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REVIEW

PINK1-PRKN mediated mitophagy: differences between *in vitro* and *in vivo* models

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ABSTRACT
Mitophagy is a key intracellular process that selectively removes damaged mitochondria to prevent their accumulation that can cause neuronal degeneration. During mitophagy, PINK1 (PTEN induced kinase 1), a serine/threonine kinase, works with PRKN/parkin, an E3 ubiquitin ligase, to target damaged mitochondria to the lysosome for degradation. Mutations in the *PINK1* and *PRKN* genes cause early-onset Parkinson disease that is also associated with mitochondrial dysfunction. There are a large number of reports indicating the critical role of PINK1 in mitophagy. However, most of these findings were obtained from *in vitro* experiments with exogenous *PINK1* expression and acute damage of mitochondria by toxins. Recent studies using novel animal models suggest that PINK1-PRKN can also function independent of mitochondria. In this review, we highlight the major differences between *in vitro* and *in vivo* models for investigating PINK1 and discuss the potential mechanisms underlying these differences with the aim of understanding how PINK1 functions under different circumstances.
Abbreviations: AAV: adeno-associated viruses;AD: Alzheimer disease; CCCP: carbonyl cyanidem-chlorophenyl hydrazone; HD: Huntington disease; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTS: mitochondrial targeting sequence; PD: Parkinson diseases; PINK1: PTEN induced kinase 1; PRKN: parkin RBR E3 ubiquitin protein ligase; ROS: reactive oxygen species; UIM, ubiquitin interacting motif.

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Introduction

Mitochondria perform a diverse range of critical cellular functions, including producing energy, buffering cytosolic calcium flux, synthesizing lipids, and participating in apoptosis-programmed cell death [1]. Since mitochondria are involved in energy production through oxidative phosphorylation, cellular redox balances, and apoptosis [1], mitochondrial dysfunction can result in increased oxidative stress, reduced ATP production, and decreased cell survival, therefore playing a vital role in many neurodegenerative disorders. In addition, mitochondria are prone to damage, and this damage results in the release of toxic levels of reactive oxygen species (ROS) to cause cell death. To combat this, there are a number of quality control systems to repair damaged mitochondria and to protect the overall integrity of the mitochondria network. However, when mitochondria damage is too severe to repair, mitochondria can be selectively degraded via the autophagy pathway, a process referred to as mitophagy [2].

Mitochondria dysfunction, including impaired fission/fusion, altered transport and morphology, and abnormal mitochondrial protein levels and activity, is a common feature of many neurodegenerative disorders such as Parkinson (PD), Alzheimer (AD) and Huntington (HD) diseases [3–6]. Mutations in two PD related genes have been reported to play key roles in mitochondria quality control: *PINK1*, encoding a serine/threonine kinase; and *PRKN/parkin*, encoding a cytosolic ubiquitin ligase. A number of *in vitro* studies have demonstrated that PINK1 works together with PRKN to remove unhealthy mitochondria by lysosomes through the mitophagy process. These studies have expanded the roles of mitochondrial dysfunction and mitophagy in PD and provided a wealth of information about how PINK1-PRKN pathway regulates mitochondria homeostasis.

Although there is wide acceptance for the role of PINK1-PRKN-mediated mitophagy, it is notable that PINK1-PRKN independent mitophagy also plays significant physiological roles and involves various receptors such as cardiolipin, a phospholipid that is protective against neural apoptosis [7,8]. It is also important to recognize the limitations of the studies that support the current prevailing model for PINK1-PRKN-mediated mitophagy. In particular, much of the work that supports this model relied on the use of mitochondria toxins, overexpressed PINK1 or PRKN, and immortalized cell lines that do not exactly mimic the physiological or pathological conditions *in vivo*. There is still lack of *in vivo* evidence for PINK1-PRKN-mediated mitophagy in animal models. Furthermore, non-mitochondrial dependent functions of PINK1 and PRKN have also been reported, but whether the mitochondria-independent function of these two proteins is related to PD pathogenesis or other pathological conditions remains elusive. Thus, in this review, we will discuss the differences in PINK1-PRKN-mediated mitophagy between *in vitro* and *in vivo* models, aiming at elucidating how PINK1 and PRKN function *in vivo* and how their dysfunction is involved in PD and other physiological or pathological conditions.

