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PINK1 and Parkin: emerging themes in mitochondrial homeostasis

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The Parkinson's disease (PD)-associated protein kinase, PTEN-induced putative kinase1 (PINK1), and ubiquitin E3 ligase Parkin, function in a common signalling pathway known to regulate mitochondrial network homeostasis and quality control, including mitophagy. The multistep activation of this pathway, as well as an unexpected convergence between the post-translational modifications of ubiquitylation and phosphorylation, has added breadth to our understanding of cellular damage responses during human disease. In concert with these new insights in signal transduction, unique modalities and signatures of vertebrate mitophagy have been unravelled *in vivo* for the very first time. The cell biology of mammalian mitophagy, and the roles of PINK1-Parkin signalling *in vivo* have emerged to be more complex than previously thought.

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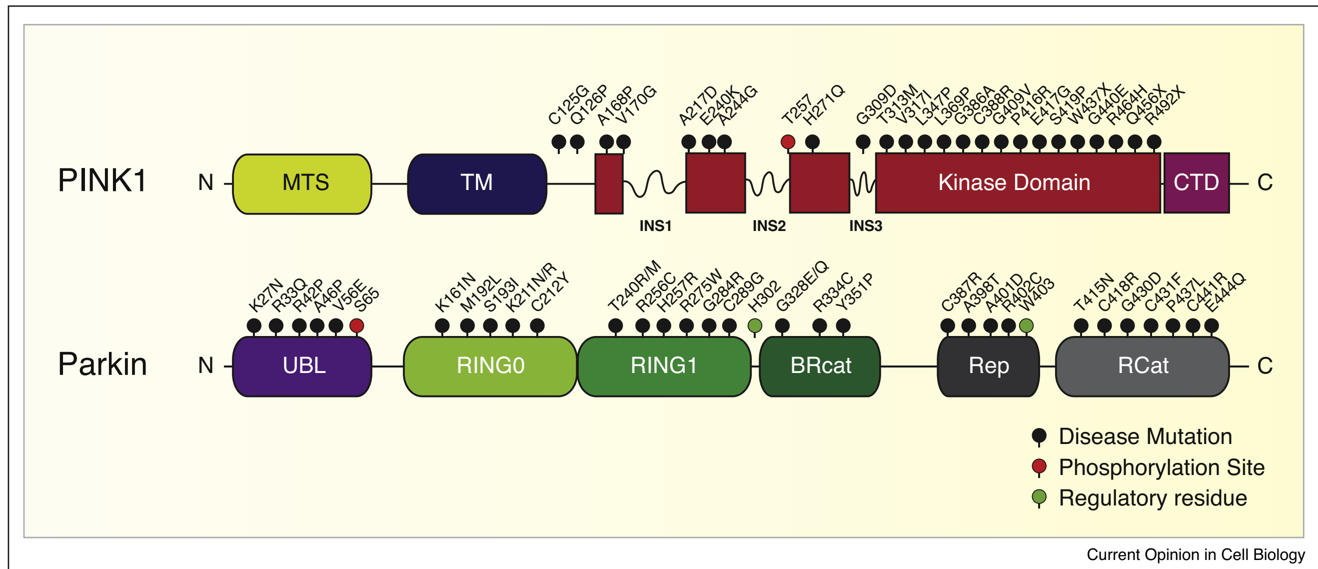
Introduction: an evolving view of mitochondrial homeostasis

Whilst mitochondria may have granted eukaryotes the ultimate survival advantage during evolution, the dysfunction of these dynamic and functionally pleiotropic organelles confers a potentially devastating vulnerability to mammalian cells. This is particularly relevant in the context of terminally differentiated or long-lived cells such as neurons, cardiomyocytes and renal tubular cells. Complex pathways have evolved to sense, label and sequester damaged or dysfunctional mitochondria to neutralise their cytotoxic potential. One such mechanism is the delivery of compromised mitochondria or associated mitochondrial components to the lysosome for elimination, *via* the autophagy pathway [1]. Mitochondrial autophagy or 'mitophagy' is receiving increased attention as a major pathway that sustains mitochondrial network homeostasis, by regulating mitochondrial number and protecting cells from the deleterious effects of 'mitotoxicity'. Mutations in human PTEN-induced kinase 1 (PINK1) and the RING-IBR-RING (RBR) E3-ubiquitin ligase Parkin (encoded by *PARK6* and *PARK2* genes, respectively) result in early-onset Parkinson's disease (PD) (Figure 1) [2]. Initial discoveries in *Drosophila melanogaster* revealed both of these proteins function within a common, evolutionarily conserved mitochondrial homeostasis pathway. Landmark cell-based studies demonstrated that PINK1-Parkin signalling regulates stimulus-induced mitochondrial clearance *in vitro*, and research on the molecular regulation of this and related responses has proliferated dramatically over the past decade. Whilst the 'PINK1-Parkin axis' has received the great majority of attention, it is important to note that PINK1-Parkin-independent mitophagy is emerging as a burgeoning area of research. Reciprocally, mitophagy-independent functions of PINK1/Parkin are also widely observed. Recent breakthroughs have redefined our view of vertebrate mitophagy *in vivo*, and provoke a reassessment in our understanding of how mitochondrial quality control is regulated in a variety of contexts.

