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Review

Mitochondrial Dysfunction and Mitophagy in Parkinson's Disease: From Mechanism to Therapy

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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 PTEN-induced putative kinase 1 (*PINK1*) and parkin (*PRKN*) 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 noncanonical 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 PD (sPD), opening opportunities for therapeutically targeting mitochondria in PD.

Highlights

Mitochondrial dysfunction is implicated in PD through both environmental exposure and genetic factors. Increased understanding of mitochondrial dysfunction and mitophagy in PD has identified new mechanisms and therapeutic opportunities.

Identification of the up- and downstream regulators of PINK1/parkin-dependent mitophagy has highlighted tight regulation of ubiquitin phosphorylation as well as roles of parkin independent of PINK1.

There has been increased understanding of the mitochondrial roles of familial PD genes such as the disruption of mitophagy by *LRRK2* mutations and observations that α-synuclein oligomers and aggregates interact with outer mitochondrial membrane substrates, inducing mitochondrial dysfunction.

Recent studies of large numbers of patients with sPD demonstrated peripheral mitochondrial and lysosomal dysfunction, and, importantly, overlaps with phenotypes observed in familial disease.

Enhanced understanding of the mechanisms regulating mitophagy and the causes of mitochondrial dysfunction in PD have led to a range of novel therapeutic opportunities.

Mitochondrial Dysfunction Lies at the Heart of 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 tissues from patients with sPD, and the familial autosomal recessive PD genes ***PINK1*** and *PRKN* have been discovered to have a key role in mitochondrial quality control.

In this review, we explore recent progress in understanding the regulation of the PINK1/**parkin** pathway in PD pathogenesis, the roles of established PD genes, such as *SNCA* and **Leucine-rich repeat kinase 2** (*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

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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 patients with PD 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 depolarisation, PINK1 is stabilised at the outer mitochondrial membrane (OMM), where it phosphorylates pre-existing **ubiquitin** (Ub) at serine 65 residues (**Phospho-serine65 Ub**; pSer65Ub) [5]. Auto-inhibited cytosolic parkin is then recruited to sites of PINK1 activity, where RING1 of the parkin domain binds pSer65Ub, initiating a series of conformational changes [6]. Notably, parkin remains in a partially autoinhibited 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 (~4400-fold activation) Ub chain assembly (as reviewed in [8]). Evidence of increased pSer65Ub formation has been observed in postmortem PD brain and diminished in patients with *PINK1/PRKN* mutations, indicating the relevance of this pathway in disease [9,10].

Box 1. Elucidating the Role of the PINK1/Parkin Pathway in Basal Mitophagy and its Relevance to PD

Most 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. Although informative, the large-scale loss of mitochondrial function in the cell after such chemical inhibition represents suprapathophysiological conditions. In addition, much research has relied on expression/overexpression of parkin to observe mitophagy-related phenomena, such as parkin translocation, TOM20 loss, and lysosomal cargo delivery. 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 [83], mtKeima [84], and recently, mito-SRAI [85]. Data from *Drosophila* expressing mitoQC or mtKeima and from mouse dopaminergic neurons expressing mitoQC demonstrated that basal mitophagy is not affected by the loss of PINK1 or parkin [86,87]. These data appear to contradict experiments using mtKeima demonstrating a role for PINK1 in basal mitophagy [88], leaving the relative contribution of the PINK1/parkin pathway to basal mitophagy unclear. However, given the localisation of mitoQC to the 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 [85], suggesting multiple tools and approaches will 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 [89], suggest that PINK1/parkin have a role. In addition, assays such as TOM20/LC3 co-localisation provide alternative methods to assess mitochondrial turnover [90].

Together, these observations highlight not only a wealth of tools and models for assessing mitophagy, but also the need to consider the exact experimental paradigm and model used to make conclusions in cell models and *in vivo*.

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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 wild-type Ub conformation [11]. The Ub-CR conformation has an extended Ser65 loop that promotes its binding to the kinase-activation segment of PINK1, whereas nonphosphorylated 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 stabilisation 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 has been demonstrated as an additional mechanism 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

Several **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 that 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 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) suppressed 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].

