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Current Opinion in Genetics & Development

PINK1/Parkin mitophagy and neurodegeneration—what do we really know *in vivo*?

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Mitochondria are essential organelles that provide cellular energy and buffer cytoplasmic calcium. At the same time they produce damaging reactive oxygen species and sequester pro-apoptotic factors. Hence, eukaryotes have evolved exquisite homeostatic processes that maintain mitochondrial integrity, or ultimately remove damaged organelles. This subject has garnered intense interest recently following the discovery that two Parkinson's disease genes, *PINK1* and *parkin*, regulate mitochondrial degradation (mitophagy). The molecular details of PINK1/Parkin-induced mitophagy are emerging but much of our insight derives from work using cultured cells and potent mitochondrial toxins, raising questions about the physiological significance of these findings. Here we review the evidence supporting PINK1/Parkin mitophagy *in vivo* and its causative role in neurodegeneration, and outline outstanding questions for future investigations.

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Current Opinion in Genetics & Development 2017, **44**:47–53

This review comes from a themed issue on **Molecular and genetic bases of disease**

Edited by **Nancy M Bonini**, **Edward B Lee**, **Wilma Wasco** and **Allen Roses**

For a complete overview see the Issue and the Editorial

Available online 16th February 2017

http://dx.doi.org/10.1016/j.gde.2017.01.016

The PINK1–Parkin pathway

Mitochondria are a nexus of life and death of eukaryotic cells, performing a number of critical functions, including generating cellular energy, buffering cytosolic calcium flux, promoting lipid metabolism, and sequestering the cell death machinery. It has long been thought that malfunction of the mechanisms that regulate mitochondrial quality control (mitoQC) are a major driving force of normal ageing [1]. Moreover, failure of mitoQC mechanisms, leading to elevated oxidative stress, is strongly linked to age-related conditions such as neurodegeneration [2,3].

Most of the cellular chemical energy, in the form of adenosine triphosphate (ATP), is produced in the mitochondrial matrix via the process of oxidative phosphorylation (OXPHOS). This process relies on electron flow via the electron transport chain components in the inner mitochondrial membrane, culminating in the reduction of oxygen in the matrix and the generation of a membrane potential across the inner membrane ($\Delta\Psi m$). However, a deleterious side-product of this process is the generation of highly reactive free radicals, termed reactive oxygen species (ROS), that have the potential to damage macromolecules including proteins, lipids and nucleic acids. To combat this, there are multiple intervention points to protect the overall integrity of the mitochondrial network. First, mitochondria contain an abundance of anti-oxidants such as superoxide dismutase and glutathione to prevent ROS-induced damage. Second, there are a broad collection of cellular factors that repair or replace damaged mitochondrial components, including mitochondrial proteases, mitochondrial chaperones, DNA repair enzymes and the ubiquitin–proteasome system. Finally, when mitochondrial damage becomes too extensive the entire mitochondrion can be selectively degraded in the lysosome through a process referred to as mitophagy.

Mitophagy was little studied before 2008, when seminal work in the laboratory of Richard Youle showed that two proteins linked to Parkinson's disease (PD), Parkin, a cytosolic ubiquitin ligase, and PINK, a mitochondrially targeted kinase, play key roles in this process. The current prevailing model posits that under basal conditions, 'healthy' mitochondria, that is those with a high $\Delta\Psi m$, import and degrade PINK1 [4,5]. This process serves to constitutively repress a key degradation signal. Upon mitochondrial damage, typically modelled by the dissipation of $\Delta\Psi m$ with uncoupling agents or mitochondrial poisons, PINK1 import is blocked and instead accumulates on the outer mitochondrial membrane (OMM). PINK1 then phosphorylates ubiquitin (likely residual ubiquitin-modified OMM proteins) at Ser65 [6•,7•,8•]. This stimulates the recruitment of cytosolic Parkin to the mitochondrial surface [9] via the direct binding of Parkin to phospho-ubiquitin chains. Parkin is then also phosphorylated by PINK1 at an equivalent Ser65 residue within its N-terminal ubiquitin-like domain [10]. Both events stimulate the ubiquitin-ligase activity of Parkin allowing it to ubiquitinate a host of

OMM targets [11,12]. The additional supply of local ubiquitin provides further substrate for PINK1, promoting additional Parkin recruitment in a feed-forward mechanism [12]. The high density of ubiquitinated OMM proteins leads ultimately to the recruitment of ubiquitin adaptor proteins, which in turn promote engulfment of the depolarized mitochondria by autophagosomes [13,14].