PINK1, phospho-ubiquitin (p-Ub), Parkin and the regulation of stimulus-induced mitophagy

Groundbreaking work by the Youle laboratory initially linked Parkin to mitophagy, and subsequent contributions from many other laboratories have defined a central role for PINK1 in regulating Parkin following mitochondrial damage. Under steady state conditions, PINK1 is constitutively imported into mitochondria, cleaved and degraded *via* the N-end rule pathway. The proteolysis of PINK1 is mediated by the inner mitochondrial membrane associated PARL protease, and regulated by the recently described SPY complex [3]. Upon the loss of mitochondrial membrane potential that can be induced artificially by mitochondrial uncouplers (*e.g.* carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)), PINK1 becomes stabilised and activated on the outer mitochondrial membrane (OMM) by mechanisms that are not yet fully elaborated [4,5]. *Drosophila* studies suggest PINK1 levels on 'healthy mitochondria' are regulated by the LON protease, however evidence for this in mammalian cells remains unclear [6]. Cytosolic Parkin is recruited to damaged mitochondria where it becomes activated [5,7•]. Both Parkin translocation and its E3-ligase activity are

Figure 1



Domain architecture of PINK1 and Parkin.

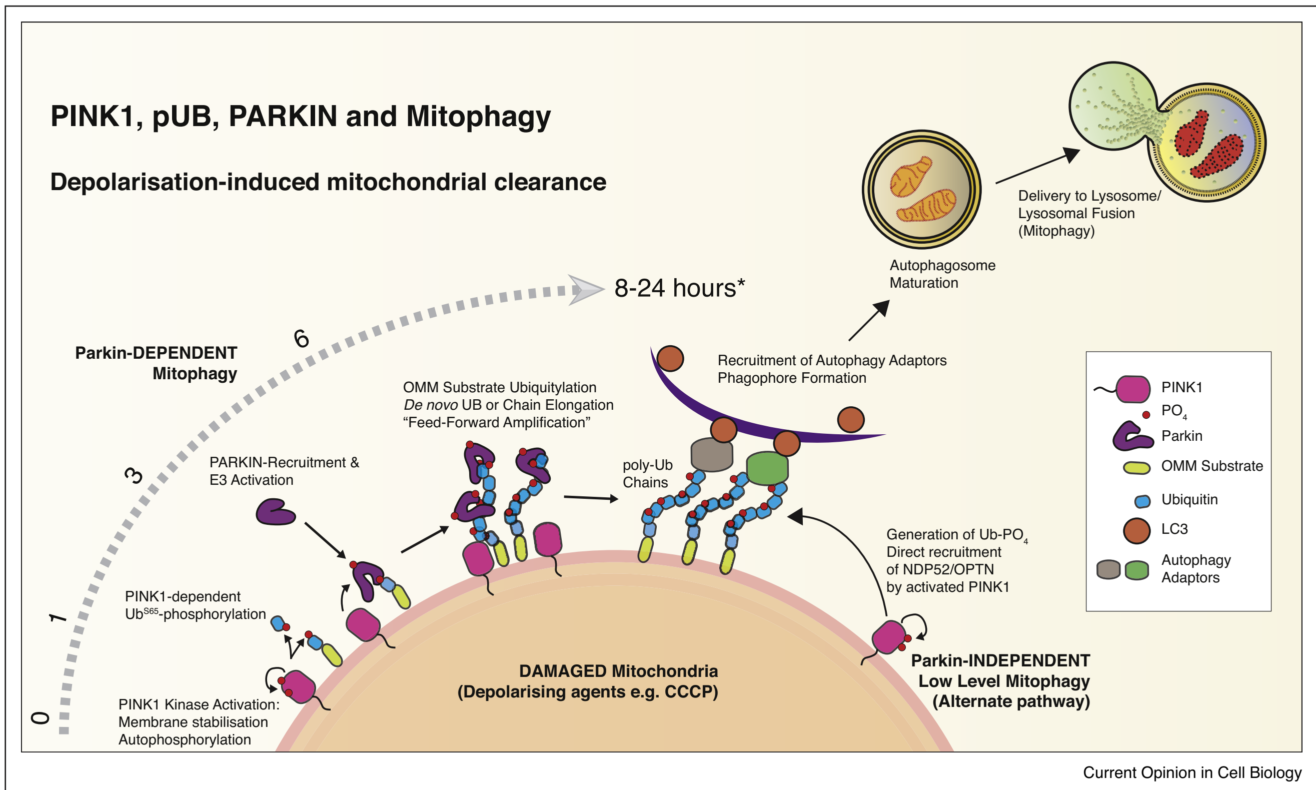
Schematic depicting the major regions of the mitochondrial-associated kinase PINK1, and the RBR-E3 Ubiquitin Ligase Parkin. Selected human disease mutations are shown in black, key phospho-sites in red and regulatory residues in green. MTS: mitochondrial targeting sequence, TM: transmembrane domain, INS: insertion, CTD: C-terminal domain, UBL: ubiquitin-like domain, RING: really interesting new gene domain, BRcat: benign-catalytic domain, Rep: repressor element, Rcat: required for catalysis domain.