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Association of PD with mitochondrial dysfunction

Parkinson disease (PD) is the second most common neurodegenerative disorder that affects more than 1% people over age 60 [9]. Clinical signs of PD include tremor, bradykinesia, postural instability, and rigidity. Pathologically, PD is characterized by age-dependent and progressive loss of dopamine neurons in the substantia nigra and accumulation of α-synuclein positive inclusions (Lewy bodies) [10]. The idea that mitochondrial dysfunction may be involved in the selective neurodegeneration of PD was first suggested by the discovery of toxic effects of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). Accidental exposure to MPTP was found to cause parkinsonism and DA neurodegeneration [11]. Follow-up studies revealed that MPTP is metabolized to MPP^+, which is a mitochondrial complex I inhibitor and is selectively taken up into DA neurons [12–14]. In addition to MPTP, other mitochondrial toxins (such as pesticide rotenone and Paraquat) have been shown to cause or correlate with increased risk of PD [15–17]. Importantly, postmortem PD patient brain tissues display well-documented evidence of mitochondrial dysfunction including complex I deficiency [18,19], bioenergetics defects, age-dependent accumulation of mtDNA deletions [20,21], and dysregulation in the expression of various mitochondrial proteins [22]. The strong link between mitochondrial dysfunction and PD indicates that DA neurons is sensitive to mitochondrial dysfunction and has stimulated interest in studying the association of mitochondrial dysfunction with genetic mutations that also cause PD.

Although the majority of PD is sporadic, specific genetic mutations have been reported in rare familial cases. Among the dozens of genes responsible for familial PD, *PINK1* and *PRKN* are two most extensively characterized genes whose mutations were found to result in early-onset (before the age of 45) and autosomal recessive PD that is also featured by neurodegeneration in association with mitochondria dysfunction [23–25]. PINK1 is a mitochondrial kinase encoding a 581-aa protein with an N-terminal mitochondrial targeting sequence (MTS) followed by a putative transmembrane domain and a serine/threonine kinase domain. On the other hand, PRKN is a 465-aa E3 ubiquitin ligase that contains an N-terminal ubiquitin-like (UBL) domain and C-terminal double Ring finger motifs connected by an IBR domain [26]. *Drosophila* genetic studies have revealed that PINK1 and PRKN function in the same biological pathway and that PRKN acts downstream of PINK1 [27–29]. Further biochemical analysis of PINK1-PRKN uncovers their function in detecting mitochondrial damage and recruiting ubiquitin machinery to remove dysfunctional mitochondria via lysosomes, establishing the prevalent theory that PINK1-PRKN work together in the same pathway to protect mitochondria.

In vitro study of the PINK1-PRKN pathway and mitophagy

Mitophagy is the selective degradation of damaged mitochondria by targeting them to the lysosomes, a critical intracellular process for maintaining mitochondria homeostasis and preventing neuronal death. Mounting evidence from biochemical and *in vitro* studies demonstrated that PINK1 and PRKN work together to induce mitophagy, as both proteins target damaged mitochondria to the lysosomes for clearance of the unhealthy mitochondria [24]. Full-length PINK1 is a 581-aa Ser/Thr kinase consisting of a N-terminal mitochondria/matrix-targeting signal (1–34 aa), a putative transmembrane (TM) domain (85–110 aa), a ser/ther kinase domain (156–511 aa) and a C-terminal extension (512–581 aa, highly conserved) [24]. *In vitro* studies of cell lines revealed that endogenous PINK1 is synthesized constitutively in the cytosol as a full-length precursor (~63-68 kDa). In healthy cells, the full-length 63-kDa PINK1 is transferred across the outer mitochondrial membrane and then cleaved at A103 and F104 to a 52-kDa isoform by inner mitochondrial membrane-bound proteases. The N-terminal cleaved 52-kDa isoform of PINK1 is then released into cytosol and degraded via ubiquitin-proteasome system [24,30,31]. As such, endogenous PINK1 is undetectable in cells with physiologically polarized mitochondria. However, when mitochondria are damaged, the full-length PINK1 is accumulated and stabilized on the mitochondrial membrane to phosphorylate PRKN (an E3 ubiquitin-ligase) and ubiquitin, leading to activation of mitochondrial quality control pathways [32]. During this process, PINK1 acts as a key sensor of mitochondrial damage.

