Ubiquitination at the Mitochondria in Neuronal Health and Disease

Christian Covill-Cooke[a, b], Jack Howden[a], Nicol Birsa[c] and Josef Kittler[a, *]

[a]Neuroscience, Pharmacology and Physiology Department, University College London, Gower Street, London, WC1E 6BT, UK.

[b]MRC Laboratory for Molecular Cell Biology, University College London, Gower Street, London, WC1E 6BT, UK.

[c]UCL Institute of Neurology, Queen Square, London, WC1N 3BG, UK.

*Corresponding author: j.kittler@ucl.ac.uk

Abstract

The preservation of mitochondrial function is of particular importance in neurons given the high energy requirements of action potential propagation and synaptic transmission. Indeed, disruptions in mitochondrial dynamics and quality control are linked to cellular pathology in neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. Here, we will discuss the role of ubiquitination by the E3 ligases: Parkin, MARCH5 and Mul1, and how they regulate mitochondrial homeostasis. Furthermore, given the role of Parkin and Mul1 in the formation of mitochondria-derived vesicles we give an overview of this area of mitochondrial homeostasis. We highlight how through the activity of these enzymes and MDV formation, multiple facets of mitochondrial biology can be regulated, ensuring the functionality of the mitochondrial network thus preserving neuronal health.

Keywords: Mitochondria; Ubiquitin; Neurodegeneration; E3 ligase; Mitochondria-derived vesicles

Abbreviations: PD: Parkinson's disease, MDV: mitochondria-derived vesicle, DA: dopaminergic, SN: substantia nigra, GWAS: genome-wide association study, OMM: outer mitochondrial membrane, OMMAD: outer mitochondrial membrane protein associated degradation, DUB: deubiquitinase, KO: knockout, ER: endoplasmic reticulum, ALS: amyotrophic lateral sclerosis, MEFs: mouse embryonic fibroblasts, mtDNA: mitochondrially DNA.

Neuronal function requires a dynamic and healthy mitochondrial network

Mitochondria play an essential role in ATP generation, calcium buffering and apoptotic signalling. In morphologically complex, postmitotic cells such as neurons, mitochondrial trafficking is crucial to match mitochondrial positioning to localised energy demands, while correct mitochondrial dynamics and quality control systems are essential to maintain a functional mitochondrial network (Birsa et al., 2013; Devine et al., 2015; MacAskill and Kittler, 2010; Schwarz, 2013; Sheng, 2017). More specifically, mitochondria employ a series of quality control mechanisms, including: i) the local degradation of misfolded proteins by proteases; ii) the utilisation of fission and fusion events to ensure mtDNA fidelity; and iii) the lysosomal degradation of mitochondrial contents to homeostatically regulate mitochondrial health (Mishra and Chan, 2016). The importance of these processes is supported by pathological mutations in the machinery of all three of them being associated with neurodegenerative disorders (DuBoff et al., 2013; MacAskill et al., 2010; Martinelli and Rugarli, 2010; Pickrell and Youle, 2015). As a result, understanding how these homeostatic systems are coordinated in a manner that ensures cellular health is vital to our understanding of neuronal function and pathology.

The integration of the diverse mechanisms of mitochondrial homeostasis can be assured by intracellular messengers. One such messenger that is particularly important in the context of mitochondrial quality control is the small polypeptide ubiquitin. This review will discuss a variety of mitochondrial quality control mechanisms with special attention given to how ubiquitin and associated ubiquitin E3 ligases regulate them and how they have significance in the health of the nervous system. More specifically the following will be discussed: 1) PINK1/Parkin-mediated mitophagy and its role in Parkinson's disease (PD), 2) the function of mitochondrially-targeted E3 ligases (namely, MARCH5 and Mul1), 3) the formation and role of mitochondria-derived vesicles (MDVs). Examples of neuronal pathologies arising from defects in mitochondrial quality control will be mentioned throughout.

PINK1/Parkin-mediated mitophagy and Parkinson's disease

Parkinson's disease is the most prevalent neurodegenerative movement disorder affecting up to 1% of people over the age of 65 worldwide (Savitt et al., 2006). Characteristic motor deficits, including slowness of movement (bradykinesia), rigidity and tremors, are the result of the selective loss of dopaminergic (DA) neurons from the substantia nigra (SN) in the midbrain and its projections to the dorsal striatum (Houlden and Singleton, 2012). Initially thought of as a sporadic disease of unknown aetiology, genetic advances in the past two decades have shown that a substantial proportion (~10%) of cases are a result of inherited genetic mutations (Lesage and Brice, 2009).

Following identification of the first PD-associated mutation in the *SNCA* gene almost 20 years ago, numerous candidate gene studies have revealed that changes in several chromosomal loci are associated with an increased risk of PD (Lill et al., 2012; Polymeropoulos et al., 1997). More recently, genome-wide association studies (GWAS) and subsequent GWAS meta-analyses have identified 28

discrete loci as PD risk factors (Nalls et al., 2014). Carrying a genetic variant in any individual locus confers an increased risk of around 15-30% for the development of PD, and this risk is considerably higher when carrying two or more mutations (Nalls et al., 2014).

Mutations in *PARK2* and *PARK6*, which encode the proteins Parkin and PINK1 (PTEN-induced putative kinase 1) respectively, can lead to recessive forms of early onset PD (Kitada et al., 1998; Valente et al., 2004); with Parkin and PINK1 mutations accounting for up to 9% and 4% of early onset (<50 years) cases of PD, respectively (Kilarski et al., 2012). It is now known that Parkin, a cytosolic multifunctional ubiquitin E3 ligase, and PINK1, a serine/threonine-protein kinase with a mitochondrial targeting sequence, function in a common mitochondrial quality control pathway; specifically, the process of mitochondrial autophagy (mitophagy) with PINK1 acting upstream of Parkin (Narendra et al., 2008; Narendra et al., 2010).