dependent upon the enzymatic activity of PINK1. Over the past two years, the multistep mechanistic regulation of Parkin activation has been elaborated in great detail (Figure 2) [5].

Parkin exists in a native autoinhibited conformation [8]. Upon mitochondrial depolarisation, activated PINK1 phosphorylates both Ubiquitin and Parkin at their respective Ser65 residues [7•,9••,10••,11••,12,]. Detailed structural and biophysical characterisation by independent laboratories demonstrated that phospho-ubiquitin (p-Ub) binds with high affinity to phosphorylated Parkin to allosterically induce conformational changes that promote recruitment of its cognate charged E2, and stimulation of Parkin activity [13–17]. Active Parkin is reported to ubiquitylate myriad putative substrates that reside in the OMM, by elongating pre-existing ubiquitin chains attached to OMM proteins or ubiquitylating substrates *de novo*. Whilst the role of p-Ub as an allosteric activator is evident, whether p-Ub may play other physiological roles is unclear. It has been reported that poly-p-Ub chains are more resistant to proteolysis by several deubiquitinases, suggesting that p-Ub may act as an important signal to maintain Parkin-directed ubiquitylation [12]. Given the pleiotropy of substrates, it is also possible that p-Ub may function as a semaphore to direct or guide Parkin to selected substrates. In this regard, it will prove exciting to determine if other proteins contain similar p-Ub binding pockets akin to that found on Parkin [5,18]. A leading hypothesis suggests particular combinations of poly-p-Ub chain topologies may represent a distinct molecular code required for the selective recruitment of autophagy machinery. Quantitative proteomics revealed damaged mitochondria become decorated with a diverse array of polyubiquitin linkages (Lys 6, 11, 48, 63) [7•]. These OMM-polyubiquitin chains serve as substrates for PINK1 phosphorylation and adaptors for Parkin, resulting in a 'feed-forward' amplification loop that drives the clearance of the damaged organelle and ultimately, the completion of mitophagy (Figure 2) [7•,19,20].

The recruitment of ubiquitin-binding autophagy receptor proteins is necessary for local phagophore formation at damaged mitochondria and, to date six such receptors have been identified: NBR1, NDP52, OPTN, p62/SQSTM1, TAX1BP1 and TOLLIP [5]. Converging studies using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) genome editing suggest NDP52/OPTN are essential for PINK1-Parkin-dependent mitophagy, and TAX1BP1 to a lesser extent [21•,22•]. Furthermore, TBK1 phosphorylation of these receptors enhances interactions between LC3 and ubiquitin [22•,23]. Interestingly, PINK1 may also generate p-Ub and recruit NDP52/

Figure 2



PINK1, p-Ub, Parkin and mitophagy.
The molecular regulation of depolarisation-induced mitochondrial clearance is well established. PINK1-Parkin-dependent signalling triggers cycles of ubiquitylation on the OMM, which result in the recruitment of the autophagy machinery, autophagosome formation and eventual clearance of the damaged organelle. Times are approximate and refer to observations from *in vitro* studies. PINK1 can also drive low-level mitophagy, independently of Parkin.

