitophagic conditions Parkin is located in the cytosol and assumes an autoinhibitory state, where its ubiquitin-like (UBL) domain occludes the ligase active domain. Upon Pink1 accumulation on the MOM and its autoactivation, Ser65 in the UBL domain of Parkin is phosphorylated and activated by Pink1 (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012; Lazarou et al., 2013). Yet, this signal is not enough to fully induce mitophagy, giving rise to the idea that Pink1 has another important substrate. Several groups could show that ubiquitin is this second essential substrate of Pink1 and that the Ser65 phosphorylation of ubiquitin and polyubiquitin chains is a major signal promoting ubiquitylation

of distinct substrates and mitophagy (Kane et al., 2014; Kazlauskaitė et al., 2014; Koyano et al., 2014; Shiba-Fukushima et al., 2014a). Phosphoubiquitin has a high affinity to Parkin and induces a displacement of the UBL of Parkin, leading to the unveiling of its ligase active domain (Kazlauskaitė et al., 2015; Kumar et al., 2015; Sauve et al., 2015; Wauer et al., 2015a; Yamano et al., 2015; Aguirre et al., 2017). The displaced UBL is now more accessible for the Pink1-driven phosphorylation, stabilizing the ‘open’ state of Parkin. The fact that linear phosphomimetic (S65D) ubiquitin chains, but not WT ubiquitin chains, could recruit Parkin in the absence of Pink1, provides additional evidence for the role of phosphoubiquitin as the genuine recruitment signal (Okatsu et al., 2015). Interestingly, in *in vitro* experiments, phosphoubiquitin was hardly conjugated to substrates, but served solely as an allosteric activator (Kazlauskaitė et al., 2014; Koyano et al., 2014).

Thus, two phosphorylation events catalyzed by Pink1 essentially create a fast feed forward mechanism. First, Pink1 phosphorylates free ubiquitin as well as (poly-) ubiquitin chains, which are already present at MOM proteins and maybe also a small amount of Parkin. Second, Parkin (and phospho-Parkin) binds with high affinity to phosphoubiquitin chains, activating itself and becoming more accessible to phosphorylation by Pink1. Subsequently, Parkin synthesizes poly-ubiquitin chains on MOM proteins which are phosphorylated by Pink1 and which in turn recruit and activate additional Parkin molecules. While ubiquitin-phosphorylation is not absolutely necessary, an S65A-ubiquitin mutation hinders the progression of the mitophagy cascade, since Parkin is poorly retained at mitochondria and polyubiquitin chains are not built up efficiently (Ordureau et al., 2015).

### Polyubiquitin chains – marking mitochondria for removal

When Parkin is fully activated, it polyubiquitylates several MOM proteins including Mfn1/2, TOM20/40/70, and voltage-dependent anion channel (VDAC) 1 (Geisler et al., 2010; Sarraf et al., 2013). The substrates of Parkin are linked to important mitochondrial functions. The ubiquitylation of these substrates ensure the isolation of mitochondria by inhibiting fusion and promoting fission (substrates: Mfn1/2 and Drp1) (Gegg et al., 2010; Rakovic et al., 2013; Pryde et al., 2016), and by interfering with mitochondrial transport [substrate: MIRO (Mitochondrial Rho GTPase 1)] (Shlevkov et al., 2016). The formation of polyubiquitin chains has been shown to occur

via K6-, K11-, K27-, K48- and K63-linkage (Geisler et al., 2010; Narendra et al., 2010; Geisler et al., 2014; Cunningham et al., 2015). While the K48-linkage is generally associated with degradation by the UPS, the roles of other types of linkage is less clear. Although K63-linkages were shown to be assembled on substrates such as VDAC (Geisler et al., 2010) and were able to bind Parkin (Zheng and Hunter, 2013), the suppression of K63-linked ubiquitylation by deletion of Ubc13, an E2 ligase specific for K63 linkages, did not affect mitophagy (Shiba-Fukushima et al., 2014b). A recent multiplexed large scale ubiquitylome analysis showed that K63-linked ubiquitin chains were in general increased during Pink1/Parkin mitophagy (Rose et al., 2016). Still, the exact roles of distinct K-linkages for mitophagy have to be resolved as K6- (Durcan et al., 2014), K48- (Chan et al., 2011), as well as K63-linkages (Narendra et al., 2010; Chan et al., 2011; Cornelissen et al., 2014) have been reported to be required for efficient induction of mitophagy.