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 PD.
CHCHD2: a mitochondrial protein and familial PD risk gene that regulates mitochondrial function.
Cyanide m-chlorophenyl hydrazine (CCCP): a protonophore and a potent mitochondrial uncoupler that depolarises the mitochondrial membrane and induces mitophagy; CCCP is used extensively in the study of PINK1/Parkin-dependent mitophagy
Dopaminergic neurons: neurons that 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 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.
Leucine-rich repeat kinase 2 (LRRK2): a widely expressed multidomain kinase; mutations in *LRRK2* are the most common form of autosomal dominant PD.
Lewy bodies: intracellular protein aggregates comprising misfolded proteins, of which α-synuclein is a prominent component; Lewy bodies are the defining pathological feature of postmortem PD brain
Mitophagy: 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; mutations in *PRKN* cause autosomal recessive PD.
Parkinson's disease (PD): a progressive, neurodegenerative disease characterised by both motor and non-motor symptoms; pathologically,

Furthermore, two classes of ubiquitylation event were identified to be more abundant in USP30-depleted iPSC-derived induced neurons [27]: (i) OMM protein ubiquitylation was elevated under basal conditions but not during depolarisation (e.g., TOM40 and VDAC2); and (ii) OMM protein ubiquitylation was elevated under basal conditions and further increased during depolarisation (e.g., TOM20, TOM5, and VDAC1). Interestingly, these classes of substrate 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 **translocase of the outer mitochondrial membrane (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 multifaceted 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]. Therefore, dephosphorylation of pSer65Ub is 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 localise to the OMM and maintain mitochondrial quality control in both neurons and astrocytes *in vitro* and *in vivo* [31,32]. In addition, recent reports demonstrated that PINK1 phosphorylates Ub in astrocytes to a greater extent than in neurons [33]. Although further studies are needed, this raises the possibility that phosphorylation and dephosphorylation of mitochondrial proteins have 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 antagonise 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 [31]. Similar to PTEN-L, PPEF2 overexpression significantly reduced the rate of pSer65Ub formation in cortical neuronal cultures. However, in parkin-overexpressing models, the effect of PPEF2 was significantly diminished [31] because 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 that 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 patients with PD, as

the disease is characterised by the aggregation of α-synuclein into Lewy bodies and the preferential degeneration of dopaminergic neurons of the SNpc.
Phospho-serine65 Ub (pSer65Ub): PINK1 phosphorylates the serine 65 residue on Ub chains on the OMM, forming pSer65Ub; pSer65Ub has a crucial role in the amplification of the PINK1/parkin pathway.
PTEN-induced putative kinase 1 (PINK1): a serine/threonine-protein kinase with a central role in mitophagy following mitochondrial membrane depolarisation phosphorylating both parkin and Ub; mutations in PINK1 cause autosomal recessive PD.
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.
Translocase of the outer mitochondrial membrane (TOM) complex: a complex of proteins (e.g., TOM20 and TOM40) that regulate protein transport across the OMM.
***Trans*-mitophagy:** degradation of mitochondria in a noncell-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.