OPTN independently of Parkin to drive low-level mitophagy, implying the involvement of additional ubiquitin E3 ligases [21•]. Until recently, it was widely assumed that the ATG-conjugation machinery is crucial for autophagosome biogenesis and cargo incorporation. A new study has cast doubt on this, suggesting that while LC3 is essential for autophagosome-endolysosome fusion, it is dispensable for the autophagic encapsulation of damaged mitochondria [24]. Consistent with a role outside of autophagosome biogenesis, other recent observations indicate LC3 conjugation is instead required to orchestrate the degradation of the inner autolysosomal membrane following autophagosome-lysosome fusion [25].

It has recently been reported that the mitochondrial ubiquitin ligase 1 (MUL1; also known as MULAN and MAPL) functions with Parkin to regulate degradation of paternal mitochondria from sperm, but the mechanism by which MUL1 induces mitophagy is unknown [26]. Rab GTPase proteins have also been implicated in mitophagy, and two RAB-GAPs (TBC1D15 and TBC1D17) modulate the activity of Rab7 to regulate encapsulation of damaged mitochondria by the autophagosome [27]. Recently published phosphoproteomic analyses demonstrated that Rab8A, 8B and 13 are indirect substrates of PINK1 [28•]. The temporal dynamics of Rab phosphorylation following mitochondrial insult is consistent with their potential contribution to the later stages of mitophagy [28•]. The links between PINK1, TBK1 and Optineurin signalling are intriguing, given previous reports that Optineurin is an effector protein of Rab8A, suggesting a potential nexus. Given the function of NDP52/OPTN/TBK1/Rab7 in xenophagy and the ancient origins of mitochondria from α-proteobacteria, interesting parallels have also been drawn between mitophagy and xenophagy [5,29].

The idea that dysregulated PINK1-Parkin signalling leads to impaired mitophagy is an attractive hypothesis, however there has been no clear validation of this mechanism in the aetiology of PD. Although PINK1-Parkin mediated mitophagy is highly reproducible *in vitro*, it is difficult to reconcile the actual contribution of PINK1-

Parkin signalling to mitochondrial homeostasis *in vivo* due to some potentially confounding factors. (1) The majority of studies utilise proliferating cell lines and although such systems are unrivalled for the tractable molecular dissection of signalling pathways *in vitro*, they relate poorly to the post-mitotic and complex A9 mesencephalic dopaminergic (mesDA) nigrostriatal projection neurons that selectively degenerate in PD. (2) High levels of PINK1/Parkin expression are usually required to detect a robust induction of mitophagy. (3) It is difficult to relate the cytotoxic induction stimuli (*e.g.* CCCP) to an analogous physiological correlate that evokes a similar level of mitochondrial clearance *in vivo*. Additionally, genetic ablation of germ-line *Pink1* or *Parkin* in mice does not recapitulate the overt neuropathology or motor dysfunction that manifests in PD.

However, a recent proof-of-concept study has highlighted the importance of endogenous *Parkin* in mediating neural mitochondrial homeostasis *in vivo* [30•]. To simulate conditions of mitochondrial stress in the absence of endogenous Parkin and investigate the effect of this on nigrostriatal pathway integrity, the authors crossed *Parkin*-deficient mice with mitochondrial *Polg*^{D257A} 'mutator' mice. The well-characterised mutator model accumulates mitochondrial DNA mutations because of defective mitochondrial proofreading by polymerase γ. Although individual mutants (*Parkin*-deficient or *Polg*^{D257A} mice) alone did not exhibit gross dopamine/dopaminergic (DA) neuropathology or behavioural abnormalities, double-mutant animals with loss of *Parkin* under conditions of constitutive mitochondrial stress *in vivo* exhibited age-dependent DA neuron loss and levodopa-responsive motor dysfunction [30•]. Whilst this important study demonstrated the requirement of Parkin for DA neuronal homeostasis under conditions of mitochondrial stress *in vivo*, it remains unclear if this pathology is a consequence of dysregulated mitophagy in double-mutant mice. Nevertheless, this important *in vivo* finding demonstrates the contribution of endogenous Parkin to mesDA neural integrity under conditions of stress *in vivo*. Interestingly and unlike *Pink1* knockout (KO) mice, a recently generated *Pink1* KO rat model exhibits locomotor dysfunction and age-related DA cell loss, in addition to altered neural metabolomics [31,32]. In contrast, *Parkin* KO rats do not exhibit significant PD-related pathology due to probable redundancy of E3 ubiquitin ligases, for example MUL1 [26].

