Mitochondrial dysfunction and mitophagy in Parkinson's disease: from mechanism to therapy

Ana Belen Malpartida[1], Matthew Williamson[1], Derek P Narendra[2], Richard Wade-Martins*[1] and Brent J Ryan*[1]

1. Oxford Parkinson's Disease Centre, Department of Physiology, Anatomy and Genetics, Le Gros Clark Building, University of Oxford, South Parks Road, Oxford, OX1 3QX.
2. Inherited Movement Disorders Unit, National Institute of Neurological Disorders and Stroke, Bethesda, MD.

*To whom correspondence should be addressed
Brent.ryan@dpag.ox.ac.uk or Richard.wade-martins@dpag.ox.ac.uk

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Abstract
Mitochondrial dysfunction has been associated with neurodegeneration in Parkinson's disease (PD) for over 30 years. Despite this, the role of mitochondrial dysfunction as an initiator, propagator, or bystander remains undetermined. The discovery of the role of the PD familial genes *PINK1* and *PRKN* (parkin) in mediating mitochondrial degradation (mitophagy) reaffirmed the importance of this process in PD aetiology. Recently, progress has been made in understanding the upstream and downstream regulators of canonical PINK1/parkin-mediated mitophagy, alongside non-canonical PINK1/parkin mitophagy, in response to mitochondrial damage. Progress has also been made in understanding the role of PD-associated genes such as *SNCA*, *LRRK2*, and *CHCHD2* in mitochondrial dysfunction and their overlap with sporadic disease, opening up opportunities for therapeutically targeting mitochondria in PD.

Mitochondrial dysfunction lies at the heart of Parkinson's disease

Parkinson's disease (**PD**) (see Glossary) is a common neurodegenerative disorder characterised by loss of **dopaminergic neurons** in the **substantia nigra pars compacta** (**SNpc**). This results in the loss of dopaminergic inputs in the striatum and the characteristic motor symptoms associated with the disease. Accumulation of aggregated **α-synuclein** in **Lewy bodies** are the pathological hallmark of PD in the SNpc, but can also be observed in other affected brain regions.

Mitochondrial dysfunction has been recognised as an important initiating factor in dopaminergic neuronal loss for over 30 years given that toxins inhibiting complex I induce dopaminergic cell loss and parkinsonism, complex I is dysfunctional in sporadic PD patient tissues, and the familial autosomal recessive PD genes ***PINK1*** and *PRKN* (**parkin**) have been discovered to play a key role in mitochondrial quality control.

This review explores the recent progress in understanding the regulation of the PINK1/parkin pathway in PD pathogenesis, the roles of established PD genes such as *SNCA* and ***LRRK2*** in mitochondrial function, and the discovery of new PD-associated genes with mitochondrial roles, such as ***CHCHD2***. Additionally, we discuss recent data highlighting commonalities in mitochondrial dysfunction in sporadic disease and monogenic PD, in addition to presenting the therapeutic opportunities presented by recognition of mitochondrial dysfunction and impaired **mitophagy** in PD.

Regulation of the PINK1/parkin pathway and its role in mitophagy

Mutations in the PINK1 (*PARK6*) and parkin (*PARK2*) genes were among the first to be linked to autosomal recessive early-onset PD [1, 2]. Although mutations in both genes presented with similar clinical phenotypes, their contribution to PD pathology remained undefined until the role of PINK1 and parkin in mitophagy was described in 2010 [3].

The mechanism of PINK1/parkin activation has been thoroughly investigated and reviewed in detail [4]. However, recent advances have added further to our knowledge of both inducers and inhibitors of this pathway (**Box 1**). These new findings have implications for PD pathophysiology and therapeutic developments for PD patients both with and without *PINK1* or *PRKN* mutations.

Structural information provides insights into the mechanism of PINK1/parkin activation

The most up-to-date model for parkin activation by PINK1 suggests that following mitochondrial membrane depolarization, PINK1 is stabilized at the outer mitochondrial membrane (OMM) where it phosphorylates pre-existing **ubiquitin** (Ub) at serine 65 residues (**pSer65Ub**) [5]. Auto-inhibited cytosolic parkin is then recruited to sites of PINK1 activity where RING1 of parkin domain binds pSer65Ub initiating a series of conformational changes

[6]. Notably, parkin remains in a partially auto-inhibited form once bound to pSer65Ub and is only fully activated by phosphorylation of its Ub-like domain by PINK1 [7] (**Figure 1**).

The role of phosphorylation of ubiquitin at serine 65 is increasingly appreciated as a marker of PINK1-induced pathway activation, leading to the recruitment of parkin in a feedforward mechanism (**Figure 1**). Once active, parkin synthesises Ub chains on OMM proteins, providing more substrates for PINK1 ubiquitin phosphorylation, which in turns recruits more parkin, ultimately leading to maximally active (~4,400-fold activation) Ub chain assembly (as reviewed in [8]). Evidence of increased pSer65Ub formation has been observed in post-mortem PD brain and diminished in patients with *PINK1*/*PRKN* mutations, indicating the relevance of this pathway in disease [9, 10].

Although a structure of fully active parkin has not yet been reported, the structure of PINK1, alone and in complex with Ub, has been thoroughly investigated. It has been shown that PINK1 preferentially binds to a Ub conformation with a retracted C-terminus (Ub-CR) at a 50-fold higher rate than the wildtype Ub conformation [11]. The Ub-CR conformation has an extended Ser65 loop that promotes its binding to the kinase-activation segment of PINK1 whereas non-phosphorylated Ub is unable to bind to this domain directly. However, it has been suggested that unmodified Ub can bind to PINK1 and be transformed to the Ub-CR conformation while bound, stabilizing the complex and promoting subsequent phosphorylation of Ser65 residues [11].

Regulators of PINK1 and parkin

Regulators of PINK1 and parkin protein levels have been recently identified, offering mechanistic and therapeutic insights. For example, genome-wide CRISPR screens have been used to identify a transcriptional repressor network, including THAP11, which negatively regulates parkin levels and mitophagy [12], and the adenine nucleotide translocator (ANT) complex which regulates TIM23-mediated translocation and stabilization of PINK1, upstream of depolarisation-induced mitophagy [13]. Similarly, Gas7 is another regulator found to act upstream of PINK1, regulating PINK1 protein levels and pSer65Ub formation, with the loss of Gas7 impairing mitophagic flux [14].