PRKN, a cytosolic E3 Ub ligase, acts downstream of PINK1 when its S65 residue is phosphorylated by PINK1 [33]. The UBL domain of PRKN also plays a special role in PRKN activation, which involves substrate recognition, recruiting and binding to SH3 or ubiquitin interacting motif (UIM) domains, proteasome association, and regulation of cellular PRKN levels and activity [34]. Structural and biochemical analysis of PRKN showed that PRKN exists in a compact and autoinhibited conformation in the basal, unstimulated state, where the UBL domain exerts an inhibitory effect on both E2 binding and transthiolation [35–39]. Upon being phosphorylated and activated by PINK1 at Ser65, PRKN modifies various outer mitochondrial membrane proteins, predominantly with K11- and K63-linked UB chains [40], and thus increases the pool of phospho-ubiquitin to recruit autophagy receptors such as SQSTM1/p62, OPTN, and MAP1LC3/LC3 [41–44] to damaged mitochondria, resulting in the removal of damaged mitochondria via mitophagy. It has been extensively shown that PRKN ubiquitinates a wide variety of cytosolic and outer mitochondrial membrane proteins upon mitochondrial depolarization [45,46].

In numerous cell lines and mouse embryonic fibroblasts, exposure to mitochondrial toxins such as carbonyl cyanide m-chlorophenyl hydrazone (CCCP), antimycin A, and rotenone, triggers the accumulation of PINK1 on mitochondria and then signals PRKN and ubiquitin by phosphorylation to ubiquitinate the damaged mitochondrial proteins for removing the damaged mitochondria by the lysosomes [24,43,47–50]. Analysis of the crystal structure of insect PINK1 bound to ubiquitin further provides a structural base for the interactions of PINK1 with PRKN and ubiquitin [51,52]. All together, these findings strongly indicate that PINK1 confers protection against mitochondrial dysfunction-dependent cell death induced by both intrinsic stress and environmental insults [53–56] (Figure 1).

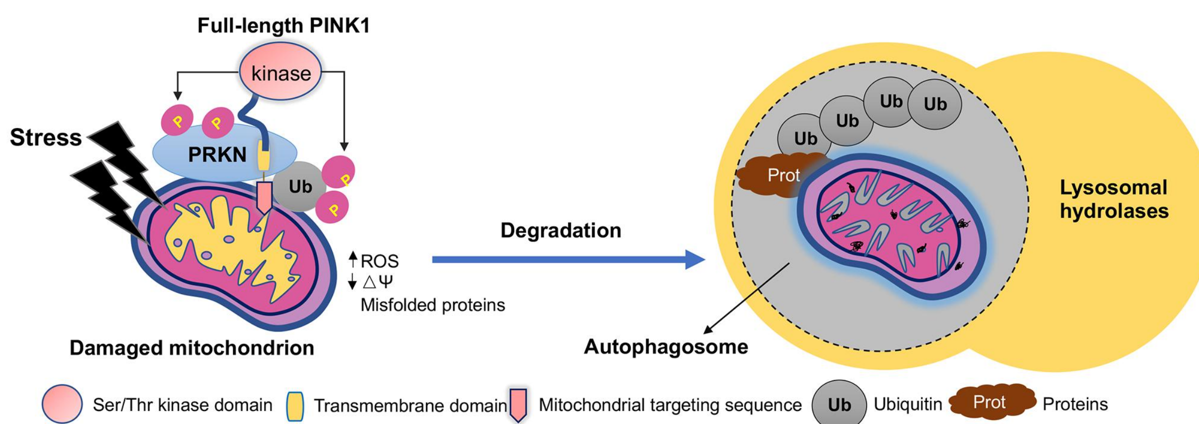

Figure 1. *In vitro* studies of PINK1-PRKN-mediated mitophagy. In cultured cells stressed by mitochondrial toxin CCCP, full-length PINK1 is targeted to damaged mitochondria. PINK1 then phosphorylates PRKN and ubiquitin to form a complex that add polyubiquitination chains to mitochondrial proteins, followed by degradation by lysosomes. The ubiquitinated proteins could also undergo degradation by the proteasome.