### Adapter proteins – ensuring the autophagosomal removal of mitochondria

The bulk ubiquitylation of MOM proteins facilitates two main downstream events: recruitment of adapter proteins and activation of the UPS. While the role of the UPS during mitophagy is not fully understood yet, it is accepted that the proteasome is partly recruited to dysfunctional mitochondria, where it degrades extracted MOM proteins promoting rupture of the MOM (Yoshii et al., 2011). According to that study both processes, however, were not necessary for the progression of mitophagy whereas in another report the UPS-dependent degradation of MOM proteins was found to be essential for mitophagy (Chan et al., 2011). Adapter proteins such as p62 (sometimes referred to as receptors) are interacting on one side with the polyubiquitin chains directly and on the other side with Atg8-like proteins such as LC3s or GABARAPs (reviewed in Stolz et al., 2014). Initially p62 was identified as the main adapter for Pink1/Parkin-mediated mitophagy (Geisler et al., 2010), but p62 was also reported to be dispensable for mitophagy itself though necessary for the clustering of dysfunctional mitochondria (Narendra et al., 2010). A more recent, comprehensive study analyzed the importance of all five known adapters TAX1BP1, NDP52, NBR1, p62 and OPTN, revealing OPTN and NDP52 to be the most important adapters for Pink1/Parkin-dependent mitophagy (Lazarou et al., 2015). This was presumably not recognized earlier as OPTN and NDP52 can, at



least partially, compensate each other. Their recruitment to dysfunctional mitochondria was independent of Parkin suggesting that Pink1 is able to directly recruit them. Besides OPTN and NDP52, TAX1BP1 was capable of LC3 binding, but neither of the three could bind to GABARAPs. On the other hand, the study also links the two proteins OPTN and NDP52 to ULK1 and hence to the initiation of autophagy (Lazarou et al., 2015). The dispensability of NBR1 was confirmed in the presence and absence of p62 (Shi et al., 2015). Another study on OPTN and NDP52 showed that TBK1 is activated upon their recruitment to mitochondria and that OPTN gets phosphorylated by TBK1 at Ser473 and Ser513, which enhances ubiquitin-binding and mitophagy (Heo et al., 2015). TBK1 additionally phosphorylates p62, NDP52 and TAX1BP1 on different domains and OPTN at Ser177, influencing the binding capacities and the localizations of the proteins (Richter et al., 2016). Still, an important role for p62 as a mitophagy adapter protein is supported by its enhanced expression via the Nrf2 and TFEB pathways during Pink1/Parkin mitophagy (Ivankovic et al., 2016).

Taken together, although there are many open questions remaining, it is likely that the five known adapter proteins each play distinct as well as partly overlapping roles in the recruitment of Atg8-like proteins ensuring the selective engulfment of mitochondrial into autophagosomes.

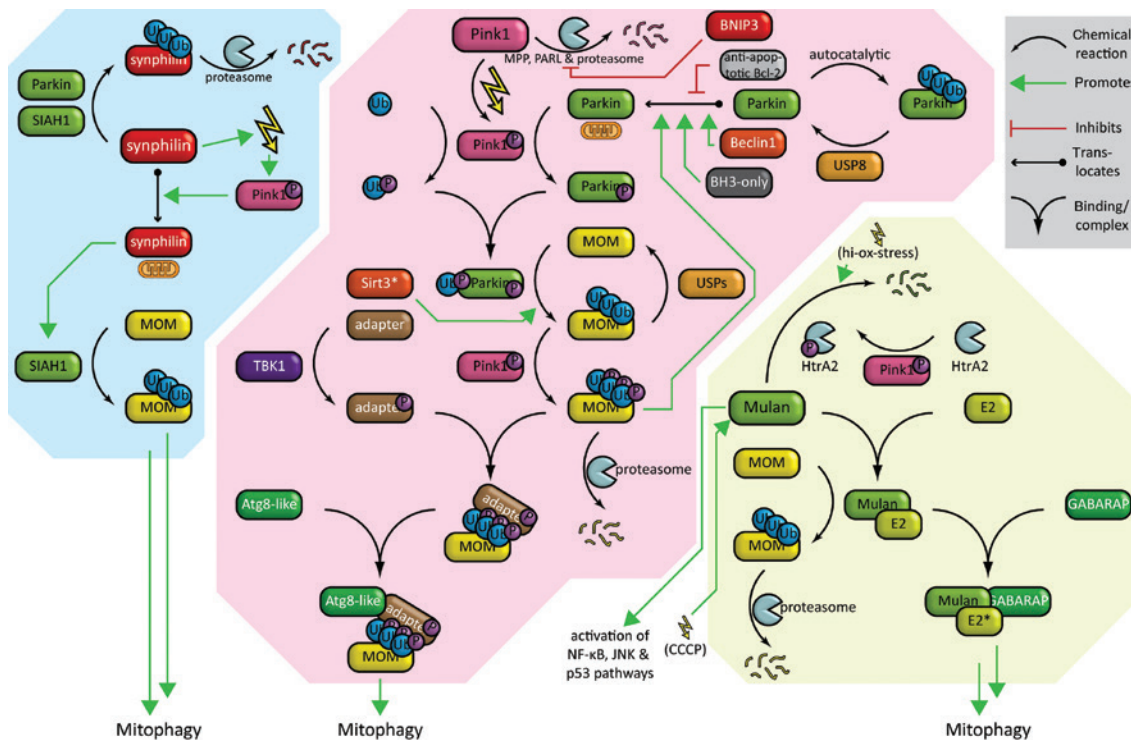
## Regulation of Pink1/Parkin mitophagy and crosstalk to other pathways