Post-translational regulation of both PINK1 and parkin have been demonstrated as additional mechanisms to regulate their activity. Several kinases, including AKT, have been proposed to initiate PINK1/parkin dependent mitophagy in various cell models, including induced pluripotent stem cell (iPSC)-derived dopaminergic neurons [15], linking upstream signalling pathways in cell stress to the initiation of mitophagy. Both ageing and α-synuclein oligomers have been demonstrated to increase parkin S-nitrosylation, increasing parkin auto-ubiquitination and subsequent degradation [16, 17], highlighting S-nitrosylation as an important regulator of mitophagy. Excessive PINK1 S-nitrosylation can impair PINK1 kinase

activity resulting in increased neuronal death in both iPSC-derived dopaminergic neurons and α-synuclein transgenic PD mice [18, 19].

Deubiquitylases as gatekeepers of mitophagy

A number of **deubiquitylases** (**DUBs**), including USP8, USP14, USP15, USP35, and most prominently USP30, have been found to regulate mitophagy by antagonising parkin activity, acting either directly on parkin or its substrates [8, 20].

USP30 is an established OMM-localised enzyme which negatively regulates PINK1/parkin-dependent mitophagy through deubiquitylation of several OMM substrates including RHOTs, VDACs, MFNs, and components of the OMM translocon [21, 22] (**Figure 1**). *In vitro* studies have demonstrated that USP30 shows selectivity for cleavage of K6- and K11-linked Ub chains [21, 23] with increased binding to K6-linked Ub chains, and increased hydrolysis of K11-linked Ub chain stoichiometry. USP30 demonstrates a lower affinity for cleavage of pSer65Ub chains allowing the synthesis of phosphorylated ubiquitin chains during PINK1/parkin activation [17, 23, 24]. However, the role of USP30 in maintaining mitochondrial quality control in dopaminergic neurons in response to physiological stimuli remains to be investigated.

Recent studies have also established a role for USP30 in regulating mitophagy under basal conditions independently of PINK1/parkin activity (**Box 2**) [22, 25]. Interestingly, knockdown of PINK1 (but not parkin) was able to suppress the enhancement of mitophagy seen upon USP30 knockdown, suggesting that USP30 acts upstream of PINK1 to set the threshold for initiating mitophagy [22, 25, 26]. Furthermore, two classes of ubiquitylation events were identified to be more abundant in USP30 depleted iPSC-derived induced neurons [27] being: (1) OMM protein ubiquitylation was elevated under basal conditions but not during depolarisation (e.g TOM40 and VDAC2); and (2) OMM protein ubiquitylation was elevated under basal conditions and further increased during depolarisation (e.g TOM20, TOM5 and VDAC1). Interestingly, these classes of substrates may represent targets of PINK1-independent and PINK1-dependent mitophagy, respectively, giving an insight into the role of USP30 in regulating mitochondrial homeostasis and mitophagy.

Under basal conditions, USP30 knockout (KO) neurons display increased ubiquitylation of proteins normally imported into the mitochondrial matrix or inner membrane via the **TOM complex** [27, 28]. Constitutive ubiquitylation during import of mitochondrial proteins relies on the E3 Ub ligase March5 and is antagonised by USP30, thereby dynamically regulating mitochondrial protein import [28]. However, March5 knockdown did not fully eliminate intramitochondrial protein ubiquitination, suggesting that additional E3 Ub ligases function upstream of USP30 [28].

USP30 has a multi-faceted role in mitochondrial quality control, making it an attractive target for therapeutic intervention in diseases characterised by mitochondrial dysfunction. Further

elucidation of the interplay between USP30, PINK1, parkin and other E3 Ub ligases in complex neuronal models, especially in the context of PD, are needed.

Identification of pSer65Ub phosphatases as negative regulators of mitophagy

Ub phosphorylated at Ser65 by PINK1 undergoes structural changes to become resistant to DUB activity [29]. Dephosphorylation of pSer65Ub is, therefore, a potentially critical negative regulatory mechanism in the modulation of mitophagy (**Figure 1**).

Recently, phosphatases such as PTEN-Long (PTEN-L or PTEN-α) and PPEF2 have been demonstrated to regulate pSer65Ub phosphorylation and act as negative regulators of mitophagy [30, 31]. These phosphatases have been demonstrated to localise to the OMM and maintain mitochondrial quality control in both neurons and astrocytes *in vitro* and *in vivo* [32, 33]. In addition, recent reports demonstrated that PINK1 phosphorylates Ub in astrocytes to a greater extent than in neurons [34]. Although further studies are needed, this raises the possibility that phosphorylation and dephosphorylation of mitochondrial proteins may play a role in modulating mitochondrial dysfunction primarily in non-neuronal cells.

PTEN-L is a translational variant of PTEN and was the first specific phosphatase described to antagonize PINK1 function by acting on pSer65Ub chains. PTEN-L knockdown significantly increasing Ub phosphorylation at Ser65 in a parkin-dependent manner [30]. More recently, PPEF2 was identified as another phosphatase antagonizing PINK1 activity [33]. Like PTEN-L, PPEF2 overexpression was shown to significantly reduce the rate of pSer65Ub formation in cortical neuronal cultures. However, in parkin-overexpressing models the effect of PPEF2 was significantly diminished [33] as the strength of the PINK1/parkin feedforward mechanism appeared to outweigh the rate of dephosphorylation. Additionally, PTEN-L KO mice displayed a *decrease* in mitophagy shown by impaired parkin recruitment in cardiomyocytes [32], suggesting that increased phosphorylation of Ub is not the sole determinant for increasing mitophagy.

Together, these recent advances in understanding the mechanisms which promote and restrain PINK1/parkin mitophagy have provided a clearer picture of mitochondrial homeostasis and canonical PINK1/parkin mitophagy. This information allows an understanding of the likely phenotypic consequences of mutations in different regions of *PINK1* or *parkin* for PD patients, as well as understanding the potential therapeutic opportunities to enhance PINK1/parkin mitophagy by increasing expression levels or activity.

Roles of PINK1 and parkin beyond canonical PINK1/parkin-mediated mitophagy

Loss-of-function mutations in either PINK1 or parkin are associated with the development of autosomal recessive familial PD in humans. It is therefore important to understand the role of PINK1 and parkin in PD beyond canonical PINK1/parkin-dependent mitophagy (**Box 2**). Early *Pink1/Prkn* KO mouse models failed to recapitulate PD pathology, and mitochondrial defects

were only found in midbrain-specific KO models [35]. However, locomotor deficits and dopaminergic neuron loss have recently been observed in two-year-old *Prkn* KO mice [36]. These observations suggest a subtle role for mitophagy in age-dependent neurodegeneration and further emphasise the preferential vulnerability of dopaminergic neurons (**Box 3**).