Fiesel and Springer provided intriguing evidence for the physiological relevance of canonical PINK1-Parkin signalling in humans by demonstrating the age-dependent accumulation of p-Ub in post-mortem brains, and its reciprocal absence in SNpc sections from a patient with a compound heterozygous *PARK6* mutation [33]. Although studies in cultured cells and post-mortem human brain suggest PINK1 to be the ubiquitin kinase for Ser65-Ub, this does not preclude a role for other protein kinases that may generate p-Ub *in vivo*, as ubiquitin can be phosphorylated at several other sites. Recent advances in cell reprogramming enabled Chung *et al.* to recapitulate the molecular pathophysiology of PD *in vitro* using induced pluripotent stem cell (iPSC)-derived DA neurons from PINK1/Parkin-patients [34]. Reprogrammed patient neurons exhibited aberrant mitochondrial morphology as well as a propensity to accumulate α-synuclein [34]. Importantly, this study also demonstrated the influence of culture conditions on modelling molecular pathophysiology *in vitro*, however the contribution of impaired mitochondrial turnover to the observed pathology remains unclear.

Alternate signals: PINK1 and PARKIN-independent mitophagy

Because of their striking roles in mediating stimulus-induced mitochondrial clearance *in vitro*, PINK1 and Parkin tend to reap the limelight in most mitophagy-related discussions. However, it should be noted that PINK1-p-Ub-Parkin-independent mechanisms of mitophagy exist and these constitute an equally exciting and emerging area of research. These could also be viewed as ubiquitin-dependent and ubiquitin-independent mechanisms of selective autophagy, reviewed in Ref. [35]. The first such mechanism described was NIX-dependent mitophagy (originally reported by the Ney laboratory and shown to be an ATG8 receptor by Dikic and colleagues) [1]. NIX and the closely related BNIP3 are induced by hypoxia, trigger autophagy and have apoptotic-related functions. They both localise to the OMM and contain LC3-interation motifs (LIR), thus acting as autophagy receptors. Upon activation, BNIP3 and NIX prompt opening of the mitochondrial permeability transition pore (mPTP), depolarisation and recruitment of LC3/GABARAPs for autophagosome formation. NIX has been shown to be important for mitochondrial elimination during developmental erythrocyte maturation. Cross talk between NIX/BNIP3 and PINK1-Parkin mitophagy pathways is unclear, although NIX has been implicated in the mitochondrial translocation of Parkin.

The OMM-protein FUNDC1 (Fun14 Domain Containing 1) has also been implicated as a receptor in hypoxia-induced mitophagy. The affinity of FUNDC1 with LC3 is controlled by Src-mediated phosphorylation of Tyr^{18} within its N-terminal LIR. Under hypoxia, de-phosphorylation of Tyr^{18} enhances the FUNDC1-LC3 interaction and evokes mitophagy. The Ser-Thr protein kinase ULK1 has been reported to phosphorylate FUNDC1 to regulate mitophagy [1].

Iron chelation *via* deferiprone has also been shown to induce mitophagy in a PINK1-Parkin-independent manner. This is particularly intriguing, as dysregulated iron metabolism has been linked to neurodegenerative

disease and clinical trials with deferiprone and PD are currently underway. It should be noted that the signalling pathway regulating deferiprone-induced mitophagy remains to be elucidated and it will be interesting to test the contribution of other ubiquitin E3 ligases, such as the recently described MUL1 E3 ligase [26]. Similarly ubiquitin can be phosphorylated at several other sites and their relevance to mitophagy and the identity of the upstream kinases regulating these residues remains unknown [36].

Orchestrating mitochondrial quality control *in vivo*

A major challenge in the field has been to accurately and faithfully measure mitophagy *in vivo*. The inability to measure this process in mammalian tissues has provoked great suspense surrounding the *bona fide* contribution of PINK1-pUB-Parkin signalling to mitochondrial turnover *in vivo*.