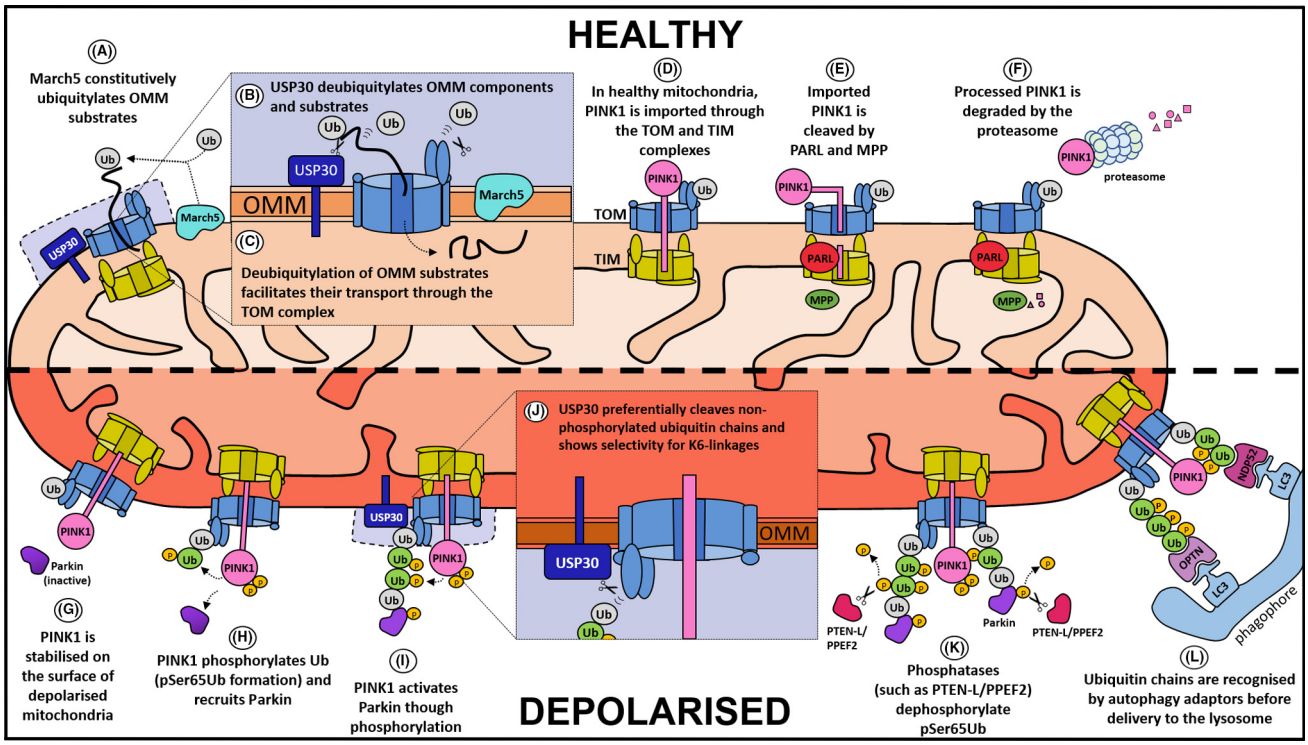

Figure 1. USP30 Functions as a Gatekeeper to Activating the PINK1/Parkin Mitophagy Pathway. (A) E3 Ubiquitin (Ub) ligases, such as March5, ubiquitylate outer mitochondrial membrane (OMM) substrates, which act as 'seeds' for PINK1/parkin-dependent mitophagy. (B) In spatially restricted regions, USP30 reduces ubiquitylation on OMM substrates under basal conditions. (C) USP30 deubiquitylates mitochondrial proteins, allowing their transport through the translocase of the outer mitochondrial membrane (TOM) complex (blue). (D) In healthy mitochondria and under basal conditions, PINK1 is imported through the TOM and translocase of the inner membrane (TIM) complexes (yellow). (E) While spanning the inner mitochondrial membrane (IMM), PINK1 is cleaved by PARL and MPP. (F) Processed PINK1 is then targeted to the proteasome for degradation. (G) When mitochondrial import is compromised, PINK1 becomes stabilised on the OMM. (H) PINK1 phosphorylates Ub (pSer65Ub), which marks OMM substrates. PINK1 activity also recruits parkin. (I) PINK1 also phosphorylates parkin, causing a conformational change resulting in increased parkin activity. (J) USP30 preferentially cleaves nonphosphorylated Ub chains and shows selectivity for K6-linkages. (K) PTEN-L and PPEF2 antagonise PINK1 activity by dephosphorylating parkin and Ub chains on the OMM. (L) 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.

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. Therefore, it is 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 [34]. However, locomotor deficits and dopaminergic neuron loss were recently observed in 2-year-old *Prkn* KO mice [35]. 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 [36]. In addition, loss of *PRKN* decreases the activity

Box 2. PINK1/parkin-Independent Mitophagy Pathways

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

Several PINK1-independent pathways mediated by OMM proteins that can interact with Atg8 homologues at the autophagosome through an LIR-interacting domain (LC3-interacting domain) have been identified, including Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3), BNIP3-like (BNIP3L; NIX), FUN14 Domain Containing 1 (FUNDC1), and AMBRA1. Additionally, several E3 Ub ligases other than parkin have been demonstrated to ubiquitinate OMM proteins, including MUL1 [91] and SIAH1 [92], 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 [93]. Mutations in *FBXO7* impair FBXO7–parkin interactions, resulting in mitochondrial protein misfolding and impaired mitophagy [93,94]

Post-translational regulation of OMM-LIR domain proteins occurs in response to a range of stimuli beyond the loss of membrane potential (ψ_m) (reviewed in [95]). 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 [96]. Interestingly, MARCH-5 ubiquitinates FUNDC1, targeting it for degradation [97], 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 that are delivered to the lysosome [98]. This process is dependent on PINK1 and parkin, but is independent of canonical macroautophagy machinery [98]. Parkin, via Tollip, was recently hypothesised to act as a switch controlling the trafficking of MDVs toward the lysosome or towards extracellular vesicles facilitated by sorting nexin 9, OPA-1, and VPS35 [67,99,100]. 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/NF-κB pathway [99]. 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 [101].