Isogenic *PRKN* KO iPSC-derived dopaminergic neurons have been demonstrated to have substantial changes in both their native and post-translational proteome, resulting in disruption of both mitochondrial and non-mitochondrial phenotypes including impaired RhoA signalling which results in impaired neurite outgrowth [37]. In addition, loss of *PRKN* decreases the activity of lysosomal enzymes, including GBA (GCase). Given that parkin also targets several vacuolar ATPases for degradation [38], together these data suggest a loss of PRKN has an impact downstream of mitochondria [39] affecting cellular processes beyond mitochondrial homeostasis. iPSC-derived dopaminergic neurons from PD patients with compound heterozygous *PRKN* mutations demonstrate a loss of parkin and an inability to release ER-mitochondrial contact sites in response to mitochondrial depolarisation [40]. Additionally, a comparison of several monogenic PD rat models using *in vivo* microdialysis has demonstrated alterations in dopamine and/or dopamine metabolites as well as alterations in other neurotransmitters in *PINK1* and *parkin* KO rats [41].

Furthermore, PINK1/parkin modulates inflammation in response to activation of the STING pathway induced by mitochondrial DNA damage [42]. Loss of *Pink1* has also been shown to increase mitochondrial antigen presentation (MiTAP) with bacterial challenge of *Pink1* KO mice demonstrating cytotoxic (CD8+) T-cell responses in both the gut and brain [43]. Given that MITAP induction correlated with loss of dopaminergic neuronal axonal varicosities in the striatum [43], the role of MITAP and peripheral inflammation in PD and particularly PINK1/parkin mutation patients is of great interest.

The effects of mutations in PD causing genes on mitochondrial function and mitophagy

Recent findings have greatly increased our understanding of the mechanisms by which established monogenic PD-associated proteins alter mitochondrial function and mitophagy, providing interesting insights into overlap between mechanisms involved in monogenic PD. In addition to the long-established autosomal dominant familial mutations in *SNCA* and *LRRK2*, other genes implicated in mitochondrial function/dysfunction carry inherited PD mutations, such as *CHCHD2 (PARK22)*, *GCH1*, and *VPS35* (*PARK17*), and, in some cases, also show association with PD in genome-wide association studies (GWAS). Understanding how these pathways trigger nigral degeneration will inform on mechanisms in both familial and sporadic PD.

α-synuclein

α-synuclein, the main component of Lewy bodies, has long been associated with disruption of mitochondrial function but without a clear mechanistic rationale. In recent years, significant developments have been made in assessing the mitochondrial targets affected by α-synuclein and the α-synuclein species responsible for these effects (**Figure 2**). For instance, overexpression of α-synuclein or the addition of exogenous aggregated α-synuclein species has shown α-synuclein to interact with several OMM components including TOM20 [44, 45], VDAC [46], and F_1F_o-ATP synthase, causing mitochondrial permeability transition pore (MPTP) opening [47]. Accumulated pathological α-synuclein, or dopamine-modified or phospho-mimetic species mimicking pathological forms of the protein, have been shown to preferentially bind to mitochondria [44, 48], inhibiting mitochondrial protein import and leading to mitochondrial membrane depolarization and impaired cellular respiration [48].

Models of seeded α-synuclein aggregation demonstrated decreased levels of MFN-2 and OPA1 levels [49], consistent with previous reports of α-synuclein-induced mitochondrial fragmentation and bioenergetic alterations in iPSC-derived dopaminergic neurons [45]. Proteomic analyses revealed significant recruitment of proteins involved in oxidative phosphorylation into aggregates consistent with impairments in respiration. Furthermore, recent studies have demonstrated that the process of Lewy body formation, rather than aggregation itself, is the key driver for dysfunction induced by α-synuclein [49] Mitochondrial dysfunction and complex I deficits are temporally separated from pS129 formation induced by α-synuclein fibrils in hippocampal neurons, suggesting additional mechanisms beyond the interaction between α-synuclein and the mitochondria are needed for dysfunction [49]. Indeed, Lewy bodies from PD patients have been found to contain fragmented mitochondria crowded with lipids and lysosomes as well as α-synuclein [50], again suggesting that mitochondrial dysfunction, α-synuclein aggregation and Lewy body formation are intrinsically linked.

LRRK2

A growing body of evidence links LRRK2 mutations to PD-associated mitochondrial dysfunction (**Figure 3**). It has been shown in iPSC-derived dopamine neurons that LRRK2 can regulate mitophagy by removing the OMM adaptor protein MIRO from the MIRO/MILTON/KINESIN motor complex resulting in reduced mitochondrial transport along the cytoskeleton, reducing engulfment by autophagosomes and subsequent degradation [51]. The pathogenic G2019S mutation in the kinase domain of LRRK2 disrupts this function, slowing the initiation of mitophagy [51]. These observations are in agreement with previous reports of impaired mitochondrial trafficking in *LRRK2*-R1441C mutant rat neurons [52]. Importantly, these phenotypes have also been observed in neuronal cultures from idiopathic

PD patients, strengthening the argument for a more general role of compromised mitophagy in the pathology of PD [51].

Furthermore, two independent studies have reported that mutant LRRK2 can impair PINK1/parkin-dependent mitophagy via independent mechanisms [53, 54]. The increased kinase activity of the LRRK2-G2019S mutant was shown to disrupt protein-protein interactions on the OMM early in PINK1/parkin-dependent mitophagy, including the recruitment of the mitochondrial fission protein Drp1 and parkin [53]. In addition, both the G2019S and R1441C mutations in LRRK2 impair later stages of PINK1/parkin-dependent mitophagy through increased phosphorylation of the small GTPase RAB10 inhibiting its interaction with the autophagy receptor OPTN and reducing accumulation on depolarised mitochondria [54]. However, given the differential expression of LRRK2 between brain regions and cell types [55], the role of LRRK2 in initiating mitochondrial dysfunction solely in neurons cannot be assumed.

Further advances have identified mitochondrial roles for additional PD-associated genes. Whilst many of the details of the mechanisms of these dysfunctions remain to be elucidated, including the precise effects of these mutations on mitochondrial turnover, the convergence of these dysfunctions are of great interest to improve our understanding of PD pathology.

GBA

Heterozygous mutations in *GBA*, which encodes the lysosomal enzyme GCase, responsible for degrading glucosylceramide, are the strongest genetic risk factors for PD. Recently, analysis of post-mortem brain tissue from PD patients carrying heterozygous *GBA* mutations revealed increased mitochondrial content, increased mitochondrial oxidative stress, and impaired autophagy [56]. These findings mirror previous observations in iPSC-derived dopaminergic neuronal cultures from patients with *GBA*-L444P mutations and *GBA* KO neurons [57]. Furthermore, primary hippocampal neurons from *Gba*$^{L444P/WT}$ knock-in mice displayed abnormal mitochondrial morphology, increased mitochondrial oxidative stress, and defects in basal and PINK1/parkin-dependent mitophagy [56], again pointing toward a more general defect in mitophagy in the pathology of PD.