Mechanism of PINK1/Parkin-mediated mitophagy

The subcellular localisation and turnover of PINK1 is known to be an important step in sensing the loss of mitochondrial membrane potential and the subsequent activation of damaged-induced mitophagy. Under basal conditions PINK1 is imported into the mitochondria in a membrane potential-dependent mechanism and subsequently processed and degraded by a wide range of mitochondrial peptidases, including: MPP, PARL (an Alzheimer's disease-associated protein), m-AAA and ClpXP (Greene et al., 2012; Lazarou et al., 2012). However, upon loss of the membrane potential during damage, PINK1 becomes stabilised in the outer mitochondrial membrane (OMM) leading to the recruitment of Parkin from the cytosol to the mitochondria (figure 1).

Recently PINK1 was identified as the first kinase to phosphorylate ubiquitin (Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). Once phosphorylated at S65, ubiquitin undergoes a conformational change and has been shown to bind to and induce the release of the ubiquitin-like domain of Parkin from the core of the protein. This releases Parkin auto-inhibition, and favours PINK1-dependent phosphorylation of Parkin at S65 mitochondria (Kazlauskaite et al., 2015; McWilliams and Muqit, 2017; Sauvé et al., 2015; Wauer et al., 2015a, 2015b). The importance of this phosphorylation in the initiation of mitophagy is emphasised by the ectopic recruitment of Parkin to the mitochondria in the absence of PINK1 activity being unable to lead to the ubiquitination and lysosomal clearance of mitochondria (Lazarou et al., 2012). Instead, the accumulation of PINK1 on the OMM as a preceding step is necessary. Indeed the phosphorylation of ubiquitin through the kinase activity of PINK1 is required for the subsequent recruitment of the autophagy receptors Optineurin and NDP52 and phosphorylated ubiquitin levels have been shown to increase following mitochondrial damage (Fiesel et al., 2015; Lazarou et al., 2015).

Once Parkin is fully activated and stabilised on damaged mitochondria, it can ubiquitinate a myriad of substrates. Proteomic studies revealed that Parkin may directly or indirectly regulate ubiquitination of more than 100 mitochondrial proteins upon mitochondrial depolarisation (Sarraf et al., 2013), of which the best characterised are Mfn1 and Mfn2, Miro1, VDAC1, Drp1, Tom20 and 40 (Birsa et al., 2014;

Geisler et al., 2010; Poole et al., 2010; Wang et al., 2011; Ziviani et al., 2010). More specifically, the mitofusins have been shown to be rapidly ubiquitinated by Parkin following mitochondrial damage (Gegg et al., 2010; Ziviani et al., 2010) in a process promoted by the PD-associated protein Fbxo7 (Burchell et al., 2013). Following ubiquitination, mitofusin is retrotranslocated from the OMM by the AAA+ ATPase VCP/p97 followed by proteasomal degradation in a process known as OMMAD (outer mitochondrial membrane protein associated degradation) (Kim et al., 2013; Tanaka et al., 2010; Xu et al., 2011). As proteins essential for OMM fusion, damaged-induced degradation of Mfn1 and Mfn2 is proposed to separate dysfunctional mitochondria, therefore preserving the function of the entire mitochondrial network and the cell (Youle and Narendra, 2011).

Work in the heart has also shown that Mfn2, but not Mfn1, is essential for Parkin translocation to the mitochondria during damage (Chen and Dorn, 2013). Furthermore, PINK1 phosphorylation of Mfn2 at T111 and S442 is required for the Parkin-Mfn2 interaction and the subsequent ubiquitination of Mfn2 (Chen and Dorn, 2013; Gong et al., 2015). Indeed a reduction or complete loss of Mfn2 in dopaminergic neurons leads to an accumulation of damaged mitochondria and neurodegeneration (Lee et al., 2012; Tang et al., 2015). Furthermore, mutations in Mfn2 lead to Charcot-Marie-Tooth disease type 2A2 and hereditary motor and sensory neuropathy. Despite the evidence of functional Mfn2 being required for neuronal health, downregulation of Mfn2 - or the Drosophila homologue – in PINK1 or Parkin KO models can alleviate pathology (Celardo et al., 2016; Deng et al., 2008; Gautier et al., 2016; Poole et al., 2008). As a result, it is clear that Mfn2 levels must be regulated in a manner to preserve neuronal health.

Another example of a protein whose ubiquitination and degradation has been shown to be of particular importance in mitophagy is Miro1. As a protein critical for mitochondrial trafficking, Miro1 must be regulated to allow the optimal positioning of the mitochondrial network in the complex, polar morphology of a neuron (Guo et al., 2005; Macaskill et al., 2009; MacAskill et al., 2009; Norkett et al., 2016; Russo et al., 2009; Stephen et al., 2015; Wang and Schwarz, 2009). Mitochondrial trafficking arrests during mitochondrial damage in axons, preceding mitophagy, and is caused by Parkin-dependent degradation of Miro1 (Ashrafi et al., 2014; Hsieh et al., 2016; Wang et al., 2011). Miro1 phosphorylation is proposed to be important for this process, with reports showing Miro can be phosphorylated by PINK1 (Wang et al., 2011; Weihofen et al., 2009).