Despite convincing *in vitro* studies indicating the critical role of PINK1 in mitophagy, it seems that the regulation of mitophagy by PINK1 is also cell type dependent. The study of cultured mouse cortical neurons with PRKN overexpression revealed that PRKN recruitment and translocation to depolarized mitochondria occurred only after 12 h of treatment with mitochondrial toxins, suggesting that PINK1-PRKN-mediated mitophagy in neurons is a quite slower process than that in non-neuronal cells [57]. Different types of neurons show variability in the intracellular environment and mitochondria stresses, which may complicate the study of PINK1-PRKN-mediated mitophagy in neurons. Immortalized cells typically rely on glycolysis and could potentially tolerate substantial loss of mitochondria, whereas neurons rely primarily on oxidative phosphorylation for energy production [58–60], implying that additional mechanisms regulate mitophagy in neurons. Beyond mitophagy, overexpression of PINK1 or PRKN in rat hippocampal axons decreases mitochondrial movement, suggesting that PINK1-PRKN also mediate mitochondrial motility in cultured neurons [54].

In vivo investigation of PINK1-PRKN-associated mitochondrial function

Although current evidence from cultured cells does provide strong support for PINK1-PRKN-mediated mitophagy, it is also important to recognize the limitations of the *in vitro* work that relies on PRKN or PINK1 overexpression and acute mitochondrial injury. In particular, there is a pressing need for obtaining *in vivo* evidence to support the role of PINK1-PRKN-mediated mitophagy. To this end, a variety of animal models have been established to investigate the *in vivo* function of PINK1 and PRKN (Table 1). The first *in vivo* evidence indicating that PINK1 and PRKN could work together to regulate mitochondrial quality control came from genetic complementation studies in *Drosophila*. In 2003, *Drosophila* genetic studies revealed that *prkn* null mutant flies exhibit locomotor defects, muscle degeneration, and mitochondrial dysfunction, thereby providing *in vivo* fact for involvement of PRKN in mitochondrial quality control [27]. Then in 2006, other genetic studies further revealed that *Pink1* null mutant flies also showed apoptotic muscle degeneration, mitochondrial defects, and male sterility as seen in *park* mutant flies [28,29]. Notably, park overexpression rescues male sterility and mitochondrial defects in *Pink1* mutant flies, but overexpression of Pink1 does not rescue the phenotypes of *park* mutants, suggesting that Pink1 and park function in the same pathway and that park acts downstream of Pink1. Furthermore, overexpression of Drp1 (Dynamin related protein 1) or inhibition of Marf/MFN, which is involved in mitochondrial fusion-fission dynamics, also recovered mitochondrial defects and muscle degeneration in the *park* and *Pink1* mutant flies [61–63]. These findings indicated that in *Drosophila* Pink1-park maintain mitochondrial integrity when dealing with energetically demanding tissues.

The *in vitro* studies and fly model investigation have made incredible progress in understanding the roles of PINK1-PRKN and mitophagy, but a major challenge remains in the field is to accurately and faithfully detect PINK1-PRKN-mediated mitophagy in the mammalian brain. A number of *pink1 prkn* knockout mouse models have been established for investigating the effects of PINK1-PRKN on mitochondria and neuronal survival. However, these *pink1 prkn* knockout mouse models fail to replicate selective and overt neurodegeneration seen in PD [66]. Even 2-year-old triple knockout mice that lack *Pink1, Prkn* and *Park7/DJ-1*, which are three main genes causative of early onset PD, do not show obvious neurodegeneration [67]. Absence of neuronal loss in these knockout mice could be due to the existence of compensatory mechanisms that are able to cope with PINK1-PRKN-PARK7 /DJ-1 deficiency in mouse models or the life span of mouse that is too short to reach a threshold of damage sufficient to induce an overt phenotype.

To address the above possibilities, conditional *prkn* knockout mice, which were established via AAV-mediated Cre-loxp induction, showed DA neuronal loss in aged mice [68]. However, a recent study reported that AAV-mediated Cre expression would induce a massive decrease in neuronal populations of substantia nigra [69]. It seems that additional

Table 1. *PINK1* or *PRKN* knockout animal models.