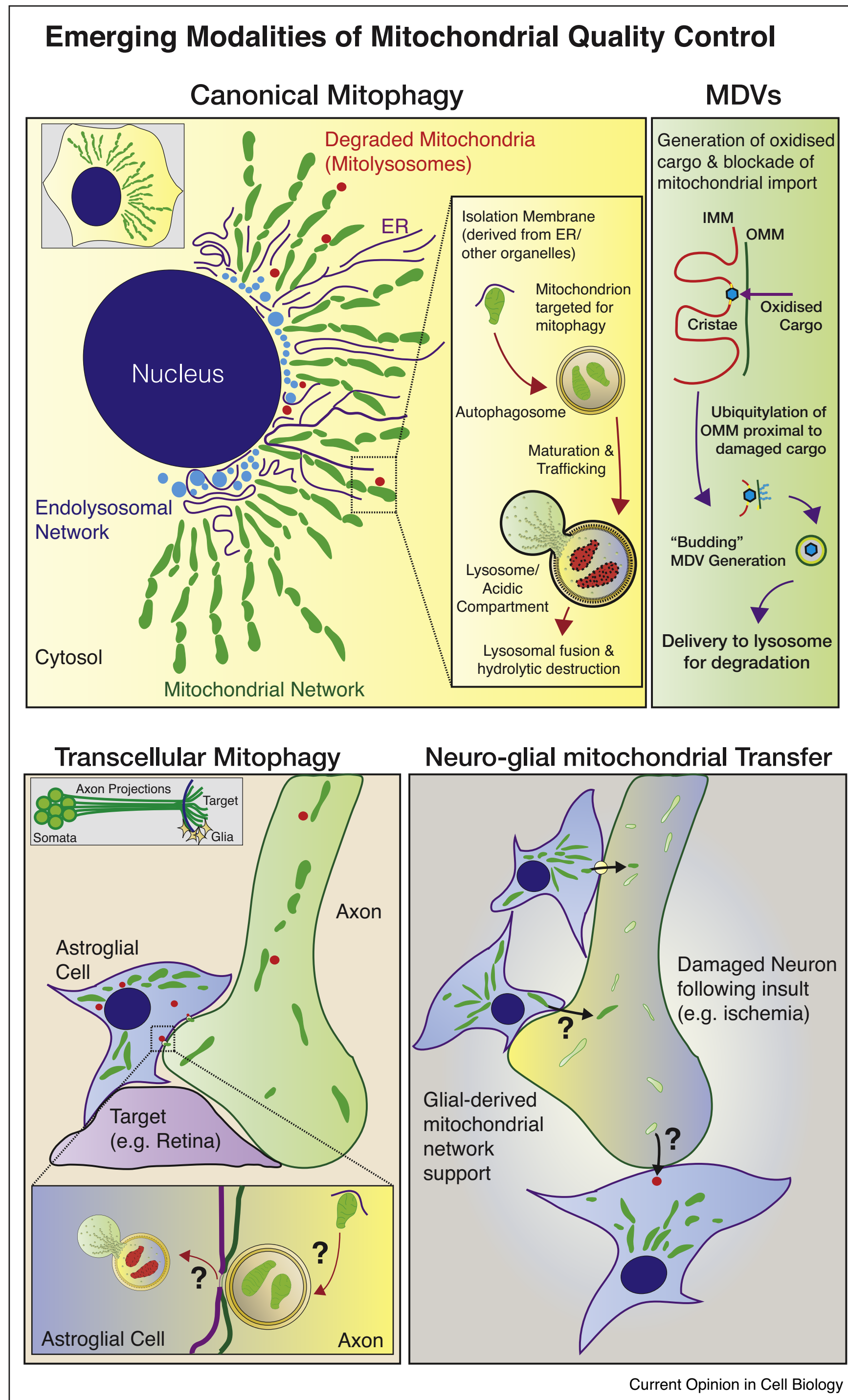
Powerful insights into eukaryotic mitophagy have been obtained from budding yeast, from the initial *in vivo* studies by the Lemasters laboratory to more recent work on p-Ub signalling. Intriguingly, although there is no known PINK1 orthologue in yeast, Swaney *et al.* observed the presence of Ser65-p-Ub in *S. cerevisiae* upon exposure to oxidative stress [37]. This raises the exciting possibility that an alternate, PINK1-like ubiquitin kinase may exist. Invertebrate *in vivo* mitophagy has also been investigated in novel reporter assays using *C. elegans*, where *Pink1* and *Parkin* were reported to orchestrate ageing-associated mitophagy [38]. However, these studies relied upon induction stimuli, as the basal rate of turnover in nematodes appears low. Fly genetics has also provided important insights into the regulation of mitochondrial homeostasis by PINK1 and Parkin [39].