Phosphorylation of Miro1 at S156 – and the corresponding site in dMiro in Drosophila – was shown to be required for optimal Parkin-dependent degradation of Miro1 during damage (Shlevkov et al., 2016; Tsai et al., 2014; Wang et al., 2011). Furthermore, phosphorylation at T298/T299 was shown to dominantly abolish the effects of S156 phosphorylation, preventing the Parkin ubiquitination and degradation of Miro1 (Shlevkov et al., 2016). Despite these findings, contradictory data suggest that PINK1 does not phosphorylate Miro and S156 of Miro1 does not affect its degradation, perhaps owing to differences in cell-type and protein expression levels (Birsa et al., 2014; Kondapalli et al., 2012; Liu et al., 2012). The degradation of Miro1 has been shown to have particular significance in neurodegeneration with knockdown of Miro in PD models leading to an alleviation of PD pathology (Hsieh et al., 2016; Liu et al., 2012). However, complete loss of Miro1 has been associated with

neurodegeneration in the central and peripheral nervous system, thereby highlighting the need for tight regulation of Miro1 levels in neuronal health (López-Doménech et al., 2016; Nguyen et al., 2014).

Beyond simply causing the degradation of the mitochondrial substrate, mass mitochondrial ubiquitination has been shown to engage the autophagy machinery. To achieve this, autophagy receptors have been proposed to bridge ubiquitinated cargoes and LC3 decorated autophagosomal membranes via their ubiquitin binding and LC3 interacting regions (figure 2). Although many autophagy receptors have been identified, only Optineurin and NDP52 are required for the clearance of mitochondria following damage (Khaminets et al., 2016; Lazarou et al., 2015; McWilliams and Muqit, 2017). Coupled to the role of Optineurin and NDP52 in LC3 recruitment they have also been shown to recruit proteins required in the early stages of autophagy. The role for the other receptors is not yet known; however, p62 has been found to have a role in perinuclear aggregation of mitochondria following damage (Narendra et al., 2010).

In antagonism to this widespread ubiquitination, several deubiquitinases (DUBs) have been identified which act to regulate the progression of Parkin-mediated mitophagy, via two mechanisms. Firstly, Ataxin-3 and USP8 regulate auto-ubiquitination of Parkin, a process which controls the turnover and mitochondrial recruitment of Parkin (Durcan and Fon, 2011; Durcan et al., 2014). Secondly, USP15 and USP30 are thought to directly antagonise the ubiquitination of mitochondrial substrates, suggesting the existence of a balance of ubiquitin levels on the mitochondrion, which can be shifted upon Parkin activation during mitochondrial damage (Bingol et al., 2014; Cornelissen et al., 2014; Cunningham et al., 2015). Silencing of USP15 and USP30 has been shown to favour lysosomal clearance of mitochondria during damage and therefore pharmacologically inhibiting these proteins may serve as potential therapeutic targets for PD (Bingol et al., 2014; Cornelissen et al., 2014).

Mitochondrial dysfunction, PINK1/Parkin-mediated mitophagy and neurodegeneration

Whilst much of the work in elucidating the mechanism of PINK1/Parkin-mediated mitophagy has been carried out in a variety of cell types, work in neurons has led to some important insights into mitophagy in the brain and the aetiology of PD. One important question that arises from this is to what extent mitophagy occurs *in vivo* (Whitworth and Pallanck, 2017). To address this, the use of transgenic animals has been informative. The development of mouse lines with fluorescent mitophagy markers – namely: mito-mKeima and mito-QC - have shown that mitophagy does indeed occur in a wide variety of tissues including many regions of the brain (McWilliams et al., 2016; Sun et al., 2015). Furthermore, phosphorylated-ubiquitin has also been observed in the brains of mice and of a post-mortem PD patient, providing supporting evidence of mitophagy *in vivo* (Fiesel et al., 2015; Pickrell et al., 2015).

The identification of loss-of-function mutations in PINK1 and Parkin in familial forms of PD has given important insights into the aetiology of the disease. Therefore, it is surprising that Parkin null mice show no or limited signs of a Parkinsonian phenotype with little or no motor defects and in some cases slight alteration in dopamine metabolism (Goldberg et al., 2003; Itier et al., 2003; Perez and

Palmiter, 2005; Von Coelln et al., 2004). This is also mirrored with the PINK1 KO mice (Akundi et al., 2011; Kitada et al., 2007). Similarly, work in Drosophila has found that Parkin KO leads to motor coordination deficits, however, little DA neuron degeneration is observed (Cha et al., 2005; Greene et al., 2003; Pesah et al., 2004; Whitworth et al., 2005). Recent work has given some explanations for the lack of pathology in these model systems. One explanation is that Mul1 (a mitochondrially-targeted E3 ligase discussed below) is required for efficient mitophagy in mice (Rojansky et al., 2016). Another explanation is that in mice the genetic insults required to lead to defective mitophagy and subsequent dopaminergic neuron degeneration are different. Indeed, PINK1 or Parkin KO mice overexpressing human A53T α-synuclein exhibit a phenotype reminiscent of PD (Chen et al., 2015; Gispert et al., 2015). Thirdly, as mtDNA mutations accumulate with age, the lifespan of a human is sufficient to amass pathological mutations in the mtDNA (Bender et al., 2006; Kraytsberg et al., 2006). Indeed patients with mutations in POLG (the mitochondrial DNA polymerase) exhibit loss of DA neurons of the SN (Reeve et al., 2013). In support of this idea, a cross between the Mutator mouse (a mouse model with increased mutations rates in mtDNA) and Parkin KO was found to have neurodegeneration in DA neurons of the SN. These data, coupled with decreased rates of mitophagy being observed with age has highlighted how a model for the sporadic forms of PD can be assimilated from the familial forms (Sun et al., 2015).