Genetic anomaly	Species	Modifications Approach	Pathology and phenotypes	Reference
Pink1	*Drosophila*	KO	-Male sterility -apoptotic muscle degeneration - defects in mitochondrial morphology and increased sensitivity to multiple stresses including oxidative stress.	[28]
Pink1 and *park*	*Drosophila*	*Pink1*(B9); *park*(25)	-Basal mitophagy is highly prevalent in multiple Drosophila tissues -Loss of Pink1 or park does not affect basal mitophagy in adult flies	[77]
Pink1 and *park*	*Drosophila*	*Pink1*(B9); *park* RNAi	-Loss of Pink1 or park does not affect mitophagy in 1-week-old adult flies -Mitophagy increases with aging, and this age-dependent rise is abrogated by Pink1 or park deficiency.	[81]
park	*Drosophila*	KO	-Male sterility -Reduced lifespan, locomotor defects, apoptotic muscle degeneration -Defects in mitochondrial morphology	[27]
pink1	Mouse	KO	-In absence of overt neuronal death -Increasing mitochondrial dysfunction	[64]
prkn	Mouse	KO	-In absence of overt neuronal death	[65]
pink1-prkn-park7/DJ-1	Mouse	KO	-No obvious neuronal loss -Normal behavior	[67]
pink1	Mouse	KO	-No obvious neuronal loss -The endogenous PINK1 is dispensable for basal mitophagy in vivo using Mito-QC reporter.	[76]
pink1	Rat	KO	-Age-dependent loss of tyrosine hydroxylase (TH)-positive neurons -Motor deficits	[74]
pink1	Rat	KO	-Developed behavior deficits -No loss of dopaminergic neurons	[75]
prkn	Rat	KO	-No obvious neuronal loss -Normal behavior	[74]
PINK1-PRKN-PARK7/DJ-1	Pig	KO	-No obvious neuronal loss -Normal behavior	[86]
PINK1-PRKN	Pig	KO	-No obvious neuronal loss -Normal behavior	[87]
PINK1	Rhesus monkeys	KO (targeting *PINK1* exon2 and exon4)	-Severe neuronal loss -Reduced motor activity	[90]
PINK1	Rhesus monkeys	KO (target *PINK1* exon2 alone)	-Neuronal loss not detected in monkey brain -*PINK1* mutant fibroblast-derived DA neurons did not show specific PD-associated phenotypes	[91]
PINK1-PARK7/DJ-1	Rhesus monkeys	KO (adult)	-Classic PD symptoms, -Severe nigral dopaminergic neuron loss - a-synuclein pathology	[92]
PINK1	Rhesus monkeys and Cynomolgus monkeys	KO (adult)	-Severe neuronal loss -Motor deficits	[93]

stress and insults need to be added to induce neurodegeneration in *pink1-prkn* knockout mice. For example, crossing the POLG (mtDNA mutator) mouse with *prkn* knockouts produced offspring with selective degeneration of dopaminergic neurons and locomotor deficits, though loss of PRKN did not affect overall levels of mtDNA somatic mutations [70]. Furthermore, dopaminergic neuronal loss was observed in *pink1* knockout mice in the presence of α-synuclein or MPTP induced toxicity [71,72]. Intestinal bacterial pathogens were also recently found to promote neurodegeneration in *pink1* knockout mice [73]. *Pink1* KO rats were reported to have age-dependent loss of TH (tyrosine hydroxylase)-positive neurons [74], but this mild degeneration could not be confirmed by other studies [75]. These findings indicate that the rodent brains are more resistant to *pink1* mutations when compared to the human brain since a patient carrying homozygous point mutations in either the *PINK1* or *PRKN* gene would suffer neurodegeneration.

The *pink1* or *prkn* KO mouse models are also unable to show striking defects in mitophagy. Two reporter systems, mito-QC and mt-Keima, have contributed to our understanding of physiological mitophagy. Recent studies using Mito-QC mouse and Drosophila models demonstrated that basal mitophagy activity is not affected by the loss of PINK1 [76,77]. Although mt-Keima is more sensitive than mito-QC to detect PINK1-PRKN-mediated mitophagy in mice after exhaustive exercise, the basal level of mitophagy is independent of PINK1 in the absence of exhaustive exercise [78]. Furthermore, using both Keima- and tandem mCherry-GFP-based reporters in zebrafish revealed that disruption of *pink1* or *prkn* had no effect on mitophagy [79]. In Drosophila, however, PINK1 was found to be essential for the induction of mitophagy in response to hypoxic exposure and rotenone treatment [80] and to be required for an age-dependent increase in mitophagy [81]. It is possible that PINK1-PRKN-mediated mitophagy is species dependent and occurs under pathological conditions. A knockin mouse model, in which PRKN cannot be phosphorylated by PINK1, also showed no clear neurodegeneration or nigrostriatal mitophagy impairment [82]. In addition, mass spectrometry analysis of *pink1* knockout rodents did not show significant alterations in the expression levels of mitochondrial proteins [83], and inconsistent or mild alterations of mitochondrial function were found among the *PINK1* KO animal models [84,85]. All these raise an important issue of whether PINK1 acts differentially *in vitro* and *in vivo* to regulate mitochondrial function.

