PINK1- and Parkin-mediated mitophagy at a glance

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PTEN-induced kinase 1 (PINK1) and Parkin are mutated in many cases of early-onset familial Parkinson's disease (PD), but how these proteins are related to the pathogenesis of PD was unclear. Recently, work in cultured cells has shown that Parkin can mediate autophagy of damaged mitochondria (termed mitophagy) (Narendra et al., 2008) downstream of PINK1 (Geisler et al., 2010a; Matsuda et al., 2010; Narendra et al., 2010b; Vives-Bauza et al., 2010). These reports suggest that a quality control pathway maintains the mitochondrial homeostasis in many cell types, including in dopaminergic neurons, whose malfunction causes the motor deficits in PD. Here, we briefly summarize how defective mitochondria in a cell are specifically recognized and committed to mitophagy by PINK1 and Parkin.

What is mitophagy?

Mitophagy is the selective engulfment of mitochondria by autophagosomes and their subsequent catabolism by lysosomes (see Poster). Starvation induces a less-selective form of autophagy in which cytosol and a variety of organelles are engulfed into autophagosomes, which leads to their degradation in order to supply nutrients to compensate for the loss of external supplies. It was initially found that mitochondria are selectively engulfed by autophagosomes following a loss in membrane potential (Lemasters et al., 1998), suggesting that mitophagy mediates selective removal of damaged mitochondria. This idea has been supported by recent knockout mouse models, where loss-of-function of ATG7, which functions in the autophagy pathway, results in an accumulation of defective mitochondria in certain tissues (Komatsu et al., 2005; Wu et al., 2009). Among the first events in quality control by mitophagy is the distinction between damaged mitochondria and healthy mitochondria. Following their identification by PINK1, defective mitochondria are engulfed in double-membraned autophagosomes that fuse with lysosomes, thereby merging their contents
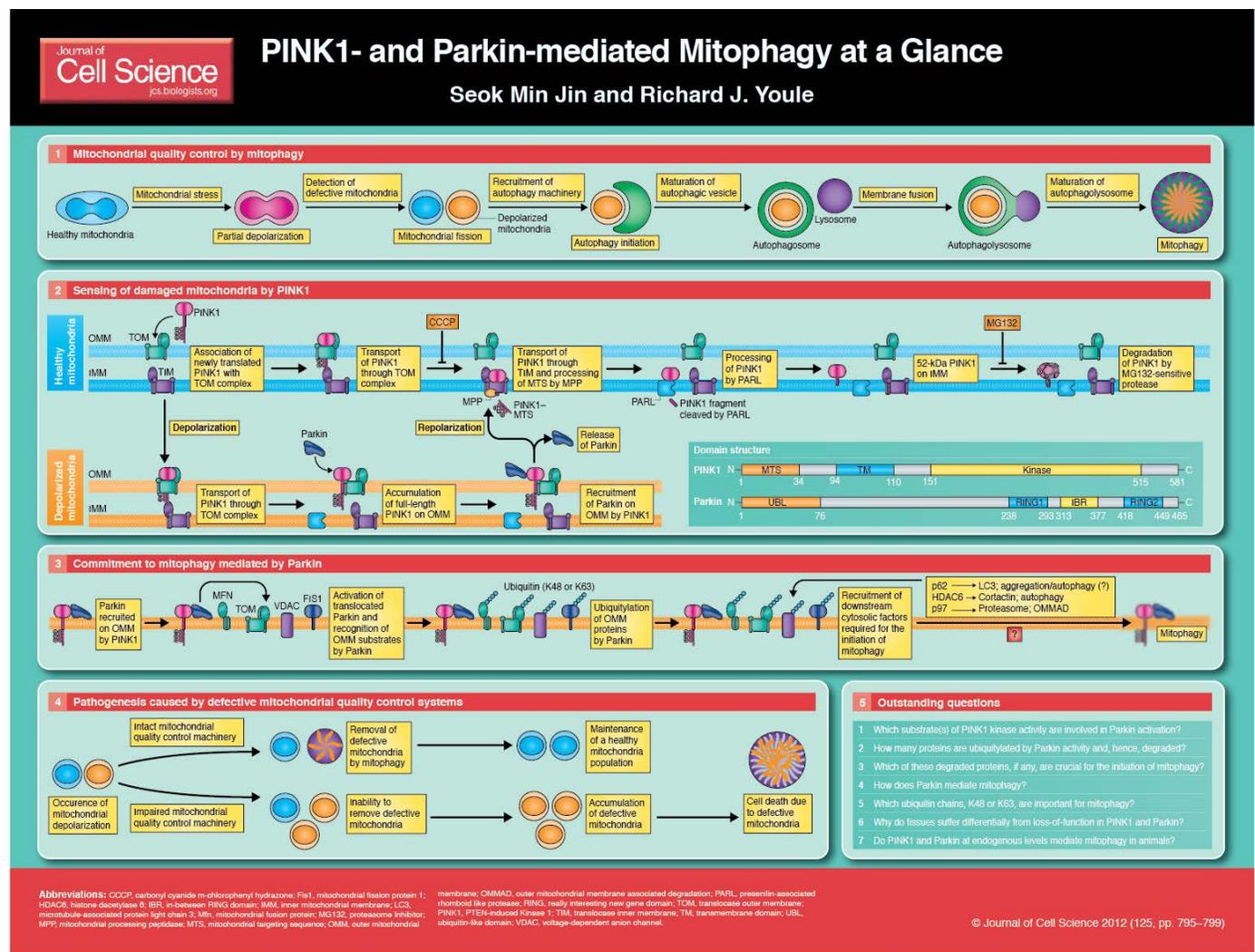
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and allowing hydrolytic degradation (reviewed by Kim et al., 2007). In addition to quality control, mitophagy occurs to regulate mitochondrial number (Zhang et al., 2009b) and eliminate mitochondria during the development of specialized cells or tissues such as reticulocytes (Kundu et al., 2008; Mortensen et al., 2010; Zhang et al., 2009a). Overexpression of Parkin has been found to induce the complete removal of mitochondria from cells by mitophagy when mitochondria lose their membrane potential (Narendra et al., 2008). As Parkin has also been found to selectively bind only to damaged mitochondria it has been suggested that Parkin might mediate a quality control pathway of mitophagy.