CHCHD2

Mutations in the *CHCHD2* gene were identified as a novel familial PD gene in 2015 [58]. *CHCHD2* encodes a protein which modulates mitochondrial function in conjunction with the ALS/FTD-associated gene *CHCHD10* [59]. Further analysis of this pathway has identified that CHCHD2 accumulates in damaged mitochondria and regulates CHCHD10 oligomerisation [60].

CHCHD2 has been shown to play a significant role in the maintenance of mitochondrial cristae [59] as well as stabilising OPA1 to promote mitochondrial fusion [61]. Mutations in CHCHD2 have been demonstrated to induce precipitation of both mutant and wild type (WT) protein

in the intermembrane space (IMS) [61] as well as cytochrome C destabilization, impaired respiration, and mitochondrial ROS generation [62]. Moreover, CHCHD2 mutations have been associated with α-synuclein aggregation and oligomerization in human post-mortem brain tissues and iPSC-derived dopaminergic neurons [63]. Although CHCHD2 has been demonstrated to play a vital role in mitochondrial health, its potential role in the induction of mitophagy remains to be elucidated.

GCH1

GTP-cyclohydrolase 1 (GCH1) is the rate-limiting enzyme in synthesis of tetrahydrobiopterin (BH4), a cofactor for enzymes including tyrosine hydroxylase and nitric oxide synthase, as well as acting as an antioxidant. Polymorphisms in the *GCH1* gene are enriched in PD patients and rare mutations have been identified in up to 0.75% of PD patients [64]. α-synuclein has also been demonstrated to alter GCH1 activity [65]. In addition to the cytoplasmic role of BH4, it has been demonstrated that BH4 plays a role in mitochondrial function and redox regulation [66]. The absence of BH4 results in decreased mitochondrial function and increased levels of DRP1 activation, proteome remodelling and metabolic alterations [66].

VPS35

VPS35 plays a role in recycling DLP1 complexes, with mutations and oxidative stress increasing VPS35 interactions and mitochondrial fission [67]. Parkin has been shown to poly-ubiquitinate VPS35 affecting endosomal sorting WASH complex-mediated retromer [38]. More generally, VPS35 mutations disrupt autophagosome formation through ATG9a, inhibiting autophagosome formation and vesicle transport between mitochondria and peroxisomes [68]. In addition, inhibition of retromer function results in accumulation of hyperactive (GTP-bound) RAB7 on lysosomes inhibiting ATG9a and autophagosome formation [69]. Given the established link between LRRK2 and VPS35 [70], the similarity between mitochondrial phenotypes in VPS35 and LRRK2 models is noteworthy.

Relevance to sporadic disease

As only ~15% of PD can be attributed to a monogenic cause, research into the molecular mechanisms of sporadic PD (sPD) is vital. A number of recent studies have investigated sporadic disease, identifying overlap between sporadic and monogenic phenotypes which may lead to a greater understanding of the interplay between dopaminergic neuronal physiology and genetics in PD.

Increased pSer65Ub has been observed in post-mortem PD brain and neuronal models of PD [9, 10, 71] but decreased in *PINK1*/*PRKN* mutation carriers, demonstrating the relevance of mitochondrial damage and mitophagy induction via the PINK1/parkin pathway in PD patients. A comprehensive study of pSer65Ub in post-mortem brain from Lewy Body Disease demonstrated a significant correlation between pSer65Ub and Braak tau tangle staging in the

hippocampus and with Lewy bodies in the amygdala, but not the substantia nigra [9]. However, how these observations apply to the general sPD population and the upstream- or downstream role of pSer65Ub in PD pathology remain important questions to be addressed.

A large study of PD patient fibroblasts identified distinct subsets of sPD patients with mitochondrial or lysosomal dysfunction [72]. Specifically, a subset of patient fibroblasts demonstrated altered complex I and IV protein levels and these deficits were enhanced after direct differentiation of dopaminergic neurons [72]. In addition, we have demonstrated that bioenergetics are altered in peripheral blood mononuclear cells from sPD patients [73]. These studies further suggest that systemic mitochondrial dysfunction, combined with the high metabolic demand of dopaminergic neurons, leads to the preferential vulnerability of dopaminergic neurons in PD (**Box 3**).

Common mechanisms between monogenic and sporadic disease have been noted, such as impaired degradation of MIRO at the mitochondrial surface, which is also observed in LRRK2 models [51, 74], and these overlaps are likely key to understanding convergent pathways in PD subgroups. Highlighting this, SNPs in a number of mitochondrial genes have been associated with the age of onset of PD [75], further suggesting a substantial role for mitochondria in sPD aetiology and the need for multiple pathways to converge to initiate disease pathology.

Therapeutics targeting mitochondrial dysfunction

Loss of mitochondrial quality control could substantially contribute to the high susceptibility of SNpc neurons to neurodegeneration in PD (**Box 3**). Recent advances in our understanding of mitochondrial homeostasis and damage in PD has led to the identification of a number of potential therapeutic avenues to promote the clearance of old or damaged mitochondria in both PD and other diseases involving mitochondrial dysfunction.

Small molecule activators of PINK1 and parkin, or inhibitors of USP30 and pSer65Ub phosphatases, are promising therapeutic targets for enhancing mitophagy in PD. Similarly, although less defined, USP8, USP14, and USP15 inhibitors represent promising routes to upregulating mitophagy [20, 76, 77].

Direct activation of PINK1 has been demonstrated by kinetin triphosphate KTP [78] and further development of bioavailable KTP precursors is underway [79]. In addition, two small-molecule activators of PINK1 which are structurally distinct from KTP have been identified by high-throughput screening and have been validated in both dopaminergic neurons and PINK1 knockdown Drosophila [80]. An orthogonal approach of directly expressing recombinant

parkin engineered for increased solubility and cell permeability has shown promise in protecting cells from both toxin and α-synuclein challenge [81].

Direct targeting of proteins impacted by autosomal dominant PD mutations offers a strategy to expand therapy beyond *PINK1*/*PRKN* patients. Encouragingly, two independent studies have demonstrated that LRRK2 kinase inhibitors are able to correct impaired RAB10-optineurin and parkin-Drp1 interactions in *LRRK2* mutant cells [53, 54]. The increased LRRK2 kinase activity in idiopathic PD has also been shown in animal models in which LRRK2 inhibition corrected mitochondrial and lysosomal dysfunction [82, 83]. LRRK2 inhibitors currently in clinical trials could therefore hold great promise for correcting mitochondrial dysfunction and mitophagy defects beyond patients with LRRK2 mutations. Similarly, the effects of augmentation of GCase activity on peripheral mitochondrial dysfunction using small molecule chaperones such as ambroxol, TFEB activators or recombinant GCase-like enzyme such as Cerezyme, will be of great interest. Moreover, nicotinamide riboside has been demonstrated to correct mitochondrial phenotype in iPSC-derived neurons carrying mutations in *GBA*, and in *Drosophila* models [57]. In addition, several small molecules, such as the repositionable drug ursodeoxycholic acid, have been demonstrated to augment mitochondrial complex activity and ATP production and to be beneficial in PD models including, importantly, in sPD fibroblasts [72].