The activation of the Pink1/Parkin mitophagy pathway represents a sensitive way to ensure mitochondrial quality control which is promoted by its feed forward mechanism of Parkin recruitment and Pink1-dependent activation. Still, there are supplementary mechanisms to fine tune the system (Figures 3 and 4). An accepted general regulatory property of ubiquitin ligases is their ability to get autoubiquitylated. For Parkin, K6-linked ubiquitin chains have been observed which are correlated to a delay in mitochondrial translocation and induction of mitophagy. This autoinactivation of Parkin is counteracted by the ubiquitin-specific protease USP8 (Durcan et al., 2014). USPs are a class of deubiquitinating enzymes (DUBs) that cleave ubiquitin moieties from polyubiquitin chains or substrate proteins mostly in a linkage-specific manner (Leestemaker et al., 2017). They fulfill regulatory functions in many processes such as proteolysis and autophagy. Three USP enzymes, USP15, USP30 and USP35, are known to counteract the progression of mitophagy by eliminating polyubiquitin chains

from known substrate proteins of Parkin (Bingol et al., 2014; Cornelissen et al., 2014; Wang et al., 2015). Another level of control is added to the network, by ensuring that Pink1- and Parkin-marked mitochondria are actually degraded as USPs are only able to cleave polyubiquitin chains but not phosphoubiquitin chains (Swaney et al., 2015; Wauer et al., 2015b). As mentioned above, adapter proteins including OPTN are regulated via phosphorylation by TBK1 increasing their affinity for polyubiquitin. In this regard, it will be interesting to find out whether there are phosphatases specific for S65p-polyubiquitin chains and adapters such as OPTN, which would add another level of regulation.

An interesting aspect of regulation suggesting a possible crosstalk between ubiquitin- and receptor-mediated mitophagy was found during a search for Pink1 interaction partners (Figure 4). The mitophagy receptor BNIP3 was shown to interact with Pink1 and to inhibit its proteolytic degradation (Zhang et al., 2016). Thereby the uncleaved form of Pink1 is stabilized, which can promote the induction of Parkin-dependent mitophagy. Proteins of the general autophagy machinery such as Beclin1 can also promote mitophagy directly by assisting in Parkin translocation to mitochondria (Choubey et al., 2014). During hypoxia-induced mitophagy Sirt3, a mitochondrial deacetylase, was found to facilitate the interaction between Parkin and VDAC1 (Qiao et al., 2016). During Pink/Parkin mitophagy general autophagy and mitochondrial biogenesis are upregulated as indicated by elevated levels of p62, lysosomal proteins, and PGC-1 $\alpha$ , a positive regulator of mitochondrial biogenesis (Ivankovic et al., 2016). A crosstalk between mitophagy and apoptosis was also suggested as the anti-apoptotic Bcl2-family proteins, Bcl-XL, Mcl-1 and Bcl-W, were found to antagonize Pink1/Parkin-dependent mitophagy. These proteins were shown to inhibit the mitochondrial translocation of Parkin and ubiquitylation of mitochondrial substrate proteins, while depolarization and Pink1 accumulation at the MOM still occurred. In line with this observation, BH3-only proteins such as Bad, Bim, Noxa and Puma enhanced Parkin translocation (Hollville et al., 2014).