Mitochondrially-targeted E3 ligases and mitochondrial homeostasis

Alongside the extensive literature on the ability of Parkin to ubiquitinate mitochondrial substrates, a role for several other E3-ligases at the mitochondria has been described, including: MARCH5, Mul1, RNF185 and the ER-localised protein, gp78. The role of the latter two at the mitochondria is somewhat under-appreciated; however, they are thought to regulate mitophagy (Fu et al., 2013; Mukherjee and Chakrabarti, 2016; Tang et al., 2011). Given the body of literature surrounding MARCH5 and Mul1, the ability of these two proteins to regulate local protein homeostasis at the mitochondrion will be discussed, coupled to the broader impacts of ubiquitination by these mitochondrial proteins.

MARCH5 in neuronal health

MARCH5 (also known as MITOL) is situated in the OMM via four transmembrane domains with E3 ligase activity facing the cytosol. The E3 ligase activity of MARCH5 has been shown to have an influence on mitochondrial dynamics through the ubiquitination of the dynamin-related GTPases: Mfn1/2 and Drp1, and the Drp1-receptor MiD49 (figure 3) (Cherok et al., 2017; Karbowski et al., 2007; Nakamura et al., 2006; Xu et al., 2016; Yonashiro et al., 2006). The subsequent proteasomal degradation of these proteins has been noted to have an impact on mitochondrial dynamics; however, the exact effect on morphology has varied between studies. The original descriptions of MARCH5 showed that it promoted elongation of the mitochondrial network (Nakamura et al., 2006; Yonashiro et al., 2006). This idea has also been supported by subsequent data highlighting that disruption of MARCH5 function leads to fragmentation of mitochondria (Cherok et al., 2017; Xu et al., 2016); (Park and Cho, 2012). Conversely, reports have shown that knockdown of MARCH5 in fact results in fusion

of the mitochondria and expression of the H43W, RING mutant in MARCH5 KO MEFs leads to elongation of the mitochondria (Cherok et al., 2017; Karbowski et al., 2007).

The role of MARCH5-dependent regulation of mitochondrial dynamics in the broader context of the cell has been shown to impinge upon a variety of cellular process. These include: cell cycle, cellular senescence, and viral signalling; however, more noteworthy examples include: apoptosis, mitophagy and ER-mitochondria contact site formation coupled with calcium dynamics (Park and Cho, 2012; Park et al., 2014, 2010). In the case of apoptosis, MARCH5 KO cells are more susceptible to antimycin A and FCCP induced cell death (Park et al., 2014; Xu et al., 2016). Mechanistically, this susceptibility was shown to occur through regulating damage-induced mitochondrial hyperfusion, where MARCH5 degrades K491 acetylated Mfn1 (Park et al., 2014). MARCH5 has also been shown to regulate the ability of a cell to respond to hypoxia. The hypoxia-induced mitophagy receptor FUNDC1 has been found to interact with, and be degraded by, MARCH5 highlighting a role for MARCH5 in negatively regulating mitophagy (Chen et al., 2017; Liu et al., 2012).

MARCH5 has also been shown to promote the relocalisation of Mfn2 to ER-mitochondria contacts sites following K63-linked polyubiquitination (Sugiura et al., 2013). The downstream consequences of this are emphasised by the knockdown of MARCH5 showing abnormal ER morphology and a significant decrease in mitochondrial calcium uptake during histamine treatment (Sugiura et al., 2013). This calcium signalling role of MARCH5 is interesting when considering what the functional consequences of MARCH5 are in a neuronal context; especially as neurons are highly dependent on functional calcium dynamics (Ross, 2012; Vaccaro et al., 2017). It is also noteworthy that MARCH5 can impinge upon the interplay between mitochondria and microtubules in neurons. The distribution of mitochondria within neurons is of great importance given the extensive polarity and long processes exhibited by this cell type. Yonashiro *et al*. (2012) propose a model whereby neuronal activity-induced increases in calcium levels can lead to the *S*-nitrosylation of MAP1B light chain-10 - a protein involved in the stabilisation of microtubules - and subsequent ubiquitination by MARCH5. The degradation of MAP1B light chain-1 that follows leads to microtubule stabilisation finally resulting in mitochondrial stress and neurodegeneration, emphasising the importance of the E3 ligase activity of MARCH5 in neuronal health (Yonashiro et al., 2012).

Alongside the ability of MARCH5 to ubiquitinate proteins central to mitochondria dynamics, MARCH5 appears to have a role in the degradation of neurodegeneration-associated, mutant proteins. The G93A mutation in SOD1 (mSOD1) is linked to familial amyotrophic lateral sclerosis (ALS); a neurodegenerative disease associated with muscular atrophy. Yonashiro *et al*. (2009) discovered that MARCH5 ubiquitinates mSOD1 at the mitochondria, leading to its subsequent proteasomal degradation. This reduction in mSOD1 protein levels, leads to a decrease in reactive oxygen species resulting in an increase in cell viability, hinting at a possible innate mechanism to clear potentially detrimental proteins by MARCH5 (Yonashiro et al., 2009). Coupled to the ubiquitination of mSOD1, MARCH5 has also been shown to ubiquitinate polyQ expanded proteins associated with Huntington's disease and ataxias. Using a polyQ expanded Ataxin-3 mutant (linked to Machado-Joseph disease) as a model, Sugiura *et al*. (2011) showed that MARCH5 can ubiquitinate polyQ expanded Ataxin-3,

leading to its degradation. This degradative targeting by MARCH5 was shown to be important in reducing large aggregates of Ataxin-3, with MARCH5 knockdown causing an increase in aggregates and ultimately a decrease in cell viability linked with poor mitochondrial health (Sugiura et al., 2011).