Understanding mitophagy pathways outside of the canonical PINK1-parkin pathway will allow greater insight into the compensation that occurs in patients with PD with *PINK1/Parkin* mutations and the specific stimuli that trigger the PINK1–parkin-independent pathway in dopaminergic neurons.

of lysosomal enzymes, including GBA (GCase). Given that parkin also targets several vacuolar ATPases for degradation [37], these data together suggest that a loss of PRKN has an impact downstream of mitochondria [38], affecting cellular processes beyond mitochondrial homeostasis. iPSC-derived dopaminergic neurons from patients with PD with compound heterozygous *PRKN* mutations demonstrate a loss of parkin and an inability to release ER-mitochondrial contact sites in response to mitochondrial depolarisation [39]. Additionally, a comparison of several monogenic PD rat models using *in vivo* microdialysis demonstrated alterations in dopamine and/or dopamine metabolites as well as alterations in other neurotransmitters in *PINK1* and *parkin*-KO rats [40].

Furthermore, PINK1/parkin modulates inflammation in response to activation of the STING pathway induced by mitochondrial DNA damage [41]. Loss of *Pink1* also increased mitochondrial antigen presentation (MiTAP), with bacterial challenge of *Pink1*-KO mice demonstrating cytotoxic (CD8+) T cell responses in both the gut and brain [42]. Given that MITAP induction correlated with loss of dopaminergic neuronal axonal varicosities in the striatum [42], the role of MITAP and peripheral inflammation in PD and particularly in patients with *PINK1/parkin* mutation is of great interest.

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

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 PD compared with 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 [102], the presence of dopamine, persistent Ca^{2+} flux driven by pace-making activity, and the postmitotic 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 [103]. It was recently 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 [104]. Although this observation provides an attractive hypothesis for increased bioenergetic demand during synaptic release/recycling, this mechanism may also contribute to reactive oxygen species (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 [105].

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 (reviewed in [106]). Mitochondria and mitochondrial-ER contact sites have 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 pace-making activity [105].

Accumulation of somatic mutations and genetic mosaicism in the midbrain of patients with PD may result from the damage of mitochondrial and nuclear DNA by ROS. mtDNA copy number is increased during healthy ageing of the SNpc, whereas nigral neurons in patients with PD have a depletion of mtDNA copy number in the absence of increased rates of mtDNA damage, suggesting that mtDNA homeostasis is impaired in PD [107]. In addition, patients with PD may have an increased *SNCA* copy number in the nucleus [108]. Somatic loss-of-function mutations or increased copy number variants 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 have a key role in propagating and being affected by mitochondrial dysfunction in PD.

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 mechanisms in both familial and sPD.

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 showed that α-synuclein interacts with several OMM components, including TOM20 [43,44], VDAC [45], and F_1F_o-ATP synthase, causing mitochondrial permeability transition pore (MPTP) opening [46]. Accumulated pathological α-synuclein, or dopamine-modified or phospho-mimetic species mimicking pathological forms of the protein, preferentially bind to mitochondria [43,47], inhibiting mitochondrial protein import and leading to mitochondrial membrane depolarisation and impaired cellular respiration [47].

Models of seeded α-synuclein aggregation demonstrated decreased levels of MFN-2 and OPA1 levels [48], consistent with previous reports of α-synuclein-induced mitochondrial fragmentation and bioenergetic alterations in iPSC-derived dopaminergic neurons [44]. Proteomic analyses