Concluding remarks

Overall, recent progress has advanced our knowledge of the mechanisms regulating PINK1/parkin-mediated mitophagy. Further understanding the relative contributions of this pathway to basal and stress-induced mitophagy in dopaminergic neurons, when compared to non-PINK1/parkin-dependent mechanisms, will aid in the development of potential therapeutic activators of this pathway. Similarly, it is imperative to clarify the role of PINK1/parkin-dependent mitophagy in non-neuronal cells, such as astrocytes and microglia in which this pathway appears to be more active, in order to determine how deficits in mitochondrial quality control in glia contribute to the pathology of PD (see Outstanding Questions).

Given the multiple cellular mechanisms that are dysfunctional in *LRRK2*, *SNCA*, and *GBA*-related PD, enhancing mitophagy and improving mitochondrial health may only partially rescue dopaminergic cell loss in non-*PINK1*/*PRKN*-related diseases. Furthermore, the expression of PD risk genes, particularly *LRRK2,* is not homogenous across brain regions or cell types, raising the possibility of an important role for non-neuronal cells in suppressing mitochondrial dysfunction through mechanisms, such as trans-mitochondrial degradation (**transmitophagy**) or by the propagation of mitochondrial damage in either a cell-autonomous manner or through inflammatory processes such as MITAP or STING-activation. Similarly, the roles of genes such as *CHCHD2* and *VPS35* in mitochondrial dysfunction in dopaminergic

neurons and their contribution (if any) to disease in the wider PD population remains to be elucidated. Regardless of the genetic background of patients, the role(s) that increased LRRK2 kinase activity and α-synuclein aggregation play in damaging mitochondria and its impact of mitophagy is likely key in understanding convergent mechanisms in PD.

Investigation of novel PD-causing genes with mitochondrial roles, combined with the investigation of mitochondrial deficits in sporadic PD, will identify novel and overlapping mechanisms of dysfunction to enhance our understanding of the role of mitochondrial dysfunction in the disease process. Given the widespread prevalence of mitochondrial damage in a range of diseases, these mechanistic insights and the resulting therapeutic opportunities may also have utility beyond PD.

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Glossary

α-synuclein: the major component of Lewy bodies and a protein with diverse roles in cellular biology; mutations in α-synuclein increase the propensity for the protein to aggregate and cause autosomal dominant familial Parkinson's disease (PD)

Cyanide m-chlorophenyl hydrazine (CCCP): is a protonophore and a potent mitochondrial uncoupler that depolarizes the mitochondrial membrane and induces mitophagy; CCCP is extensively used in the study of PINK1/Parkin-dependent mitophagy

CHCHD2: a mitochondrial protein and familial PD risk gene which regulates mitochondrial function

Dopaminergic neurons: neurons which release the neurotransmitter dopamine; the midbrain dopaminergic neurons in the substantia nigra pars compacta (SNpc) are preferentially vulnerable in PD, whereas the neighbouring dopaminergic neurons of the ventral tegmental area (VTA) are relatively spared.

Deubiquitylases (DUBs): a large group of proteins that remove ubiquitin (Ub) chains from proteins; DUBs such as USP8, USP14, USP15, USP35, and most prominently USP30 have been found to regulate mitophagy by antagonising parkin activity

Lewy bodies: intracellular protein aggregates comprised of misfolded proteins of which α-synuclein is a prominent component; Lewy bodies are the defining pathological feature of post-mortem PD brain

LRRK2: leucine-rich repeat kinase 2 (LRRK2) is a widely expressed multidomain kinase; mutations in LRRK2 are the most common form of autosomal dominant PD

Mitophagy: a form of selective autophagy targeting damaged mitochondria making use of two major degradation systems: autophagy and the ubiquitin-proteasome system; mitophagy acts as a mitochondrial quality-control mechanism

Parkin: an E3 Ub ligase catalysing the attachment of Ub chains to substrate proteins; after activation by PINK1, parkin ubiquitinates outer mitochondrial membrane proteins and mediates the clearance of damaged mitochondria, and mutations in *PRKN* cause autosomal recessive PD

Parkinson's disease (PD): a progressive, neurodegenerative disease characterized by both motor and non-motor symptoms; pathologically, the disease is characterized by the aggregation of α-synuclein into Lewy bodies and the preferential degeneration of dopaminergic neurons of the SNpc

PINK1: PTEN-induced putative kinase 1 (PINK1) is a serine/threonine-protein kinase playing a central role in mitophagy following mitochondrial membrane depolarization phosphorylating both parkin and Ub; mutations in PINK1 cause autosomal recessive PD

pSer65Ub: PINK1 phosphorylates the serine 65 residue on Ub chains on the outer mitochondrial membrane forming phospho-serine65 Ub (pSer65Ub); pSer65Ub plays a crucial role in the amplification of the PINK1/parkin pathway

Substantia nigra pars compacta (SNpc): the dopaminergic nigrostriatal neurons arising from the SNpc release dopamine in the striatum modulating motor activity; the SNpc A9 region is the major site of dopaminergic neuron loss in PD

TOM complex: the translocase of the outer mitochondrial membrane (TOM) is a complex of proteins (e.g. TOM20 and TOM40) that regulate protein transport across the outer mitochondrial membrane

Trans-mitophagy: degradation of mitochondria in a non-cell-autonomous manner, e.g. by glial cells

Ubiquitin (Ub): a small protein that can post-translationally modify proteins, influencing their function and targeting them for degradation

Ventral tegmental area (VTA): a dopaminergic region of the midbrain neighbouring the SNpc. The VTA A10 dopaminergic neurons are relatively spared in PD

Elements

BOX 1 Elucidating the role of the PINK1/parkin pathway in basal mitophagy and its relevance to PD

The majority of studies of PINK1/parkin-dependent mitophagy have relied on chemical agents to inhibit mitochondrial function. Use of the ionophore **cyanide m-chlorophenyl hydrazine** (**CCCP**), or a combination of the ATP synthase inhibitor oligomycin and the complex III inhibitor antimycin A, are the most common agents used to activate PINK1/parkin-dependent mitophagy. Whilst informative, the large-scale loss of mitochondrial function in the cell after such chemical inhibition represents supra-pathophysiological conditions. In addition, much research has relied on expression/overexpression of parkin in order to observe mitophagy-related phenomena such as parkin translocation, TOM20 loss, and lysosomal cargo delivery. The identification of the pathophysiologically-relevant activators of PINK1/parkin mitophagy remains to be elucidated.