The broad range of MARCH5 targets provides evidence for a far-reaching role of MARCH5 in protein homeostasis at the mitochondria. By affecting mitochondrial dynamics, mitophagy, mutant protein degradation and calcium signalling, MARCH5 is likely to have a large impact on neuronal health. The integration of other post-translational modifications - such as acetylation, *S*-nitrosylation and phosphorylation - and along with a wide range of stresses further supports this idea, but also provides an explanation for the conflicting data on the actual involvement of MARCH5 in mitochondrial dynamics. Perhaps the E3 ligase activity of MARCH5 regulates a wide variety of processes as a way to integrate cellular responses to best preserve neuronal health.

Mul1 and the regulation of the mitochondrial network

Mul1 (also known as MULAN, MAPL, GIDE and HADES) is a mitochondrial ubiquitin and SUMO E3 ligase containing two transmembrane domains spanning the OMM with its C-terminal RING domain facing the cytosol (Braschi et al., 2009; Li et al., 2008). The full extent of the roles identified for Mul1 are beyond the scope of this review, however, those not directly linked to mitochondrial quality control include: effects on cell growth, stimulation of apoptosis and modulation of antiviral signalling in innate immunity (Bae et al., 2012; Doiron et al., 2017; Jenkins et al., 2013; Jung et al., 2011; Prudent et al., 2015). These roles arise from, but may not be limited to, the capacity of Mul1 to negatively regulate Akt protein levels and to activate NF-κB (Bae et al., 2012; Kim et al., 2015; Zemirli et al., 2014).

Originally identified for its role in MDV formation (covered in more detail below), Mul1 has since been shown to elicit remodelling of the mitochondrial network through both its ubiquitin and SUMO E3 ligase activity (figure 3). More specifically, SUMOylation of Drp1 by Mul1 has been shown to lead to fragmentation of the mitochondrial network through stabilisation of Drp1 protein levels (Braschi et al., 2009). Moreover, an increase in Mul1 protein levels also fragments the mitochondria through the ubiquitination and subsequent proteasomal degradation of Mfn2 (Tang et al., 2015; Yun et al., 2014).

The ability of Mul1 to signal for the proteasomal degradation of Mfn2 has important implications in PD. Observations that Parkin KO mice do not show any Parkinsonian phenotype lead to the assumption that perhaps parallel pathways are employed by the cell to degrade faulty mitochondria. To this end, work in Drosophila has shown that through the negative regulation of Mfn2 by Mul1, the phenotypes associated with the loss of PINK1/Parkin in flies was rescued (Yun et al., 2014). Furthermore, it has been shown that the interplay between Mul1 and Mfn2 also has relevance in PD-associated mutations in Vps35 (Tang et al., 2015). When specifically deleted in dopaminergic neurons, Vps35 leads to an increase in Mul1 protein levels, resulting in a specific reduction in Mfn2 levels. This reduction in Mfn2 levels and subsequent mitochondrial fragmentation leads to neurodegeneration, which could be rescued with wild-type Vps35 but not PD-associated mutations, highlighting a role for Mul1 in PD pathology.

Further to the role of Mul1 in mitochondrial dynamics, there is a body of evidence suggesting Mul1 has a role in mitophagy. Mul1 has been shown to interact directly with both Ulk1 and GABARAP; two proteins with a role in autophagosomal membrane formation (though the essential role of GABARAP has recently been challenged) (Ambivero et al., 2014; Li et al., 2015; Nguyen et al., 2016). Although the functional consequences of these interactions are not fully explored, it is proposed that Mul1 can ubiquitinate Ulk1 leading to its degradation. However, as the authors also show that Mul1 protein levels promote selenite-induced mitophagy, it remains an open question what the functional significance of the Mul1-Ulk1 interaction might be (Li et al., 2015). Finally more recent work has shown that both Parkin and Mul1 are required for efficient mitophagy, with large reductions of mitophagy only being observed when loss of both proteins was examined (Rojansky et al., 2016).

With Mul1 being targeted to the mitochondria it sits at the centre of the regulatory processes that affect mitochondrial homeostasis. Through its role in mitochondrial dynamics, Mul1 can alter the morphology of the network with the possibility of engaging the mitophagic and apoptotic machinery. Notably, Mul1 appears to have some antagonistic functions to MARCH5 whereby Mul1 has a pro-mitophagy affect; however, it is currently unclear what the significance of Mul1 having both SUMO and ubiquitin E3 ligase activity. Perhaps the presence of one modification can act as a commitment to one fate instead of another. For example, SUMOylation of RIG-1 by Mul1 in antiviral signalling is suggested to be a protective mechanism against ubiquitin-induced degradation of RIG-1 (Jenkins et al., 2013).

Mitochondria-derived vesicles (MDVs)

Upon setting out to identify new mitochondrially-targeted ubiquitin and SUMO E3 ligases containing a RING domain, Neuspiel *et al*. (2008) identified a previously unannotated mitochondrial gene which they referred to as MAPL (will be called Mul1 in continuity with the previous section). When probing the function of this protein they discovered that when overexpressed, Mul1 led to Drp1-dependent fragmentation of the mitochondrial network. However, upon closer inspection, Mul1 overexpression led to the production of 70-100 nm diameter vesicular structures which were shown to persist with Drp1 knockdown or Drp1 dominant-negative expression (Neuspiel et al., 2008). The formation of these Mul1-dependent MDVs has since been shown to be dependent on members of the retromer complex – namely Vps35 and Vps26a – with them ultimately fusing with peroxisomes (Braschi et al., 2010; Wang et al., 2016) (figure 4).