How does Parkin initiate mitophagy?
PINK1 and Parkin in the same pathway
Parkin was initially identified as a cytosolic E3 ubiquitin ligase that is mutated in familial forms of PD (Kitada et al., 1998), and mutations in this protein are now known to be the most prevalent cause of autosomal recessive monogenic PD. It has been found that the gene encoding PINK1, a serine/threonine kinase, is also mutated in other autosomal recessive cases of PD (Valente et al., 2004). Parkin was first linked to mitochondrial maintenance by studies of loss-of-function mutations in *Drosophila melanogaster* (Greene et al., 2003). Remarkably, *Drosophila* that lack Parkin or Pink1 display very similar defects in mitochondria (Clark et al., 2006; Park et al., 2006). In this organism, the *Pink1*-knockout phenotype is rescued by overexpression of Parkin, whereas the *Parkin*-knockout phenotype is not rescued by PINK1 overexpression, indicating that PINK1 is an upstream regulator of Parkin function. On the basis of these reports, and the finding that Parkin translocates to depolarized mitochondria and induces mitophagy (Narendra et al., 2008), in 2010 several groups reported that the recruitment of Parkin to impaired mitochondria requires PINK1 expression and its kinase activity (Geisler et al., 2010b; Matsuda et al., 2010; Narendra et al., 2010b; Vives-Bauza et al., 2010).