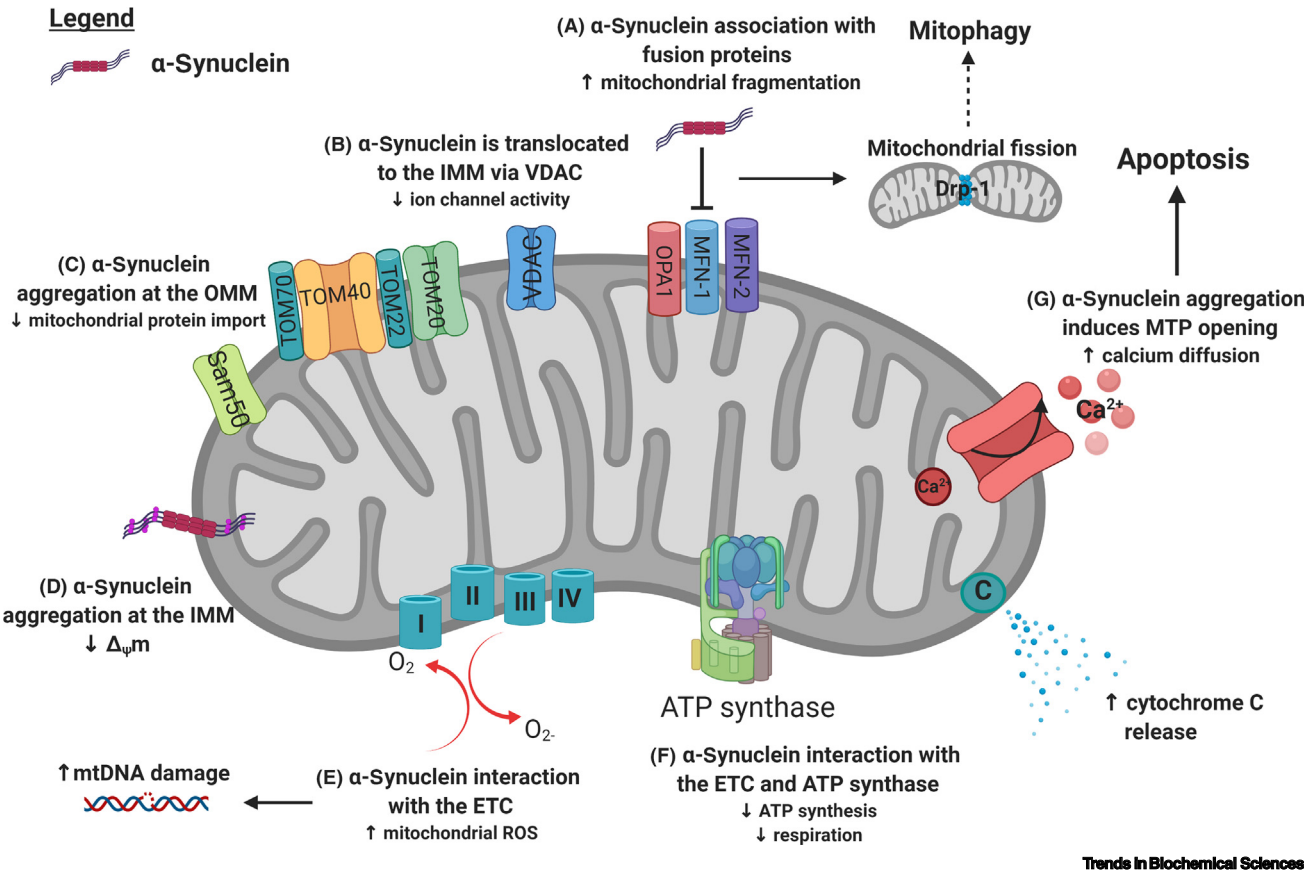

Figure 2. Aggregated and Pathogenic Forms of α-Synuclein Preferentially Bind to Mitochondria to Affect Mitochondrial Function. (A) α-Synuclein interaction with fusion proteins, such as OPA1, MFN-1, and MFN-2, has been associated with increased Drp1-mediated fission and increased mitochondrial fragmentation. (B) α-Synuclein is translocated into the inner mitochondrial membrane (IMM) via voltage-dependent anion channels (VDACs) and blocks their ion channel activity. (C) α-Synuclein aggregation at different components of the OMM, such as the translocase of the outer mitochondrial membrane (TOM) complex and the sorting and assembly machinery (SAM) complex, impairs mitochondrial protein import [109]. (D) α-Synuclein aggregation at the intermembrane space (IMS) leads to mitochondrial membrane depolarisation. (E) α-Synuclein interaction with the electron transport chain (ETC) increases the production of mitochondrial reactive oxygen species (ROS), promoting mitochondrial DNA (mtDNA) damage. (F) α-Synuclein interaction with components of the ETC results in decreased complex I activity, decreased ATP production, and decreased respiratory capacity. (G) α-Synuclein aggregation at the mitochondria induces MPTP opening, calcium diffusion, cytochrome C release, and mitochondrial swelling, ultimately leading to apoptosis.