Since this initial discovery, the McBride group has continued to characterise MDVs, leading to a broader appreciation of these vesicles. Alongside the peroxisome-destined MDVs there appears to be a heterogeneous pool of MDVs varying in cargo. Tom20-positive vesicles have been observed to be destined for the lysosome in a process distinct from autophagy and to precede fragmentation of the mitochondrial network (Soubannier et al., 2012a). Alongside this there also appears to be a Tom20-negative but matrix-positive (shown by PDH1α, but exhibiting a selective complement of matrix proteins) population of MDVs destined for the lysosome which show an enrichment of Parkin at the site of budding (McLelland et al., 2014). These are dependent on syntaxin-17 and PINK1 but not on

the E3 ligase activity of Parkin. Furthermore this subtype of MDV are produced soon after oxidative stress but not upon CCCP treatment; a widely used activator of mitophagy (figure 4) (Matheoud et al., 2016; McLelland et al., 2016, 2014; Soubannier et al., 2012a). The fusion with the lysosome is ultimately dependent on a SNARE-like mechanism, utilising SNAP29 and VAMP7 (McLelland 2016).

To identify variations in the cargoes of MDVs under different stresses, Soubannier *et al*. (2012b) developed a MDV fractionation protocol. Strikingly, the use of different drugs led to differential incorporation of mitochondrial cargoes into the vesicles. For example, antimycin A treatment was shown to increase the incorporation of complex III subunit core 2 but in contrast xanthanine oxidase (whole cell reactive oxygen species) does not, but instead incorporates oxidised VDAC1. In fact, following on from the previous idea of the dependence of oxidising drugs stimulating MDV production, one can observe an enrichment of oxidised proteins in MDVs using this fractionation approach (Soubannier et al., 2012b). What is still not clear from this data is a broader understanding of cargo incorporation as currently a low throughput analysis of cargoes has been carried out. Therefore, a comprehensive understanding of cargo incorporation under different stresses and which of these colabel will prove invaluable in unpicking the function of these vesicles.

From investigations with a combination of approaches ranging from fractionation to time lapse confocal microscopy, it is clear that MDVs act as an interesting point in mitochondrial quality control (Sugiura et al., 2014). However, what is not yet clear is the physiological role of these vesicles. Why should a subpopulation of vesicles be targeted to the peroxisome? To what extent does the proposed pruning of oxidised mitochondrial content need to occur to ensure mitochondrial health? In regard to this review it is also of great interest to know what the relevance of MDVs is in maintaining neuronal health. Parkin-dependent MDVs appear to be ubiquitin-independent and occur prior to stimulation of mitophagy in oxidative stress; therefore, what would be the significance of this in PD-associated Parkin mutants? Perhaps ubiquitin serves as a nucleation point from the movement from local quality control measures to large scale alterations in mitochondrial dynamics and ultimately degradation following mitochondrial stress.

Ubiquitin: a master regulator of a hierarchy of mitochondrial homeostasis?

The activities of E3 ligases at the mitochondria impinge upon a number of aspects of mitochondrial biology including mitochondrial quality control. It is reasonable to suppose that the pleiotropic functions of the E3 ligases could act as a way to regulate a hierarchy of mitochondrial homeostasis. Indeed, Parkin and Mul1 appear to regulate quality control from MDV formation, mitochondrial dynamics all the way up to the clearance of whole mitochondria. One key component to this multi-levelled approach to mitochondrial quality control could be the extent of ubiquitination. During the early stages of damage, MDV formation could act as the first step in mitochondrial quality control. The balance of the ubiquitination on the mitochondria by the E3 ligases and DUBs could then serve as a way to reorganise the mitochondrial network through trafficking and fission/fusion. Finally, when damage and subsequent ubiquitination surpasses a critical point, engagement of the autophagy

machinery can occur resulting in the clearance of the faulty organelle. As a result, this hierarchy preserves as much of the mitochondria in a healthy state throughout a variety of insults.

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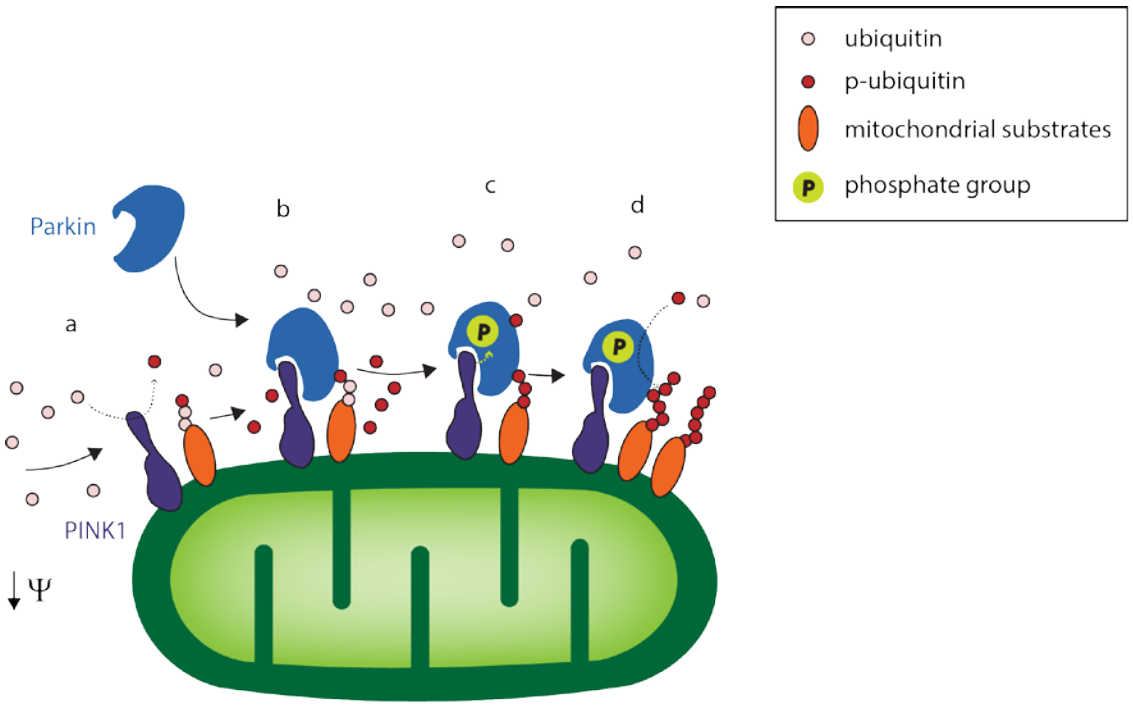