The field has been advanced by tools to measure the delivery of pH-sensitive proteins fused to mitochondrial cargo destined for lysosomes such as mitoQC [84], mtKeima [85], and recently, mito-SRAI [86]. Data from *Drosophila* expressing mitoQC or mtKeima and from mouse dopaminergic neurons expressing mitoQC have demonstrated basal mitophagy is not affected by the loss of PINK1 or parkin [87, 88]. These data seemingly contradict experiments using mtKeima demonstrating a role for PINK1 in basal mitophagy [89], leaving the relative contribution of the PINK1/parkin pathway to basal mitophagy unclear. However, given the localization of mitoQC to the outer mitochondrial membrane (OMM) and mtKeima or mito-SRAI to the mitochondrial matrix, the relative contributions of OMM protein degradation by proteasome (or MDVs) in different organisms and cell types, may be responsible for the discrepancy [86], suggesting multiple tools and approaches may be required to gain consensus.

Recently, modified versions of mitoQC, targeted to the matrix with a Flag-V5 processing sequence (mtx-XL), and mtKeima, with an additional Flag-V5 epitope (mtx-Keima^{XL}), have been generated [27], potentially closing the subtle methodological gaps between these two tools. Independent methods of assessing mitophagy, such as mitochondrial protein turnover using mass spectrometry, which identified both Atg7-dependent and independent roles of PINK1-parkin in *Drosophila* and a reduction of mitochondrial heteroplasmy with PINK1/parkin overexpression [90], suggest PINK1/parkin can play a role. In addition, assays such as TOM20/LC3 co-localisation provide alternative methods to assess mitochondrial turnover [91].

Together, these observations highlight a wealth of tools and models for assessing mitophagy but also the need to consider the exact experimental paradigm and model used to make

767 conclusions in cell models and *in vivo*.

BOX 2 PINK1/parkin-independent mitophagy pathways

While the canonical PINK1/parkin pathway is linked to Parkinson's disease (PD) through familial genetics, additional PINK1/parkin-independent mechanisms of degradation of damaged mitochondria in mammalian cells are also important in the disease.

A number of PINK1-independent pathways mediated by outer mitochondrial membrane (OMM) proteins that can interact with Atg8 homologs at the autophagosome through an LIR-interacting domain (LC3-interacting domain) have been identified, including BNIP3 (Bcl-2/adenovirus E1B 19-kDa interacting protein 3), NIX (BNIP3-like; BNIP3L), FUNDC1 (FUN14 Domain Containing 1), and AMBRA1. Additionally, a number of E3 Ub ligases other than parkin have been demonstrated to ubiquitinate OMM proteins including MUL1 [92] and SIAH1 [93], resulting in receptor-mediated mitophagy. The PD-associated gene *FBXO7* (*PARK15*) encodes an adaptor in the FBXO7-SCF E3-ligase complex and facilitates parkin translocation to mitochondria and mitophagy [94]. Mutations in *FBXO7* impair FBXO7-parkin interactions resulting in mitochondrial protein misfolding and impaired mitophagy [94, 95]

Post-translational regulation of OMM-LIR domain proteins occurs in response to a diverse range of stimuli beyond the loss of membrane potential (ψ_m) (as reviewed in [96]). For example, FUNDC1 phosphorylation status at the N-terminal LIR domain regulates its interaction with LC3 in response to the loss of ψ_m or hypoxia with the phosphoglycerate mutase family member 5 (PGAM5) acting as a phosphatase allowing the interaction of FUNDC1 with LC3 [97]. Interestingly, MARCH-5 ubiquitinates FUNDC1 targeting it for degradation [98], suggesting a coordinated interplay between the USP30-PINK1-parkin axis and (PINK1/parkin-independent) FUNDC1.

Mitochondrial derived vesicles (MDVs) are formed in response to mitochondrial damage, specifically sequestering damaged mitochondrial cargo into vesicles which are delivered to the lysosome [99]. This process is dependent on PINK1 and parkin but it is independent of canonical macroautophagy machinery [99]. Parkin, via Tollip, has recently been hypothesised to act as a switch controlling the trafficking of MDVs towards the lysosome or towards extracellular vesicles facilitated by sorting nexin 9, OPA-1 and VPS35 [68, 100, 101]. The role of MDV formation in mitochondrial homeostasis is under investigation, with the incorporation of immunogenic mitochondrial components such as mitochondrial DNA (mtDNA) into MDVs demonstrated to be less immunogenic than their release which activates the inflammasome/NFκB pathway [100]. Furthermore, there may be a non-cell-autonomous component of neuronal mitophagy, particularly in distal axons, mediated by MDVs or spheroid-like structures incorporating damaged mitochondria [102].

Understanding mitophagy pathways outside of the canonical PINK1-parkin pathway will allow greater insight into the compensation that occurs in PD patients with PINK1/parkin mutations

809 and the specific stimuli which trigger the PINK1-parkin independent pathway in dopaminergic
810 neurons.

Box 3 Mitochondria at the nexus of dopaminergic neuronal susceptibility?

The physiology, biochemistry, and anatomy of the A9 dopaminergic neurons of the substantia nigra pars compacta (SNpc) may underpin the preferential vulnerability of these neurons in Parkinson's disease (PD) compared to other neuronal types, such as neighbouring A10 dopaminergic neurons of the **Ventral tegmental area** (**VTA**). Susceptibility factors of SNpc neurons include their extensive axonal arbour and consequent high bioenergetic demand [103], the presence of dopamine, persistent Ca^{2+} flux driven by pacemaking activity, and the post-mitotic nature of neurons which render them susceptible to the accumulation of somatic mutations.

Dopamine can undergo auto-oxidation resulting in the generation of hydrogen peroxide, which can cause oxidative stress through the Fenton reaction. Therefore, any impairment of dopamine sequestration into vesicles results in increased dopamine toxicity [104]. It has recently been observed that dopamine can serve as a source of electrons in dopaminergic neurons feeding into complex IV, driving mitochondrial membrane potential and ATP production during bursts of dopaminergic neuron activity in a feed-forward mechanism [105]. Whilst this observation provides an attractive hypothesis for increased bioenergetic demand during synaptic release/recycling, this mechanism may also contribute to ROS generation and mitochondrial damage through dopamine auto-oxidation or electron leak. Indeed, evidence suggests that dopaminergic SNpc neurons undergo an increased rate of mitophagy and mitochondrial turnover relative to dopaminergic neurons in the VTA [106].

High fluxes of cytosolic Ca^{2+} through L-type calcium channels during neuronal activity are a feature of dopaminergic neurons of the SNpc in addition to efflux via the $Na^{+}/Ca^{2+}/Li^{+}$ exchanger NCLX (as reviewed in [107]). Mitochondria and mitochondrial-ER contact sites play a key role in buffering cytosolic Ca^{2+} fluxes with the CaV1 channel inhibitor isradipine having been shown to decrease mitochondrial oxidative stress in nigral dopaminergic neurons without altering pacemaking activity [106].