Parkin-mediated ubiquitylation
Following translocation to the mitochondrial surface, Parkin ubiquitylates numerous outer mitochondrial membrane (OMM) proteins, which in turn recruit other proteins to mitochondria to initiate mitophagy (Chan et al., 2011; Geisler et al., 2010a; Lee et al., 2010b; Narendra et al., 2010a; Tanaka et al., 2010; Yoshii et al., 2011) (see Poster). One proviso, in most experiments, is that overexpression of Parkin is typically used to identify mitochondrial E3 ligase substrates and to induce

mitophagy because endogenous Parkin expression is low in many cell lines. However, endogenous PINK1 has been shown to regulate mitochondrial mass in M17 and SH-SY5Y cells that express only endogenous Parkin (Gegg et al., 2010; Narendra et al., 2010b). So far, two mitochondrial substrates of endogenous Parkin have been identified, the closely related mitochondrial fusion proteins mitofusin (MFN) 1 and 2 (Gegg et al., 2010; Poole et al., 2010; Rakovic et al., 2011; Tanaka et al., 2010; Ziviani et al., 2010). Parkin is proposed to prevent mitochondrial fusion through mediating the degradation of MFN1 and MFN2, thereby segregating depolarized mitochondria from healthy mitochondria. Previously, it has been shown that inhibiting the fusion–fission cycle, by disrupting mitochondrial fission using a dominant-negative dynamin-related protein 1 (DRP1), prevents mitophagy, indicating the importance of fission in mitophagy (Twig et al., 2008). The segregated mitochondria have decreased membrane potential and reduced levels of the dynamin-related GTPase, optic atrophy 1 (OPA1), which prevents their re-fusion with healthy mitochondria. Alternatively, or additionally, the smaller size of mitochondria that results from inhibition of fusion might facilitate their encapsulation by autophagosomes. Consistently, cells (or at least mammalian cells) with elongated mitochondria due to knockdown of DRP1 (Smirnova et al., 2001) are defective in mitophagy (Tanaka et al., 2010) despite having normal Parkin translocation (Narendra et al., 2008; Tanaka et al., 2010). Apart from MFN1 and MFN2, overexpressed Parkin also mediates ubiquitylation of many other outer OMM proteins, such as voltage-dependent anion channel (VDAC), some components of the translocase of the OMM (TOM) complex (TOM70, TOM40 and TOM20), the pro-apoptotic factor BAK, mitochondrial Rho GTPases (MIRO) 1 and 2, FIS1 and others (Chan et al., 2011; Yoshii et al., 2011). However, the in vivo relevance of the degradation of these additional OMM proteins remains unclear.

Events after Parkin-mediated ubiquitylation
It has been shown that Parkin-mediated mitophagy is reduced when the proteasome is inhibited by epoxomicin or MG132 (Chan et al., 2011; Tanaka et al., 2010). Another group has reported that Parkin translocation induces OMM rupture, however, when they use another proteasomal inhibitor, lactacystin, OMM rupture is inhibited, but not mitophagy (Yoshii et al., 2011). Furthermore, the AAA$^+$ ATPase p97 (also known as VCP), which is known to be required for endoplasmic-reticulum-associated

protein degradation (ERAD) to dislocate the ubiquitylated proteins from the ER to the cytosol to make them accessible for proteasomal degradation (Rabinovich et al., 2002; Ye et al., 2001), was found to translocate to depolarized mitochondria along with Parkin. Moreover, a dominant-negative mutant of p97 inhibited degradation of MFN1 and 2, and, consequently, mitophagy (Tanaka et al., 2010). Proteasome subunits also accumulate on mitochondria during Parkin-induced mitophagy indicating that all components for the degradation of the ubiquitylated proteins are recruited to mitochondria (Chan et al., 2011). Thus, proteasomal degradation of mitochondrial substrates likely has important roles in Parkin activity (see Poster).

Considering the fact that Parkin induces an increase in ubiquitylation of OMM proteins that contain chains linked by K63 (Geisler et al., 2010a; Narendra et al., 2010a; Okatsu et al., 2010), it seems reasonable that the autophagy adaptor p62 (also known as SQSTM1) might also be involved in mitophagy as it has a K63-ubiquitin-binding domain as well as an LC3-binding domain and has a role in recruiting autophagosomes to ubiquitylated protein aggregates. However, this model is debated. Although some reports identify a decrease in mitophagy in cells upon knockdown of p62 (Geisler et al., 2010a; Lee et al., 2010b), other studies, using p62-knockout cells, find that p62 is crucial for perinuclear aggregation of damaged mitochondria, but not for mitophagy (Narendra et al., 2010a; Okatsu et al., 2010).