Figure 1 – PINK1/Parkin-mediated mitophagy

Depolarisation of mitochondria causes the stabilisation of PINK1 on the outer mitochondrial membrane. PINK1 then phosphorylates ubiquitin as well as some OMM proteins. This triggers the recruitment and phosphorylation of Parkin, which results in a feedforward mechanism to trigger OMM protein ubiquitination and ultimately mitophagy.

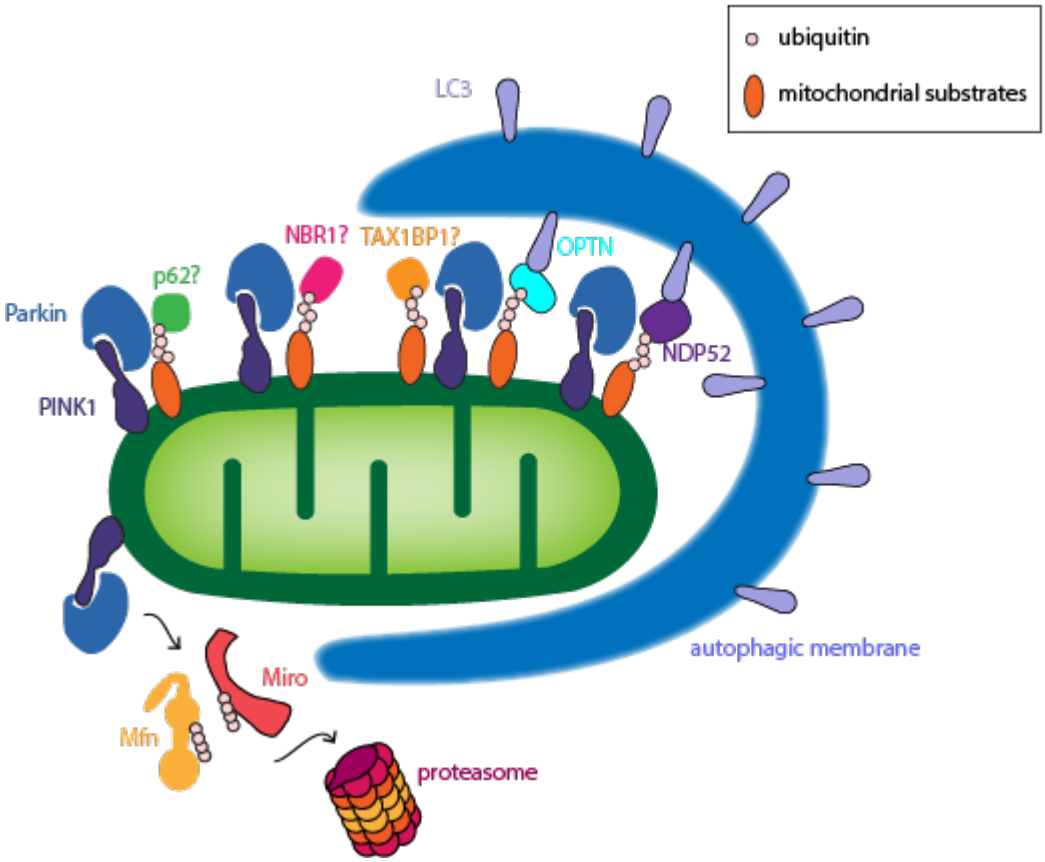

Figure 2 – Binding of damaged mitochondria to the autophagosomal membrane

Parkin-mediated ubiquitination leads to the degradation of specific mitochondrial proteins, including Miro and Mfn, via the proteasome. Concurrently mass mitochondrial ubiquitination leads to the engagement of autophagy machinery. Poly-ubiquitin chains on mitochondrial substrates bridge to LC3 decorated autophagosomal membranes via autophagy receptors, including NDP52 and OPTN, to engulf damaged mitochondria into autophagosomes.