revealed significant recruitment of proteins involved in oxidative phosphorylation into aggregates consistent with impairments in respiration. Furthermore, recent studies demonstrated that the process of Lewy body formation, rather than aggregation itself, is the key driver for dysfunction induced by α-synuclein [48] Mitochondrial dysfunction and complex I deficits are temporally separated from pS129 formation induced by α-synuclein fibrils in hippocampal neurons, suggesting that additional mechanisms beyond the interaction between α-synuclein and the mitochondria are needed for dysfunction [48]. Indeed, Lewy bodies from patients with PD were found to contain fragmented mitochondria crowded with lipids and lysosomes as well as α-synuclein [49], 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 was shown in iPSC-derived dopamine neurons that LRRK2 can regulate

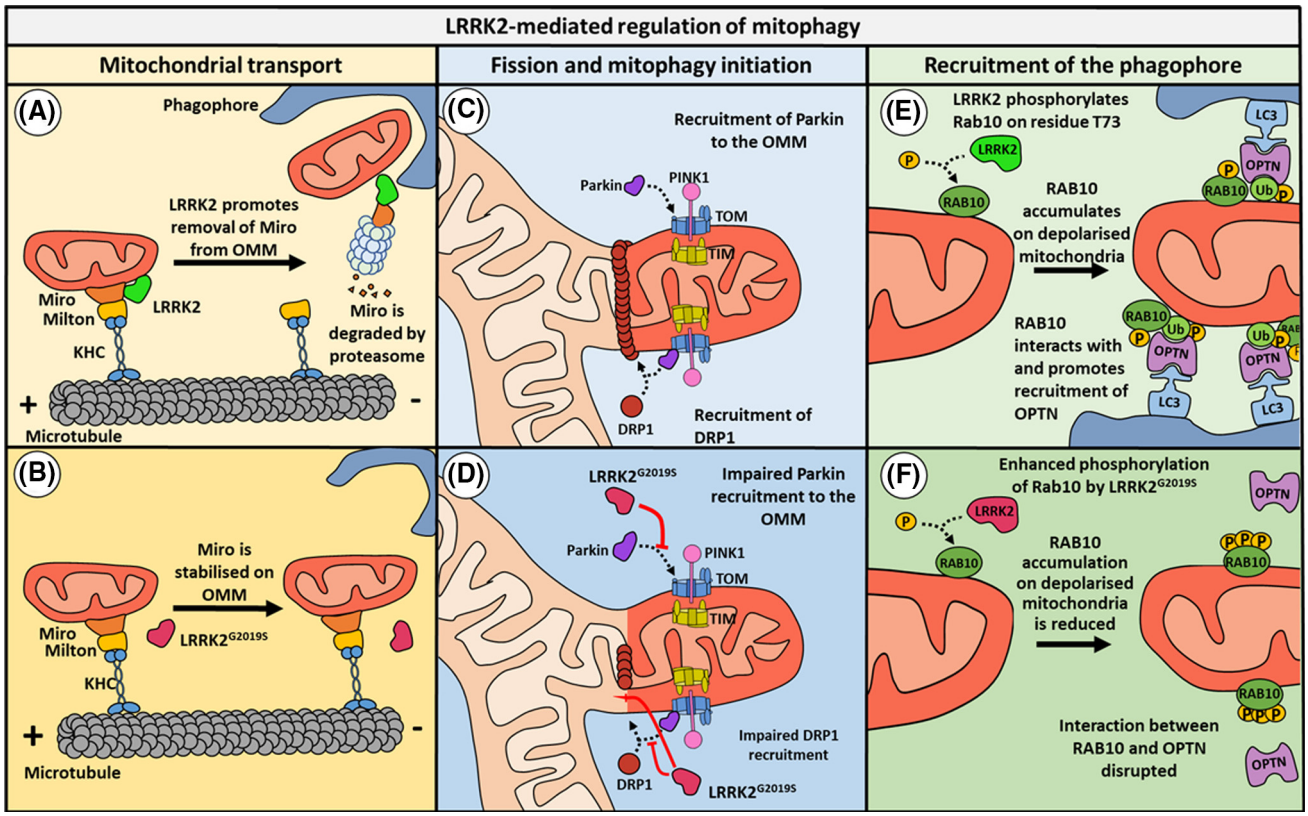

Figure 3. Normal and Disease-Associated Functions of Leucine-Rich Repeat Kinase 2 (LRRK2) in Regulating Mitophagy. (A) LRRK2 can promote mitophagy by removing MIRO from the motor complex [including Milton and kinesin (KHC)], thereby arresting mitochondrial transport and, thus, enabling engulfment by autophagosomes. (B) Pathogenic G2019S substitution in LRRK2 disrupts this function, thereby delaying the arrest of damaged mitochondria and slowing the initiation of mitophagy.(C) 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. (D) Increased LRRK2 kinase activity (overexpression of $LRRK2^{WT}$ or $LRRK2^{G2019S}$) disrupts interactions between (i) Parkin and translocase of the outer mitochondrial membrane (TOM) subunits, (ii) Parkin and Drp1, and (iii)) Drp1 and MiD51. This results in impaired PINK1/parkin-dependent mitophagy. (E) LRRK2 phosphorylates RAB10 on residue T73, leading to its accumulation on depolarised mitochondria. Here, it promotes recruitment and interacts with the ubiquitin-binding adapter OPTN, thereby promoting mitophagy. (F) 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.

mitophagy by removing the OMM adaptor protein MIRO from the MIRO/MILTON/KINESIN motor complex, resulting in reduced mitochondrial transport along the cytoskeleton and reducing engulfment by autophagosomes and subsequent degradation [50]. The pathogenic G2019S mutation in the kinase domain of LRRK2 disrupts this function, slowing the initiation of mitophagy [50]. These observations are in agreement with previous reports of impaired mitochondrial trafficking in *LRRK2*-R1441C mutant rat neurons [51]. Importantly, these phenotypes have also been observed in neuronal cultures from patients with idiopathic PD, strengthening the argument for a more general role of compromised mitophagy in the pathology of PD [50].