Another ubiquitin-binding protein, histone deacetylase 6 (HDAC6), translocates to mitochondria in a manner that is dependent on Parkin activity (Lee et al., 2010b). HDAC6 has been shown to enhance the fusion of autophagosomes and lysosomes by activating the actin-remodeling machinery (Lee et al., 2010a). Perinuclear aggregation of mitochondria and mitophagy were not observed in *Hdac6*-knockout mouse embryonic fibroblasts (MEFs) (Lee et al., 2010b). The knockdown of cortactin, which participates in HDAC6-mediated recruitment of the actin remodeling machinery (Lee et al., 2010a), has similar effects to the knockout of *Hdac6* (Lee et al., 2010b).

How are defective mitochondria detected by PINK1?
Processing of PINK1 in healthy mitochondria
Further pointing towards a role for mitochondria in parkinsonism, a predicted mitochondrial-targeting signal (MTS) was identified in the N-terminus of PINK1 (Valente et al., 2004) (see Poster). In normal mitochondria, a protein

containing a MTS and a single membrane-spanning domain is sequentially imported through the TOM complex and the translocase of inner mitochondrial membrane (TIM) complex, and then released to span the inner mitochondrial membrane (IMM) with its transmembrane (TM) domain acting as a stop transfer signal (Poster Panel 2, upper part) (reviewed by Neupert and Herrmann, 2007). It has been shown that the levels of the 52-kDa processed form of PINK1 increase upon inhibition of the proteasome (Muqit et al., 2006), whereas that the levels of the 64-kDa full-length form of PINK1 increase upon treatment with the mitochondrial depolarizing agent valinomycin (Lin and Kang, 2008). Recent work points to the following processing pathway to explain these phenomena (Jin et al., 2010; Meissner et al., 2011). Full-length 64-kDa PINK1 is imported to the IMM, and this step is associated with cleavage of the MTS sequence, to give a ~60-kDa form, by the mitochondrial processing peptidase (MPP), a general processing step for MTS-containing proteins. This MPP-cleaved form of PINK1, which now spans the IMM, is then further cleaved within or adjacent to the membrane-spanning domain by the rhomboid protease of the IMM, presenilin associated rhomboid-like protease (PARL), to give rise to the 52-kDa form (Poster Panel 2). This form is thereafter rapidly degraded by a protease that is sensitive to several different proteasome inhibitors. This pathway keeps endogenous PINK1 levels very low in polarized mitochondria to prevent mitophagy of healthy mitochondria. However, alternative hypotheses suggesting that there is a function for the 52-kDa form in this process have been presented (Deas et al., 2011; Shi et al., 2011; Whitworth et al., 2008).

Role of accumulated PINK1 in damaged mitochondria

PINK1 can act as a molecular sensor of damaged mitochondria (Geisler et al., 2010a; Matsuda et al., 2010; Narendra et al., 2010b; Vives-Bauza et al., 2010). When mitochondria are experimentally depolarized, to mimic damage, with the use of uncouplers such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) – which severely reduces the membrane potential across the IMM, which is required for TIM-mediated mitochondrial import of proteins – PINK1 no longer becomes processed by either MPP or PARL and the full-length 64-kDa form rapidly accumulates, spanning the OMM with its kinase domain facing the cytosol (Jin et al., 2010; Narendra et al., 2010b) (Poster Panel 2, lower part). This rapid accumulation of full-length PINK1 is blocked when the cells are pretreated with the

protein synthesis inhibitor cycloheximide, indicating that the accumulated full-length PINK1 is a newly translated protein and its turnover is remarkably fast (Matsuda et al., 2010; Narendra et al., 2010b). In addition, when CCCP is added together with proteasome inhibitor, the 52-kDa fragment no longer appears because the newly synthesized full-length PINK1 does not encounter the IMM proteases owing to spatial segregation (Narendra et al., 2010b). From this new OMM location, PINK1 selectively recruits Parkin to only those mitochondria within a cell that have incurred damage that leads to their uncoupling. When the N-terminal domain of PINK1, which contains the MTS and membrane-spanning regions, is replaced with an OMM-specific membrane anchor from another protein (optic atropy 3, OPA3), PINK1 avoids the degradation pathway and constitutively recruits Parkin to mitochondria, even without mitochondrial IMM depolarization, leading to mitophagy of healthy mitochondria (Narendra et al., 2010b).