Accumulation of somatic mutations and genetic mosaicism in the midbrain of PD patients may result from the damage of mitochondrial and nuclear DNA by ROS. It has been shown that mtDNA copy number is increased during healthy ageing of the SNpc whereas nigral neurons in PD patients have a depletion of mtDNA copy number in the absence of increased rates of mtDNA damage, suggesting mtDNA homeostasis is impaired in PD [108]. In addition, PD patients may have an increased *SNCA* copy number in the nucleus [109]. Somatic loss of function mutations or increased CNVs in genes such as *SNCA* may provide an alternative route to mitochondrial toxicity or protein aggregation.

Together, these data suggest mechanisms by which the unique neuronal physiology of SNpc dopaminergic neurons play a key role in propagating and being affected by mitochondrial dysfunction in PD.

Figures

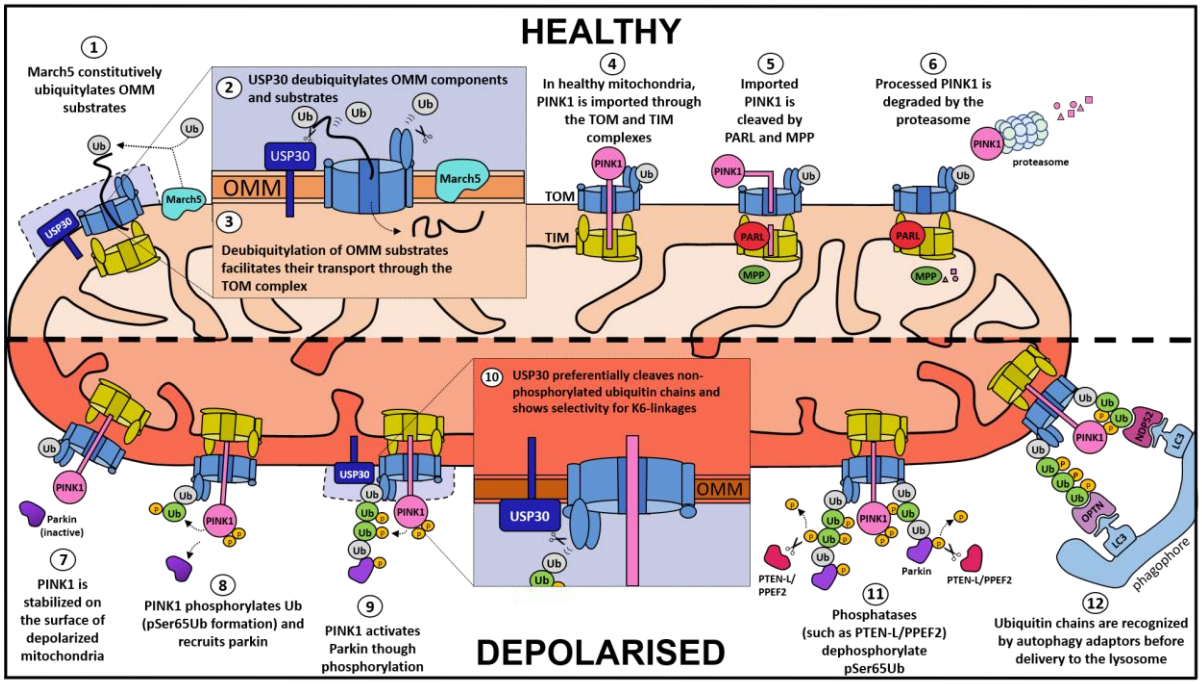

Figure 1. USP30 functions as a gatekeeper to activating the PINK1/parkin mitophagy pathway

1. E3 Ubiquitin (Ub) ligases, such as March5, ubiquitylate outer mitochondrial membrane (OMM) substrates which act as 'seeds' for PINK1/parkin-dependent mitophagy. **2.** In spatially restricted regions, USP30 reduces ubiquitylation on OMM substrates under basal conditions. **3.** USP30 deubiquitylates mitochondrial proteins, allowing their transport through the TOM complex (blue). **4.** In healthy mitochondria and under basal conditions, PINK1 is imported through the TOM and TIM complexes (yellow). **5.** While spanning the inner mitochondrial membrane (IMM), PINK1 is cleaved by PARL and MPP. **6.** Processed PINK1 is then targeted to the proteasome for degradation. **7.** When mitochondrial import is compromised, PINK1 becomes stabilised on the OMM. **8.** PINK1 phosphorylates Ub (pSer65Ub) which marks OMM substrates. PINK1 activity also recruits parkin. **9.** PINK1 also phosphorylates parkin, causing a conformational change resulting in increased parkin activity. **10.** USP30 preferentially cleaves non-phosphorylated Ub chains and shows selectivity for K6-linkages. **11.** PTEN-L and PPEF2 antagonize PINK1 activity by dephosphorylating parkin and Ub chains on the OMM. **12.** Ub chains that remain on OMM substrates mediate mitophagy via their interactions with Ub-binding adapters allowing engulfment of the mitochondrion by the autophagosome and its subsequent degradation by the lysosome.