In sum, the relationship of mitophagy and apoptosis appears to be a double edged sword. In general, mitophagy can be considered as a protective process counteracting the induction of apoptosis (Wu et al., 2015). However, excessive mitophagy can also lead to a proteasome-dependent, autophagic, non-apoptotic cell death (Akabane et al., 2016). In baker's yeast the Ubp3/Bre5 deubiquitylation complex was shown to inhibit mitophagy and proposed to antagonize excessive mitophagy (Müller et al., 2015a). This is reasonable as mitochondria are essential for cell viability and



**Figure 4:** Different proposed ubiquitin-mediated mitophagy pathways.

Regulation and interplay of the three ubiquitin-mediated pathways: the Synphilin/SIAH-1, the classical Pink1/Parkin, and Mulan/HtrA2-dependent pathways are depicted with blue, pink and yellow background color respectively. Synphilin/SIAH-1-pathway (left), on the one hand, Synphilin (bigger, middle bubble) can get ubiquitylated by Parkin and SIAH-1 promoting its degradation, on the other hand, overexpression of Synphilin triggers mitochondrial dysfunction (flash) and Pink1 accumulation. This promotes Synphilin translocation to the MOM. Synphilin recruits SIAH-1 to mitochondria which ubiquitylates unknown MOM proteins to facilitate mitophagy. Pink1/Parkin-pathway (middle), see also Figure 2; in healthy mitochondria Pink1 (bigger, upper bubble) is degraded by MPP, PARL and the proteasome. This can be blocked by the receptor BNIP3. In damaged mitochondria (flash) Pink1 autophosphorylates, accumulates at the MOM and phosphorylates ubiquitin and Parkin leading to downstream phosphoubiquitylation of MOM proteins. Several regulatory proteins are additionally involved: Parkin can autoubiquitylate which is reversed by USP8 (upper right corner). Parkin translocation to mitochondria is promoted by Beclin1, BH3-only proteins and phosphoubiquitin chains, but inhibited by anti-apoptotic Bcl-2 proteins. Sirt3 enhances binding of Parkin to one of its substrates at the MOM: VDAC. USPs 15, 30, and 35 counteract substrate ubiquitylation. The adapter proteins p62, NDP52, TAXBP1 and OPTN are activated via phosphorylation by TBK1 (left part), enhancing their affinity for polyphosphoubiquitin chains. Mulan/HtrA2-pathway (right), Mulan is a proteolytic substrate for HtrA2, which can be activated by Pink1, under high oxidative stress. Membrane potential disruption by CCCP on the other hand, leads to elevated Mulan levels. Mulan can bind different E2 enzymes and ubiquitylates Mfn2. A complex of Mulan with Ube2E3 can directly bind to GABARAP and thereby possibly facilitate mitophagy as a receptor at the MOM as well.

emphasizes that under physiological conditions it is also important to keep mitophagy at a healthy, balanced level.

## Mitophagy beyond Pink1 and Parkin

### Pink1/synphilin-1/SIAH-1-mediated mitophagy – a Parkin-independent pathway?

It is debated whether next to the receptor-mediated and the Pink1/Parkin mitophagy pathway additional pathways exist (Figure 4). Support for this possibility came from a recent

study demonstrating the ubiquitylation of MOM proteins combined with a translocation of synphilin-1 to mitochondria in a Pink1-dependent, yet Parkin-independent manner (Szargel et al., 2016). A physical interaction of Pink1 to synphilin-1 was additionally shown. Interestingly, the progression of a Parkin-independent Pink1/synphilin-mediated mitophagy was independent of external dissipation of the membrane potential. Overexpression of synphilin-1 alone triggered mitochondrial depolarization and Pink1 stabilization in the MOM. Despite the absence of Parkin in the system, ubiquitylation of MOM proteins was found to depend on seven in absentia homolog 1 (SIAH-1), an E3-ubiquitin ligase. This pathway is in principle reminiscent to the Pink1/Parkin

system, yet SIAH-1 does not appear to promote proteasomal degradation of MOM proteins (Szargel et al., 2016) which is observed during Parkin-mediated mitophagy (Yoshii et al., 2011).

Although these observations indicate an interesting alternative route downstream of Pink1, it remains unclear to which extent this pathway is of physiological importance when Parkin is expressed. It could be a backup system in the case of lack or loss-of-function of Parkin or it could be relevant in certain cell-types or tissues. Interestingly, synphilin-1 is apparently enriched in the brain. It should also be taken into account that synphilin-1 itself is a substrate for ubiquitylation by SIAH-1 (Nagano et al., 2003; Liani et al., 2004) and Parkin (Chung et al., 2001; Lim et al., 2005) leading to its subsequent degradation. The full unraveling of this pathway and its entanglement with Pink1/Parkin will be of high interest.