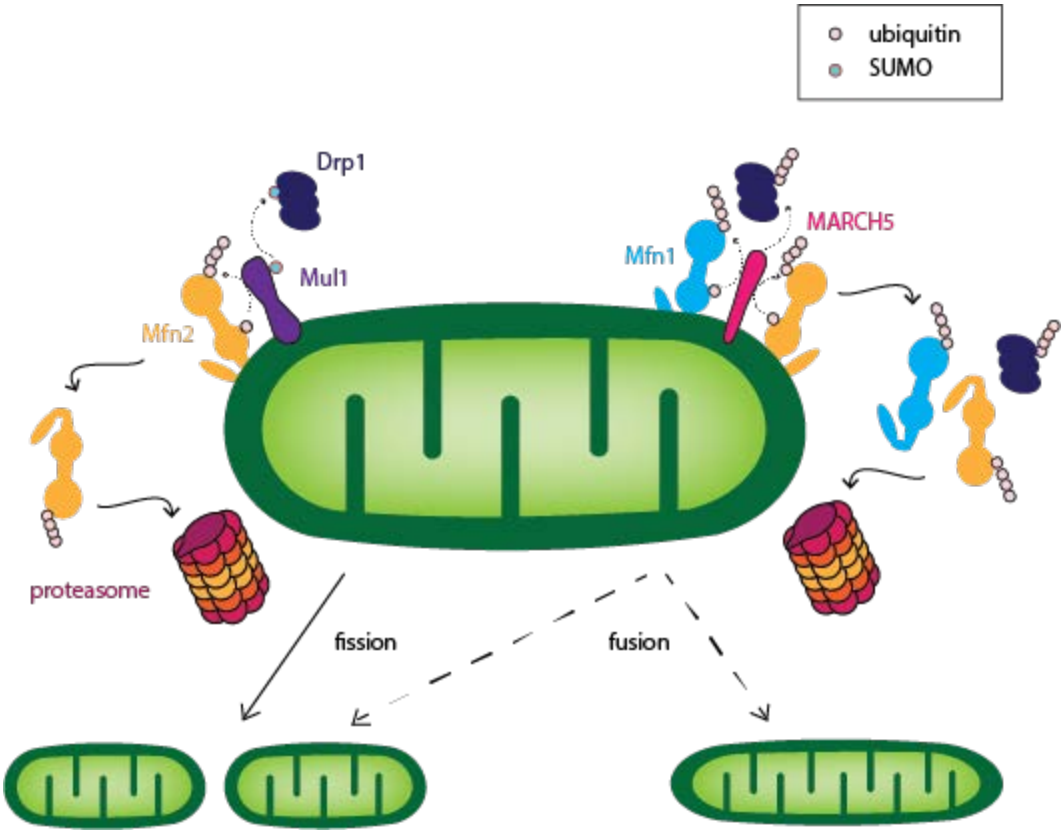

Figure 3 – Role of the E3-ligases, Mul1 and MARCH5

The E3-ligase activity of Mul1 and MARCH5 regulate the levels of the mitochondrial fission and fusion machineries. Mul1 causes fragmentation of the mitochondrial network due to its ubiquitination of Mfn2, which leads to a decrease in mitochondrial fusion. Additionally, Mul1 SUMOylates Drp1, resulting in a stabilisation of the protein and an increase in mitochondrial fission. MARCH5 is known to ubiquitinate the dynamin-related GTPases: Mfn1/2 and Drp1. The overall effect of MARCH5 E3-ligase activity is unclear as it affects the protein levels of both fission and fusion machineries

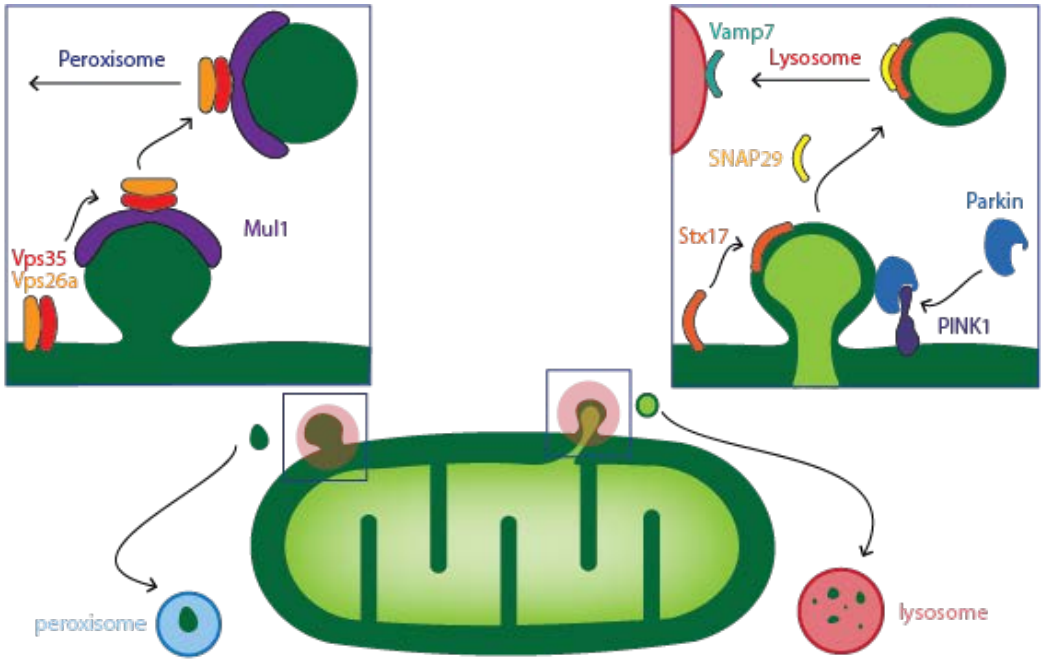

Figure 4 – Formation and degradation of MDVs

Mul1-mediated formation of MDVs is dependent on Vps35 and Vps26a, members of the retromer complex, which allow fusion with the peroxisome. Additionally, MDVs can be formed following PINK1 OMM stabilisation and Parkin recruitment to the mitochondria. These are dependent on syntaxin-17, rather than the E3-ligase activity of Parkin, and fuse with the lysosome in a SNARE-like mechanism, utilising SNAP29 and VAMP7.