The mechanism by which PINK1 and Parkin promote mitophagy, as described above, is attractive for several reasons; first, this model offers an explanation for the longstanding evidence that mitochondrial defects are central to the pathogenicity of PD, including the observations that mitochondrial toxins cause PD in humans and animal models, that PD patients have systemic mitochondrial complex I deficits, and that DA neurons in elderly individuals accumulate high levels of mtDNA mutations relative to other neuronal subtypes [15–19]. Second, these findings would explain the protective effects of PINK1 and Parkin overexpression from exposure to mitochondrial toxins [20–22]. Third, the unique physiological characteristics of substantia nigra neurons place an extreme demand on mitochondrial function to meet their high energy demand and calcium buffering requirements, which may explain the selective vulnerability of these neurons to loss of mitoQC processes [23].

While there is now wide acceptance for the role of PINK1 and Parkin in mitophagy, and the mechanisms by which these factors act in this process, it is also important to recognize the limitations of the work that support this model. In particular, much of the work that supports our current understanding of PINK1 and Parkin derives from the use of high concentrations of protonophores such as CCCP/FCCP to dissipate the mitochondrial membrane potential. Of note, these uncoupling agents have the potential to depolarise other cellular compartments such as the lysosome, and are frequently used at concentrations an order of magnitude greater than that needed to depolarise mitochondria. Such concerns were substantiated with evidence that this effect may in fact be caused by the non-specific acidification of the cytosol [24], underscoring the need for more precise targeting of mitochondria to study the cell biology. Another concern with much of the work that supports our understanding of the role of PINK1 and Parkin in mitophagy is that it derives from the use of cultured cells that overexpress these factors. While it is conceivable that these extreme measures simply amplify or accelerate normal physiological processes that would be otherwise difficult to detect, these approaches also make it unclear how such findings translate to physiological conditions. In the remainder of this article, we briefly summarize some of the findings that challenge the roles of PINK1 and Parkin in mitophagy, and the evidence supporting their role in this process using more physiologically relevant model systems.

PINK1/Parkin function in neurons

One of the early challenges to the model that PINK1 and Parkin promote mitophagy is that much of the initial data in support of this model derived from cancerous cell lines. Cancer cells typically rely on glycolysis for energy production (the Warburg effect) and could potentially tolerate substantial loss of mitochondria, whereas neurons rely heavily on OXPHOS for energy production. Indeed, cells forced to rely on OXPHOS greatly inhibits PINK1/Parkin mitophagy upon loss of $\Delta\Psi m$ [25]. Furthermore, early studies investigating PINK1/Parkin mitophagy in primary neurons failed to detect Parkin translocation and/or mitochondrial degradation upon mitochondrial depolarization, and mutations in *parkin* did not influence the pathogenicity of mitochondrial disruption in mice, which further cast doubt on the physiological relevance of mechanistic models derived from studies using cell lines [26,27]. However, subsequent studies of primary neurons have reported Parkin translocation to depolarized mitochondria, although this process appeared to be restricted to somatodendritic regions and displayed delayed kinetics relative to non-neuronal cells [28•]. Another study of hippocampal neurons reported rapid that Parkin and autophagosome recruitment could occurr in distal axons [29••]. Notably, in contrast to using pharmacological agents, Ashrafi *et al.* induced mitochondrial damage via the mtKillerRed-generated ROS. Importantly, this approach offers an alternative to chemical toxification which is more amenable to *in vivo* studies. It will be interesting to learn whether analysing PINK1/Parkin mitophagy using this approach in animals will recapitulate aspects of the current model. In summary, current evidence from cultured neurons does provide support for PINK1/Parkin mitophagy, nevertheless the still rely on Parkin overexpression.