In terms of mammalian mitophagy *in vivo*, tool development has been integral to the field and a combination of mouse genetics with fluorescent-reporter proteins has culminated in the recent rise of mitophagy reporter mouse models [40]. The recently reported *mito*-QC and mt-Keima mouse models generated by the Ganley and Finkel laboratories have unearthed the dramatic, striking and heterogeneous nature of basal mitophagy *in vivo* [41••,42••]. Although these models operate on a similar pH-based principle, the Keima protein is incompatible with tissue fixation and users are limited to making regional inferences about mitophagy *in vivo*. Conversely, the end-point *mito*-QC model facilitates the facile resolution of both mitophagy and mitochondrial network architecture within labelled subsets of cells *in vivo* at subcellular resolution. Collectively, these observations have profound implications for our view of mitochondrial turnover in mammals. The vast majority of our molecular knowledge on mitophagy is derived from studies of stimulus-induced mitophagy *in vitro*. The heterogeneity of basal turnover between tissues and even between cell types within the same organ is striking, for example in the kidney—where mitophagy between distal and proximal tubules is dramatically different. Furthermore, it will be essential to determine the contribution of developmental mitophagy to tissue function. From these findings and others, it is likely that multiple pathways regulate mitophagy in a context-dependent and cell-specific manner.

Ultimately, it will be essential to define the contribution of PINK1, p-Ub and Parkin to the regulation of basal mitophagy in vertebrates by exploiting these recently described approaches. Accurately identifying basal mitophagy within precise cellular subsets will be essential to understand if dysfunctional mitophagy underpins the selective vulnerability of particular neuronal subsets in complex degenerative disorders such as PD. Given the postulated contribution of dysregulated mitophagy to the aetiology of PD, how can we reconcile such selective pathophysiology in humans with the widespread, basal nature of mitophagy *in vivo*? If PINK1/Parkin are the master regulators of basal mitophagy, might we not expect patients to exhibit a greater degree of extra-nigral mitochondrial-associated pathology? These findings raise the interesting idea that PINK1/Parkin may be required to protect against a distinct type of stress-evoked mitophagy *in vivo*, as observed in Ref. [30•]. Collectively, these observations should stimulate an interesting debate on the cell subtype-specific functions of PINK1/Parkin *in vivo*.

Destructive and protective modalities of mitochondrial transfer have recently been reported in neurons (Figure 3). Interestingly, depolarisation-induced axonal mitophagy was described *in vitro* in neurons with overexpressed Parkin [43]. The exciting phenomenon of *trans*-cellular mitochondrial degradation (or axonal *trans*-mitophagy) has also recently been described *in vivo* within the optic nerve head [44•]. Remarkably, axonal mitochondria destined for destruction are extruded from terminal processes, engulfed by neighbouring astrocytes and eliminated within their lysosomes. Given the inordinate complexity of the mature nervous system with $\sim 10^{15}$ connections, this topography may account for how a spatial economy of destruction is achieved in mammalian neurons when faced with the challenge of distance [45]. An equally intriguing discovery with implications for mitochondrial quality control is neuro-glial mitochondrial transfer, recently described following simulated ischemic insult [46]. Might the acquisition of healthy mitochondria serve to equilibrate network homeostasis, and how is this orchestrated with basal or induced mitophagy during ageing? Furthermore, do these new mitophagy modalities rely on PINK1-Parkin signalling, and could particular permutations of poly-p-Ub chains specify these alternate delivery routes to the lysosome? Although further work is required to substantiate the frequency of such events in a

Figure 3



Emerging modalities of mitochondrial quality control.

Our knowledge of mitochondrial homeostasis has expanded, and new routes to the lysosome have been uncovered. Canonical mitophagy refers to PINK1-Parkin-dependent or independent mitophagy that has been classically studied in cultured cells. The mitochondrial-derived vesicle (MDV) pathway is emerging as a key regulator of mitochondrial quality control. Damaged mitochondria can also be extruded from cells and degraded by lysosomes in neighbouring cells (Transcellular mitophagy), and damaged neurons may acquire healthy mitochondria from glial cells (Neuro-glial mitochondrial transfer). It is unclear if these latter modes of delivery are dependent on PINK1-Parkin signalling.

variety of contexts, both discoveries have profound implications for our understanding of nervous system architecture at a subcellular level.