Lack of neuronal loss and obvious alterations in mitophagy in *pink1* knockout mice has motivated researchers to utilize large animals to create PD models. Recent development of CRISPR-Cas9 allowed for disrupting the genes of *PINK1-PRKN* in large animals for investigating the consequences of loss of function. Interestingly, CRISPR/Cas9-mediated *PINK1-PRKN* deletion in pigs did not produce any obvious neurodegeneration and severe phenotypes [86,87], like *pink1* or *prkn* KO mouse models [53,67,88]. These findings suggest that PINK1-PRKN deficiency may induce species-dependent neuropathology and underscore the importance in using non-human primates to explore the function of PINK1 and PRKN [89].

Recently, a few groups have used CRISPR-Cas9 to target the monkey *PINK1* gene to generate monkey models of PD [90–93]. When the monkey *PINK1* exon 2 and exon 4 were targeted by two gRNAs in embryos, a large *PINK1* DNA fragment was deleted, resulting in almost complete elimination of PINK1 expression in the monkey brain [90]. The newborn monkeys showed severe neurodegeneration or died postnatally, demonstrating for the first time that PINK1 is essential for neuronal survive in the developing primate brain [90]. Furthermore, disruption of *PINK1* gene via AAV-mediated CRISPR/Cas9 in adult monkey brain also led to severe neurodegeneration and motor deficits of monkeys [93]. It is worth noting that the homozygous deletion of a large region of *PINK1* gene has not been found in humans, perhaps because such deletion is embryonic lethal in humans [94]. Interestingly, using the paired gRNA-Cas9-D10A nickases to disrupt the monkey *PINK1* gene in the fertilized monkey oocytes did not model PD phenotypes in the live young monkeys [91]. On the other hand, co-editing *PINK1* and *PARK7/DJ-1* genes via AAV-delivered CRISPR-Cas9 system in adult monkey substantia nigra could mediate severe nigra dopaminergic cell loss and motor function deficits [92]. It seems likely that the phenotypes of *PINK1*-targeted monkeys rely on the extent to which *PINK1* is depleted.

The severe neuronal loss in *PINK1* mutant monkey brain is in clear contrast to the absence of neurodegeneration in mouse models that have completely deleted the *Pink1* gene, suggesting that PINK1′s function is at least species-dependent. The *PINK1* mutant monkey model also allowed for studies of the effect of PINK1 on mitochondria. Interestingly, although PINK1 deficiency could cause severe neuronal loss, there was no obvious differences in mitochondrial protein expression, morphology, and metabolic activities between wild type and *PINK1*-targeted monkey brains [93]. Instead, loss of PINK1 caused marked reduction of phosphorylation of proteins that are important for neuronal survival, which is also supported by the selective expression of PINK1 kinase form (55-kDa) that lacks N-terminal mitochondria targeting domain in the monkey brain [93]. In support of the notion that PINK1 exists as a cytoplasmic protein in the primate brain, fractionation analysis indicated that the majority of the monkey PINK1 is cytosolic [93]. Consistently, most of substrates phosphorylated by PINK1 are also cytoplasmic proteins [93]. Thus, PINK1 deficiency triggers neurodegeneration in the developing monkey brain by affecting protein phosphorylation without impacting mitochondrial homeostasis (Figure 2).

PINK1 expression levels account for different results from *in vitro* and *in vivo* models

Understanding why PINK1 acts differentially *in vitro* and *in vivo*, especially on mitophagy, is important for uncovering how PINK1 engages in important cellular processes and PD pathogenesis. A number of cell lines-based studies have revealed that PINK1-PRKN axis regulates mitochondria clearance via mitophagy upon acute mitochondrial toxin treatment that results in the dissipation of mitochondrial membrane potential. An important issue is whether acute and excessive stress on mitochondria can also occur *in vivo*. PD is