### Mulan – a Parkin-like receptor?

Mitochondrial ubiquitin ligase activator of NF- $\kappa$ B1 (Mulan or Mul1) is an E3 ligase, spanning the MOM, harboring an inter membrane space (IMS)-domain and a cytosolic RING finger domain (Figure 4). Mulan was shown to be degraded by HtrA2 (also termed Omi), a serine protease involved in apoptosis and mitochondrial homeostasis under high oxidative stress conditions (Cilenti et al., 2014). Degradation of Mulan leads to high levels of Mfn2 indicating that Mfn2 is a substrate of Mulan. Furthermore, CCCP treatment increased the amounts of Mulan in HtrA2 KO and in WT MEFs demonstrating a role during CCCP-induced mitophagy. Mulan was shown to interact with several E2 enzymes (Ube2E2, Ube2E3, Ube2G2, and Ube2L3) and a complex of Mulan and Ube2E3 was able to physically interact with GABARAP but not LC3 (Ambivero et al., 2014). In a recent study Mulan together with MARCH5 was found to ubiquitylate SLC25A46, a putative Ugo1-like fusion/fission factor, and by that facilitate the proteasomal degradation of SLC25A46 (Steffen et al., 2017). Interestingly, this selective degradation occurred independent of mitophagy and apoptosis induction.

Despite the mode of action, the HtrA2/Mulan pathway is apparently not entirely independent of Pink1/Parkin-mediated mitophagy, as HtrA2 is known to be activated by Pink1 via phosphorylation (Plun-Favreau et al., 2007). Thus, under normal conditions, when Pink1 is transported efficiently to the IMS it would ensure the inhibition of Mulan-mediated mitophagy. Therefore, it is not clear whether Mulan provides an independent, alternative receptor-mediated mitophagy pathway, or an alternative

Pink1/Parkin-dependent pathway, or whether it rather acts as a regulator of mitochondrial dynamics or different signaling pathways via, NF- $\kappa$ B, jun N-terminal kinases (JNK), p53, or Akt. The direct recruitment of an Atg8-like protein to mitochondria and the localization of Mulan at the MOM indicate a receptor-like role. Opposed to this, its enzymatic activity as an E3 ligase and its putative substrate Mfn2 point to a Parkin-like role in mitophagy. It will thus be interesting to decode the molecular mechanisms of the contribution of Mulan to mitophagy in the future.

## Ubiquitin-mediated mitophagy in yeast

Though the main focus in this review is mitophagy in mammalian cell systems, the use of the model organism *Saccharomyces cerevisiae* has driven mitophagy research considerably. While the general autophagy machinery and certain pathways in mitophagy are either conserved or they show high homologies between yeast and mammals, it is surprising that ubiquitin-mediated mitophagy was long believed to be an exclusive pathway for higher eukaryotes (e.g. *Caenorhabditis elegans*, *Drosophila melanogaster* and mammals). In a recent study looking for modulators of mitophagy in a genome-wide manner, we identified the Ubp3/Bre5 deubiquitylation complex as an inhibitor of mitophagy. This complex on one hand, inhibited mitophagy, while on the other hand, it promoted other forms of autophagy including ribophagy (Müller et al., 2015a). This not only indicates a vital role for ubiquitylation during mitophagy in yeast as well, but moreover shows an entanglement of different forms of autophagy. Hence, it is reasonable to conclude the existence of a tight regulatory network with sensors and switches balancing different forms of autophagy, depending on the overall status of the cell. In addition, overexpression of Parkin in baker's yeast was shown to increase resistance to oxidative stress and to extend chronological lifespan (Pereira et al., 2015).

These discoveries are a first step in unveiling ubiquitin-mediated processes in mitochondrial quality control in baker's yeast. Future studies using this model organism will certainly provide further insights into the regulatory and molecular mechanism of mitophagy, and will enhance our understanding of the evolution of these processes.

## Conclusions

Quality control of mitochondria is organized on multiple levels, including protein folding, proteostasis, formation of



MDVs, organelle dynamics and mitophagy. The latter is of particular importance as it helps to eliminate dysfunctional or superfluous mitochondria in many cell types. This process is mediated by at least two mechanistically very distinct pathways: (1) either utilizing direct protein-protein interactions between a receptor positioned in the MOM and downstream factors of the autophagy machinery or (2) employing the formation of ubiquitin chains at the MOM that are indirectly connected to the autophagy machinery via adapter proteins. Receptors are typically activated via (de-)phosphorylation increasing their affinity for LC3/Atg8-like proteins. While this regulation is relatively simple, the ubiquitin-mediated pathway is apparently fine-tuned at different levels by (de-) phosphorylation, (de-)ubiquitylation, and by modulating the recruitment and degradation of important factors. The two distinct pathways may act independently from one another, but are seemingly also interconnected at certain regulatory points. Despite the impressive work of many groups on this field in the recent years, we are still at the beginning of deciphering this complex regulatory network which determines if, when, and how a specific mitochondrion undergoes elimination by autophagy.

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