Furthermore, two independent studies have reported that mutant LRRK2 can impair PINK1/parkin-dependent mitophagy via independent mechanisms [52,53]. 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 [52]. 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 [53]. However, given the differential expression of LRRK2 between brain regions and cell types [54], 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. Although 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 is 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 patients with PD carrying heterozygous *GBA* mutations revealed increased mitochondrial content, increased mitochondrial oxidative stress, and impaired autophagy [55]. These findings mirror previous observations in iPSC-derived dopaminergic neuronal cultures from patients with *GBA*-L444P mutations and *GBA*-KO neurons [56]. 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 [55], again pointing toward a more general defect in mitophagy in the pathology of PD.

CHCHD2

Mutations in *CHCHD2* were identified as a novel familial PD gene in 2015 [57]. *CHCHD2* encodes a protein that modulates mitochondrial function in conjunction with the ALS/FTD-associated gene *CHCHD10* [58]. Further analysis of this pathway identified that CHCHD2 accumulates in damaged mitochondria and regulates CHCHD10 oligomerisation [59].

CHCHD2 has a significant role in the maintenance of mitochondrial cristae [58] as well as stabilising OPA1 to promote mitochondrial fusion [60]. Mutations in CHCHD2 induce precipitation of both mutant and wild-type (WT) protein in the intermembrane space (IMS) [60] as well as cytochrome C destabilisation, impaired respiration, and mitochondrial reactive oxygen species (ROS) generation [61]. Moreover, CHCHD2 mutations have been associated with α-synuclein aggregation and oligomerisation in human postmortem brain tissues and iPSC-derived dopaminergic neurons [62]. Although CHCHD2 has been demonstrated to have 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 *GCH1* are enriched in patients with PD and rare mutations have been identified in up to 0.75% of patients with PD [63]. α-Synuclein has also been demonstrated to alter GCH1 activity [64]. In addition to the cytoplasmic role of BH4, BH4 also has a role in mitochondrial function and redox regulation [65]. The absence of BH4 results in decreased mitochondrial function and increased levels of DRP1 activation, proteome remodelling and metabolic alterations [65].