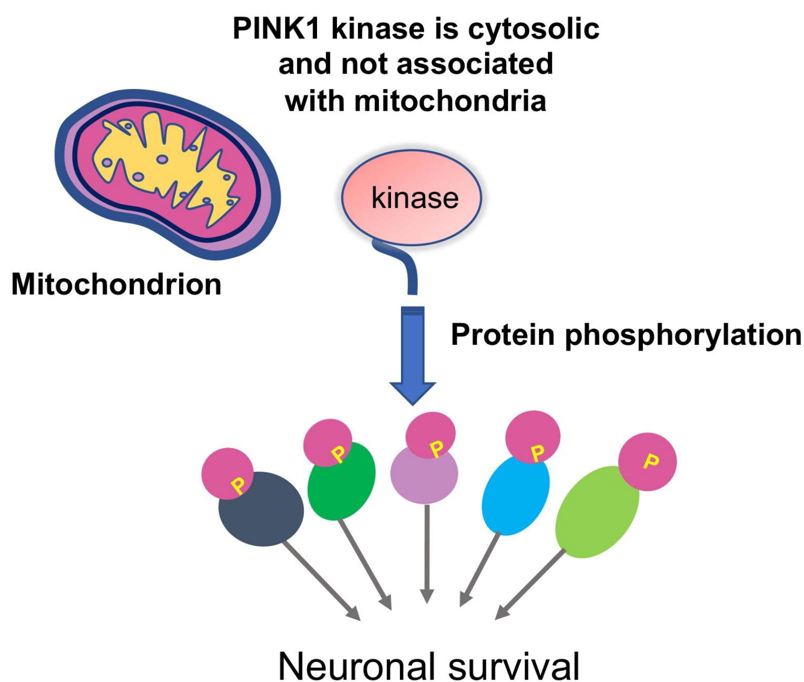

Figure 2. *In vivo* studies of PINK1. In the primate brain, PINK1 kinase (55-kDa) is stably expressed in the cytoplasm and is not associated with mitochondria. It phosphorylates a large number of proteins to maintain neuronal survival.

characterized by age-dependent neurodegeneration, a process that is likely involved in chronic and progressive damage on mitochondria. It remains to be investigated whether the effect of chronic and cumulative stress on mitochondria can trigger PINK1-PRKN-mediated mitophagy.

Also, regulation of mitochondrial metabolic function and mitophagy is not identical in different types of cells. For example, immortalized cells typically rely on glycolysis due to their origin as cancer cells and could potentially tolerate substantial loss of mitochondria, whereas neurons rely primarily on oxidative phosphorylation for energy production such that loss of entire mitochondrial pool would be fatal [58–60]. CCCP-induced mitochondrial depolarization results in mitochondria translocation of PRKN in cultured cell lines, which, however, was difficult to be seen in cultured primary neurons [95,96].

A more critical phenomenon is different expression levels of PINK1 in various types of cells and different species. Most of the *in vitro* studies used overexpression of PINK1-PRKN to investigate their translocation on mitochondria and regulation on mitophagy because endogenous PINK1 is hardly detected. The same scenario also applies to small mammals. It has been well recognized that endogenous PINK1 in rodents is at the undetectable level [30,93,97,98]. Endogenous PINK1 is thought to be synthesized constitutively in the cytosol as a full-length precursor (~63-68 kDa). Upon import into mitochondria, PINK1 is proteolytically cleaved to produce its mature form (~52-55 kDa) that is subsequently re-translocated to the cytosol, resulting in a rapid turnover and low steady state levels [30]. Thus, overexpression of PINK1 *in vitro* and endogenous level of PINK1 *in vivo* apparently account for differential effects seen *in vitro* and *in vivo*.

Establishment of *PINK1* knockout monkey models demonstrated for the first time that loss of PINK1 can mediate striking neuronal loss in the mammalian brains and also provided important insight into the selective neurodegeneration in PD. Being able to detect endogenous PINK1 expression at the protein level has been a very challenging and difficult issue in the field. Using five different antibodies against three epitopes in human PINK1, Yang et al. uncovered the expression of PINK1 that is selectively abundant in the primates [93]. PINK1 is abundantly expressed in the primate brain, but not in the peripheral tissues, and exists as a kinase form to phosphorylate a large number of neuronal proteins [90,93]. These findings strongly support the idea that PINK1 functions as a cytosolic kinase to maintain neuronal viability. In line of this concept, a number of previous *in vitro* studies have indicated that cytosolic PINK1 plays vital roles in various intracellular function and neuronal survival [99–103]. They also explain why loss of *Pink1* does not induce neurodegeneration in the mouse brain because the undetectable endogenous PINK1 is dispensable for the development and maturation of rodent neuronal cells. However, the expression of PINK1 is critical for PRKN activation, as PRKN remains inactive and becomes activated when it is phosphorylated by PINK1 [33,104,105]. Thus, the intrinsically low level of PINK1 in the rodent brain would confer a minor impact of *prkn* knockout in the mouse brain.