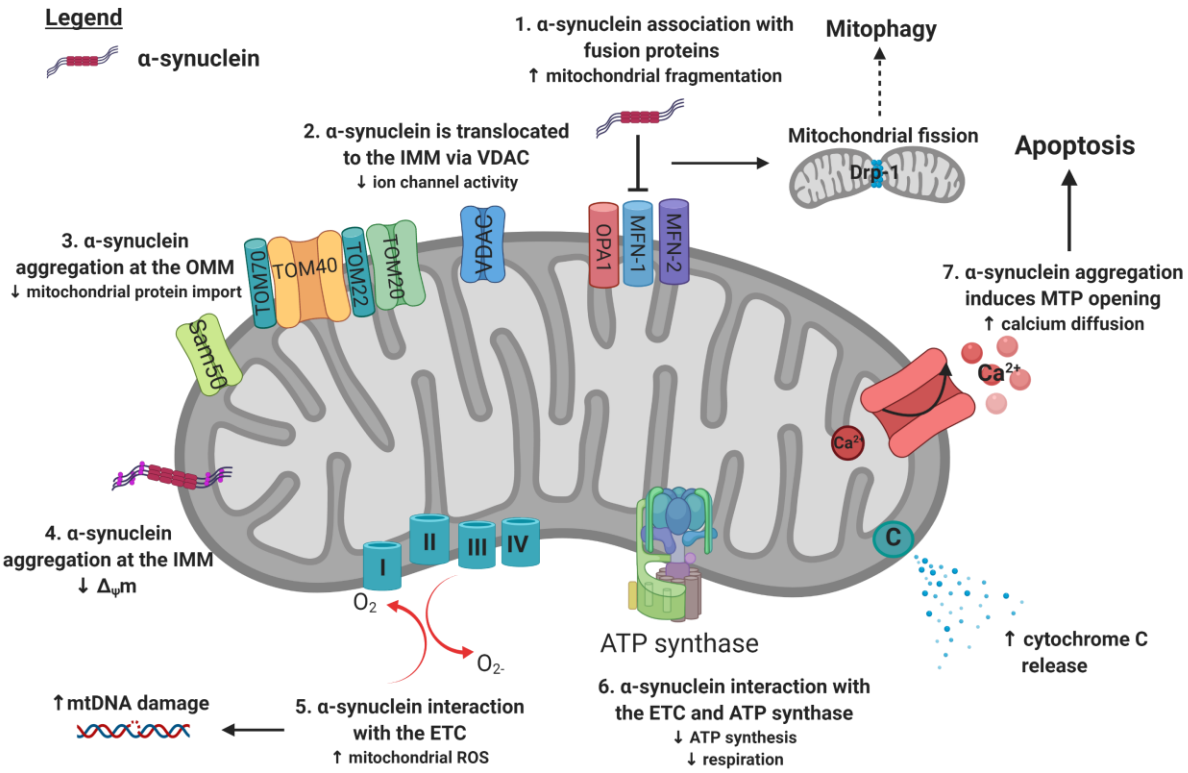

Figure 2. Aggregated and pathogenic forms of α-synuclein preferentially bind to mitochondria to affect mitochondrial function.

1) α-synuclein interaction with fusion proteins such as OPA1, MFN-1, and MFN-2 has been associated with increased Drp1-mediated fission and increased mitochondrial fragmentation. **2**) α-synuclein is translocated into the innter mitochondrial membrane (IMM) via VDAC and blocks its ion channel activity. **3**) α-synuclein aggregation at different components of the OMM, such as the TOM complex and the SAM (sorting and assembly machinery) complex, impairs mitochondrial protein import [110]. **4**) α-synuclein aggregation at the intermembrnae space (IMS) leads to mitochondrial membrane depolarization. **5**) α-synuclein interaction with the electron transport chain (ETC), specifically at complexes X and Y, increases the production of mitochondrial reactive oxygen species (ROS), promoting mitochondrial DNA (mtDNA) damage. **6**) α-synuclein interaction with components of the ETC results in decreased complex I activity, decreased ATP production, and decreased respiratory capacity. **7**) α-synuclein aggregation at the mitochondria induces MPTP opening, calcium diffusion, cytochrome C release, and mitochondrial swelling, ultimately leading to apoptosis.

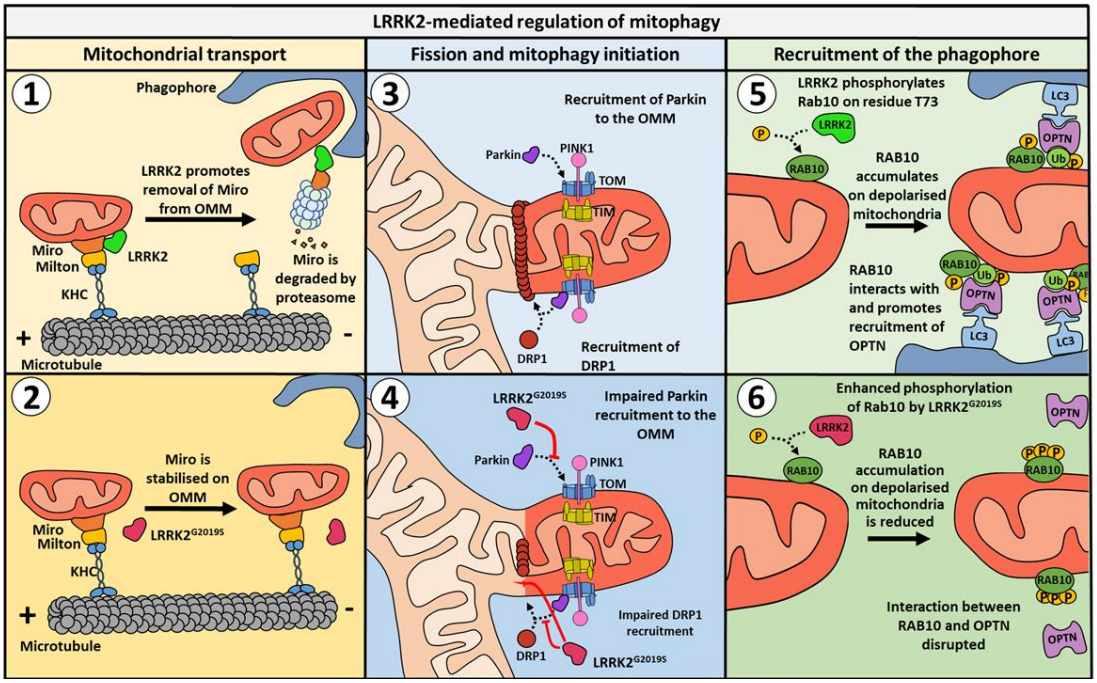

Figure 3. Normal and disease-associated functions of LRRK2 in regulating mitophagy.
1) LRRK2 can promote mitophagy by removing MIRO from the motor complex (including Milton and kinesin (KHC)), thereby arresting mitochondrial transport and thus enabling engulfment by autophagosomes. **2**) Pathogenic G2019S substitution in LRRK2 disrupts this function, thereby delaying the arrest of damaged mitochondria and slowing the initiation of mitophagy. **3**) Recruitment of parkin to the outer mitochondrial membrane (OMM) of damaged mitochondria is one of the earliest stages of mitophagy. Co-ordinated recruitment of Drp1 by PINK1 and parkin is essential for mitochondrial fission, which isolates damaged mitochondria for subsequent degradation. **4**) Increased LRRK2 kinase activity (overexpression of $LRRK2^{WT}$ or $LRRK2^{G2019S}$) disrupts interactions between a) Parkin and TOM subunits, b) Parkin and Drp1, and c) Drp1 and MiD51. This results in impaired PINK1/parkin-dependent mitophagy. **5**) LRRK2 phosphorylates RAB10 on residue T73 leading to its accumulation on depolarized mitochondria. Here, it promotes recruitment and interacts with the ubiquitin-binding adapter OPTN, thereby promoting mitophagy. **6**) LRRK2 (G2019S and R1441C) impairs later stages of PINK1/parkin-dependent mitophagy through increased phosphorylation of RAB10, which inhibits its interaction with OPTN. This reduces the accumulation of RAB10 and OPTN on depolarised mitochondria and attenuates mitophagy.