Triggers for PINK1/Parkin function

To address concerns with off-target effects of the depolarizing agents used in early studies of PINK1 and Parkin, more recent studies have used mitochondria-specific poisons such as oligomycin and antimycin A [12,30], and have observed comparable effects to CCCP/FCCP uncoupling. A study using live imaging of cultured cells potentially offers additional support for the role of PINK1 and Parkin in the degradation of depolarized mitochondria by showing that mitochondrial fission products with decreased membrane potential have a higher probability of undergoing autophagy [31]. However, whether this mitophagy is mediated by PINK1 and Parkin has not been definitively established. Moreover, the mitochondria targeted for degradation in this live cell imaging study display only a relatively small decrease in membrane potential, far below that seen in experiments involving cells treated with depolarizing agents, further

questioning the role of PINK1 and Parkin in this form of mitophagy. Finally, Ca2+-pacemaking has been shown to trigger more substantial mitochondrial depolarization [32], yet there is no evidence that this mitochondrial depolarization triggers PINK1–Parkin mediated mitophagy. Finally, recent work has shown that unfolded matrix proteins trigger PINK1–Parkin-mediated mitophagy without triggering mitochondrial depolarization [33,34]. Together, these studies pose serious challenges to the model that mitochondrial depolarization is the true physiological stimulus for PINK1–Parkin mediated mitophagy.

PINK1/Parkin in animal models: of mice and flies

PINK1 and Parkin have also been extensively studied using *in vivo* model systems. In particular, pioneering studies in the fruit fly *Drosophila* were the first to demonstrate a role for Parkin in mitochondrial maintenance [35], and to link PINK1 and Parkin in a common biological pathway [36,37]. *PINK1/parkin* deficiency in *Drosophila* triggers the accumulation of enlarged, damaged mitochondria in sperm, flight muscle and dopaminergic neurons, suggesting that this pathway influences mitochondrial integrity in energetically demanding tissues [35–37]. To directly test the hypothesis that PINK1 and Parkin promote mitophagy *in vivo* under physiological conditions, Vincow *et al.* used an innovative proteomic approach employing quantitative mass spectrometry to monitor mitochondrial protein turnover in *Drosophila* heads [38••], which has provided some compelling evidence. Working on the premise that under physiologically constant conditions mitochondrial components would be degraded and replaced at a constant rate to maintain steady-state levels, the accumulation of heavy isotope-labelled mitochondrial proteins was monitored in *Pink1* and *parkin* mutants compared to wild-type flies. These experiments revealed that the half-life of many mitochondrial proteins was significantly increased in *parkin* mutants in a pattern that closely resembled that of autophagy-defective *Atg7* mutants, thus supporting a role for Parkin in mitophagy.

While studies in *Drosophila* offer potential support for a role of PINK1 and Parkin in mitophagy, they also leave several questions unanswered, and raise new questions. In particular, flight muscle disruption is apparent in young *Pink1* and *parkin* mutants, preceding any influence of age-related mitochondrial damage, thus raising the possibility that the role of PINK1 and Parkin in flight muscle may not involve mitophagy. Similarly, mitochondrial defects in the germline of *Pink1* and *parkin* mutants also occur during the development of these tissues and seem more likely to result from altered mitochondrial fission/fusion, rather than a defect in mitophagy [39,40], again challenging the concept that these defects involve mitophagy. Moreover, it is also hard to reconcile the genetic rescue of *Pink1* mutants by *parkin* overexpression [36,37] with the apparent absolute requirement for PINK1 to promote mitophagy in cultured vertebrate cells [30]. Additionally, although *parkin* mutants showed a dramatic reduction in mitochondrial protein turnover, the influence of *Pink1* on mitochondrial turnover was restricted primarily to membrane-spanning respiratory chain components. *parkin* mutants also had a disproportionately large effect on membrane-spanning respiratory chain components, suggesting a novel mitochondrial protein-selective feature of PINK1 and Parkin on mitochondrial protein degradation.