Emerging themes from cell biology to pathology

Alternate pathways have emerged where damaged mitochondrial components can be excised and delivered to acidic endolysosomal compartments for degradation. In response to oxidative stress, the McBride and Fon laboratories have shown that PINK1 and Parkin mediate the cargo-dependent export of excised mitochondrial regions as membrane derived vesicles (MDVs) for delivery to lysosomes [47•,48]. The Qa-SNARE Syntaxin-17 was recently implicated in this process [49]. Furthermore, a physiological role for PINK1-Parkin signalling-dependent generation of MDVs has been found in mitochondrial antigen presentation (MitAP) [50]. In response to cellular stress, a novel PINK1-Parkin-mediated MDV pathway exports mitochondrial-derived self-antigens for presentation at the cell surface, triggering an immune response [50]. This unexpected discovery of MitAP provides an interesting link between autoimmunity and PD [51]. The complexity of signalling pathways regulating the delivery of damaged mitochondria to the lysosome also needs to be evaluated in light of emerging findings in organelle crosstalk [52]. A variety of mitochondria-organelle contact sites are emerging as key regulators of lipid transport, mitochondrial transport and distribution [52]. Such crosstalk is also exemplified by the recent and exciting revelation of the dual mitochondrial-ER origins of peroxisomes [53]. The development of next-generation chemical probes for monitoring Parkin-activation should also prove valuable in resolving the contribution of PINK1-Parkin signalling to these new and exciting aspects of mitochondrial cell biology [54]. Intriguing links between mitophagy and metabolic regulation are also emerging, especially with respect to cardiovascular development. The Dorn laboratory demonstrated a role for Parkin during cardiac perinatal development, by showing that cardiomyocyte-specific ablation of *Parkin* at this stage provokes a lethal cardiomyopathy caused by the retention of foetal mitochondria. A wave of mitophagy was observed in late embryonic *mito*-QC mice, suggesting that a defined window of developmental cardiomitophagy *in utero* may orchestrate an essential phase of metabolic adaption during early life [33].

Concluding remarks

Our view of mitophagy, signal transduction and its contribution to the pathophysiology of PD should be evaluated in the light of mesDA neurobiology. It is estimated that a single SN DA neuron can innervate 2.7% of the total striatal volume, synapsing with up to 75,000 medium spiny neurons [55,56]. Furthermore, our understanding of DA neuron function itself is continually evolving with the advent of more sensitive tools [57,58]. The selective nature of mitophagy, as distinct from non-selective general macro-autophagy cannot be understated. Although general macroautophagy is a catabolic process that evolved to sustain cells during instances of nutrient deprivation, basal mitophagy proceeds in cells that have ample access to nutrients, and represents a unique quality control mechanism required to sustain the integrity of the mitochondrial network. Moreover, major differences have been unearthed with respect to the regulation of this process *in vivo*. Despite major research efforts in this field, several important questions remain unanswered (Box 1). Ultimately, it will be imperative to combine state of the art signalling studies with cutting edge *in vivo* cell biology to clarify the precise contribution of mitophagy pathways to mammalian neural integrity.

Box 1 Outstanding questions in the field.

- What is the precise contribution of PINK1-Parkin-mediated mitophagy to basal mitophagy *in vivo*?
- Is a chronic 'mitotoxic' stress required to induce PINK1-Parkin mediated mitophagy *in vivo*?
- Given the prevalence of basal mitophagy in tissues, if PINK1-Parkin mediates mitophagy *in vivo*—how can this be reconciled with the highly selective degeneration observed in PD?
- What are the upstream activation stimuli of PINK1-Parkin signalling *in vivo*?
- Do PINK1, p-Ub or PARKIN play a role in trans-cellular mitochondrial trafficking, that is during axonal *trans*-mitophagy or neuroglial transfer?
- Do specific ubiquitin chain topologies or associated patterns of these act as mitophagy-associated molecular patterns for phagophore formation?
- What is the function of PINK1-Rab signalling in mitophagy?
- How are mitochondrial-organelle membrane contacts orchestrated under conditions of acute or chronic mitochondrial stress?
- Do other ubiquitin kinases/ligases regulate mitophagy in mammals?

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Due to space constraints, we were unable to provide a comprehensive citation of all the relevant primary literature. We apologise to those whom we have omitted. We thank Ian Ganley for critical reading of the manuscript.

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- •• of outstanding interest

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