The differences between *in vitro* and *in vivo* models for studying PINK1-PRKN function raise crucial issues that need to be well addressed. First, why is PINK1 undetectable in mice but abundant in the primates? The regulation of PINK1 expression is more likely mediated at translational and/or protein stability level, as *PINK1* mRNA is ubiquitously and abundantly expressed across different species [106]. Thus,

rigorous investigation of mechanisms underlying PINK1 protein expression and cleavage is required. The undetectable expression of PINK1 in the rodent models makes it difficult to use mice to perform this important investigation. Use of large mammals such as monkeys for investigation could elucidate the mechanism for species-dependent expression of PINK1 and may also explain the absence of neuropathological phenotypes of pig models that have deleted the *PINK1* gene [86,87]. For example, the primate-specific CASP4 (caspase 4), which is not expressed in the mouse brain, was able to cleave TARDBP/TDP-43 to cause truncated TARDBP to redistribute in the cytoplasm, resulting in the cytoplasmic toxicity in the monkey brain [107]. The cytoplasmic truncated TARDBP can selectively reduce SQSTM1 expression in the monkey brain by inhibiting *SQSTM1* mRNA stability via its binding to the unique 3′UTR sequence (GU/UG) of the primate *SQSTM1* transcript [108]. The findings from *TARDBP* and *PINK1* mutant monkey models indicate the same disease-causing gene may have different gene expression patterns or regulatory mechanisms, which thus contribute to different phenotypes and pathological changes in different species. Second, to what extent dose PINK1-PRKN-mediated mitophagy play a role *in vivo*, especially under pathological conditions? There is no doubt that PINK1-PRKN mediate mitophagy in cultured cells under stress conditions and may also function to protect mitochondrial integrity *in vivo* under particular conditions. Does full-length PINK1, which is able to localize to mitochondria, or cytosolic PINK1 kinase play a more important role for cell survival and mitochondrial function, and how do these different forms of PINK1 coordinate their functions *in vivo*? Although loss of PINK1 in the monkey model causes neuronal loss without significant impact on mitochondrial morphology and protein levels, it remains to be investigated whether PINK1 in the primate brain is targeted to mitochondria when chemical-induced mitochondrial damage occurs. In addition, since female *pink1* knockout rats were found to display early onset Parkinsonian behaviors [109], it would be interesting to examine whether sex also influences the severity of phenotypes in the PINK1 mutant monkey models.

Concluding remarks

The use of cell culture models and mitochondrial toxins has elucidated the molecular mechanism by which PINK1 and PRKN act together to degrade damaged mitochondria. However, more studies under the *in vivo* physiological or pathological conditions need to be conducted to validate PINK1-PRKN-mediated mitophagy and its relevance to neuropathology when both PINK1 and PRKN are expressed at the endogenous level. For example, the current non-human primate studies were focused on the aspect of neurodegeneration caused by loss of PINK1. It remains to be explored whether the established methods to measure the basal mitophagy activities can be applied to monkeys via transgenic expression of the Mito-QC reporter. There has been wealth of information from *in vitro* studies regarding PINK1′s proteolytical process, PRKN recruitment, phosphorylation of ubiquitin and mitochondria proteins. Whether these important *in vitro* events also occur in the non-human primates under physiological conditions remains to be rigorously investigated. Despite the limitations in using the non-human primate models due to their time-consuming and high-cost experiments, it is clear that the non-human primate model would be very valuable to address these outstanding issues. In addition, PINK1 expression levels appear to account for differences seen in the *in vitro* and *in vivo* models and are also found to be altered in tumor cells, and its function is not restricted to the brain and is beyond mitochondria [32]. Thus, combination of *in vitro* and *in vivo* experiments to investigate PINK1 would continuously delineate the functions of this important and multifaceted protein. Understanding how PINK1 functions differentially under divergent conditions would also help develop the therapeutics for diseases that are related to dysfunction of PINK1.

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