PINK1 and Parkin have also been studied extensively in mice. Perhaps the most obvious disconnect is the apparent lack of gross physiological, neurological or behavioral phenotypes in *PINK1/parkin* knockout mice, which is not easy to reconcile with the notion that PINK1/Parkin mitophagy is an important cell survival mechanism. However, two recent studies have reported that post-natal conditional *parkin* knockout does result in loss of nigral dopaminergic neurons [41,42], suggesting a compensatory mechanism occurs during development in germline knockouts. Although the nature of this compensatory mechanism is currently unclear, Parkin-independent mechanisms of mitophagy have been described and it is possible that the induction of one or more of these mechanisms may compensate for loss of *parkin* [43–45]. Nevertheless, this finding clearly indicates a discordance between mouse and man since *PINK1* or *parkin* loss almost invariably leads to a profound neurodegeneration in humans that can be evident as early as 3 years of age.

Although overt dopaminergic neuron degeneration is not observed upon germline knockout of mouse homologs of *PINK1*, *parkin* and other genes associated with recessive forms of PD, a recent study of a mouse strain that expresses a proof-reading defective version of the mtDNA polymerase (POLG), known as the 'mutator' mouse [46,47], provides evidence that PINK1 and Parkin play a role in mitochondrial quality control in this organism. Although mutator mice and *parkin* mutants display no loss of dopaminergic neurons, mice that are homozygous for the *parkin* knockout in the mutator background revealed a significant degeneration of dopaminergic neurons and motor deficits [48••]. Moreover, the sensitivity to this double hit showed a relative selectivity for nigral dopaminergic neurons, a cardinal feature of PD. Surprisingly however, there was no increase in the mtDNA mutation frequency in the double mutants, suggesting that the influence of *parkin* deletion in the mutator background was not a consequence of a defect in the selective elimination of mutation bearing mitochondria. While the influence of Parkin on the mutator mouse phenotype requires further examination, these findings are at least consistent with a biological role of Parkin in mitochondrial quality control.

Future directions—the when, how and where of mitophagy

What is the physiological trigger?

Although there has been incredible progress in our understanding of the biological roles of PINK1 and Parkin and mitophagy over the past 8 years following the seminal work linking these factors to mitophagy, many important questions remain about this pathway. For example, it remains unclear whether mitochondrial depolarization is the true physiological trigger of PINK1–Parkin pathway induction. The recent finding that unfolded mitochondrial matrix proteins trigger pathway induction independent of a reduction in membrane potential raises the possibility that unfolded proteins are the true physiological trigger of pathway induction [33,34]. However, it is far from clear how unfolded matrix proteins may trigger mitophagy. Moreover, substantial recent work indicates that unfolded mitochondrial matrix proteins can trigger a general cellular response to alleviate this stress [49], so the finding that the PINK1–Parkin pathway also responds to unfolded protein stress raises the question of how these two pathways are coordinated to influence the decision of whether to repair, or degrade the mitochondria that harbour unfolded proteins.

What is the precise mechanism of PINK1/Parkin mitoQC?