VPS35

VPS35 has role in recycling DLP1 complexes, with mutations and oxidative stress increasing VPS35 interactions and mitochondrial fission [66]. Parkin poly-ubiquitinates VPS35, affecting endosomal sorting WASH complex-mediated retromer [37]. More generally, VPS35 mutations disrupt autophagosome formation through ATG9a, inhibiting autophagosome formation and vesicle transport between mitochondria and peroxisomes [67]. In addition, inhibition of retromer function results in accumulation of hyperactive (GTP-bound) RAB7 on lysosomes, inhibiting ATG9a and autophagosome formation [68]. Given the established link between LRRK2 and VPS35 [69], the similarity between mitochondrial phenotypes in VPS35 and LRRK2 models is noteworthy.

Relevance to Sporadic Disease

Given that only ~15% of PD can be attributed to a monogenic cause, research into the molecular mechanisms of sPD (sPD) is vital. Several recent studies investigated sPD, 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 postmortem PD brain and neuronal models of PD [9,10,70] but decreased in *PINK1/PRKN* mutation carriers, demonstrating the relevance of mitochondrial damage and mitophagy induction via the PINK1/parkin pathway in patients with PD. A comprehensive study of pSer65Ub in postmortem brain from patients with 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.

A large study of fibroblasts from patients with PD identified distinct subsets of patients with sPD with mitochondrial or lysosomal dysfunction [71]. 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 [71]. In addition, bioenergetics are altered in peripheral blood mononuclear cells from patients with sPD [72]. 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 [50,73], and these overlaps are likely key to understanding convergent pathways in PD subgroups. Highlighting this, SNPs in several mitochondrial genes have been associated with the age of onset of PD [74], 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 have led to the identification of several 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 [20,75,76].

Direct activation of PINK1 has been demonstrated by kinetin triphosphate KTP [77] and further development of bioavailable KTP precursors is underway [78]. In addition, two small-molecule activators of PINK1 that are structurally distinct from KTP were identified by high-throughput screening and have been validated in both dopaminergic neurons and *PINK1*-knockdown *Drosophila* [79]. 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 [80].

Direct targeting of proteins impacted by autosomal dominant PD mutations offers a strategy to expand therapy beyond patients with *PINK1*/*PRKN*. Encouragingly, two independent studies demonstrated that LRRK2 kinase inhibitors can correct impaired RAB10-optineurin and parkin-Drp1 interactions in *LRRK2* mutant cells [52,53]. The increased LRRK2 kinase activity in idiopathic PD has also been shown in animal models in which LRRK2 inhibition corrected mitochondrial and lysosomal dysfunction [81,82]. Therefore, LRRK2 inhibitors currently in clinical trials could 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 enzymes, 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 [56]. 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 [71].

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, compared with non-PINK1/parkin-dependent mechanisms, will aid 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, 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 (***trans*-mitophagy**) 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 remain to be elucidated. Regardless of the genetic background of patients, the role(s) of increased LRRK2 kinase activity and α-synuclein aggregation in damaging mitochondria and its impact of mitophagy is likely key in understanding convergent mechanisms in PD.

Outstanding Questions

What is the role of PINK1/parkin pathway in homeostasis of mitochondria relative to non-PINK1/parkin dependent mitophagy? Is this different in dopaminergic neurons?

Is potentiation of the PINK1/parkin pathway a viable therapeutic strategy for PD?

What is the role of MITAP and STING-mediated inflammation in monogenic and sPD and are these key modulators of the involvement of microglia and astrocytes in PD?

Is PINK1/Parkin-dependent mitophagy more active in astrocytes/microglia than in neurons? Does the pathway function the same way in these cell types? How does impaired mitochondrial quality control in microglial cells contribute to the pathology of PD? Is this linked to inflammatory responses occurring during neurodegeneration?

What is the role of novel PD genes with roles in mitochondrial regulation, such as *CHCHD2*, *GCH1*, and *VPS35* and are these genes/proteins affected in monogenic or sporadic disease?

Is there a dominant or convergent mechanism by which α-synuclein oligomerisation and aggregation affect mitochondria? What is the role of this process in disease aetiology?

Is there sufficient evidence for LRRK2-mediated mitochondrial dysfunction to be considered an endpoint in LRRK2 inhibitor trials?

Investigation of novel PD-causing genes with mitochondrial roles, combined with the investigation of mitochondrial deficits in sPD, 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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