Another important unanswered question involves the precise role of PINK1 and Parkin in mitochondrial quality control. As mentioned above, *parkin* mutations influence the phenotype of mutator mice, but this influence appears to be independent of a role of Parkin in degrading mutation-bearing mitochondria. Moreover, proteomic studies in flies raise the possibility of a mitochondrial-protein selective feature of PINK1 and Parkin. One attractive model that offers a potential explanation for these findings derives from recent work documenting the existence of a piecemeal process of mitochondria quality control—the formation of cargo-selective mitochondria derived vesicles (MDVs) [50]. The function of MDVs appear diverse but evidence strongly implicates a role in the selective transport of oxidized mitochondrial components for degradation in lysosomes [51–53]. Importantly, PINK1 and Parkin have been linked to the formation and trafficking of MDVs [54•,55], as well as a third PD factor, Vps35 [55,56]. Although MDVs have not been analysed directly *in vivo*, and may prove extremely challenging, this model would explain the selective degradation of respiratory proteins in *Drosophila parkin* and *Pink1* mutants. Moreover, such a vesicular pathway would also potentially explain the finding that removal of *parkin* influences the phenotypes of mutator mice without affecting the frequency of mtDNA mutations. Given that the mitochondrial genome encodes membrane spanning components of the respiratory chain, the presence of Parkin may mitigate the effects of mtDNA mutations by selectively promoting the degradation of the mutationally altered products of these mutations through this vesicular pathway. Additional evidence in support of this pathway is provided by the finding that genetic studies in *Drosophila* revealed a striking interaction between *vps35* and *parkin* [57], providing strong support for a common pathway. Important future challenges involve the validation of this vesicular pathway in an *in vivo* context, and the elucidation of the regulatory mechanisms that influence the decision to activate wholesale mitochondrial degradation, or piecemeal degradation through this vesicular pathway.

What are the mechanisms of mitochondrial component turnover?

Lastly, while the proteomic assay of mitochondria protein turnover is useful for monitoring mitochondrial protein degradation, this approach is agnostic as to the precise mechanism of mitochondrial protein degradation. For many, seeing is believing, and proteomic and other biochemical methods do not allow fine spatial and temporal resolution to address where and when this turnover is occurring. Hence, the recent reports of two transgenic mouse models to monitor mitophagy *in vivo* are timely [58•,59•]. One uses a matrix-targeted pH-sensitive variant of GFP (mtKeima) while the other uses a tandem GFP-RFP fusion targeted to the OMM (called '*mito*-QC'). Both methods exploit the pH difference between relatively neutral matrix or cytosol and the acidic lysosomal lumen to induce a change in fluorescence. While these valuable tools have not yet been used in the context of PINK1/Parkin mitophagy, they have revealed that basal mitophagy is surprisingly abundant but varies widely between and even within a tissue or organ. Notably, the mtKeima reporter showed elevated mitophagy by hypoxia, mtDNA mutations and cancerous cells, but was reduced with age, a high-fat diet and expression of Huntingtin. While, there are still questions to address concerning how well these reporters mirror mitochondrial components, for example whether the reporter have a similar half-life in lysosomes compared to mitochondrial proteins and whether a proportion may be mis-localized and reporting autophagy of other compartments, the stage is set to now use these reporters to explore the *in vivo* nature of PINK1/Parkin mitophagy. It will be particularly interesting to investigate whether certain brain regions, in particular the basal ganglia, have high levels of to mitophagy which require PINK1/Parkin.

Concluding remarks

In conclusion, the use of cell culture models and chemical toxification of mitochondria has allowed the elucidation of the molecular mechanism by which PINK1 and Parkin signal the degradation of mitochondrial components, although initial attempts to translate these findings to neurons and animal models was controversial. However, there is now mounting evidence *in vivo* and importantly under physiological conditions supporting a

PINK1/Parkin-mediated mitoQC process involving turnover of mitochondria components. Nevertheless, it is still unclear exactly what are the physiological stimuli that signal the distinction between destruction and repair processes. Ultimately, it also remains to be determined whether stimulating this PINK1/Parkin mitoQC represents a viable therapeutic target. These outstanding points seed the field for vigorous and insightful research to come.

Funding

AJW acknowledges funding from the MRC (MC-A070-5PSB0) and ERC (StG 309742), and LJP acknowledges funding from the NIH (5R01GM104990).

Conflicts of interest

The authors declare no conflicts of interest.

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These two publications described the first transgenic mouse lines engineered to express fluorescent reporter constructs to monitor mitopahgy *in vivo*. These initial reports do not analyse PINK1/Parkin function but do describe a surprisingly abundant basal mitophagy that varies widely between and even within tissues.