Mutations in PINK1 that are found in PD patients and those experimentally designed to inhibit PINK1 kinase activity fail to recruit Parkin, supporting the model that Parkin recruitment is a key function of PINK1 (Geisler et al., 2010a; Geisler et al., 2010b; Matsuda et al., 2010; Narendra et al., 2010b; Vives-Bauza et al., 2010). Thus, PINK1 might serve as a flag to initiate the removal of depolarized mitochondria, which, in healthy mitochondria, is constitutively and continuously degraded by the normal membrane-potential-mediated TIM import pathway (see Poster Panel 3). However, it is still not known how PINK1 recruits Parkin to mitochondria. Some reports describe a physical interaction between PINK1 and Parkin by in vitro co-immunoprecipitation (Sha et al., 2010; Xiong et al., 2009). Other studies report the direct phosphorylation of Parkin by PINK1 using in vitro kinase assays (Kim et al., 2008; Sha et al., 2010), although this finding has been difficult to confirm in cells (Narendra et al., 2010b; Vives-Bauza et al., 2010).

What is the link between PINK1–Parkin-mediated mitophagy and PD pathogenesis?

PD is the second most common neurodegenerative disease after Alzheimer's disease. PD patients show characteristic phenotypes, such as bradykinesia, resting tremor, rigidity and postural instability owing to the selective loss of dopaminergic neurons in the pars compacta of the substantia nigra, a brain structure located in the midbrain supplying the striatum with dopamine. Although most cases of PD are believed to be caused by unknown environmental factors and genetic backgrounds,

several mutations in genes, for example, α-synuclein (Polymeropoulos et al., 1997), leucine-rich repeat kinase 2 (LRRK2) (Paisan-Ruiz et al., 2004), PINK1 (Valente et al., 2004), Parkin (Kitada et al., 1998), DJ-1 (Bonifati et al., 2003) and ATPase type 13A2 (ATP13A2) (Najim al-Din et al., 1994), have been identified as causing familial PD. Of these, mutations in α-synuclein and LRRK2 cause autosomal-dominant forms of PD through protein aggregate toxicity and gain-of-function mutations, respectively, whereas those in PINK1 and Parkin are loss-of-function mutations.

Oxidative stress and mitochondrial dysfunction have been associated with PD pathogenesis (reviewed by Arduino et al., 2011; Cardoso, 2011; Schapira, 2008). Supporting this hypothesis, PD-causing toxins, such as rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), are mitochondrial poisons that cause selective death of midbrain dopaminergic neurons. Complex 1 deficiency (Mizuno et al., 1989; Parker et al., 1989) and deletions in mitochondrial DNA (Bender et al., 2006; Kraytsberg et al., 2006) have also been identified in subtantia nigral neurons of PD patients. Although it is still not known why dopaminergic neurons appear to be more susceptible to mitochondrial damage than other tissues, one indication is that pacemaker activity causes waves of mitochondrial reactive oxygen species that might generate damage (Guzman et al., 2010).

These connections between PD and mitochondria suggest that the malfunction of the mitochondrial quality control pathway could result in the accumulation of defective mitochondria, thus leading to cell death (see Poster Panel 4). Consistent with such a housekeeping function for PINK1 and Parkin, loss-of-function mutations in PINK1 and Parkin can affect different tissues and cause different phenotypes in humans, mice and flies. However, whether the accumulation of defective mitochondria more generally underlies pathogenic mechanisms in PD remains to be elucidated.

Perspectives

Despite extensive investigation, several steps in the mitophagy pathway remain a mystery (see Poster Panel 5). One intriguing question is which substrate(s) of the PINK1 kinase activity are involved in Parkin activation. Direct phosphorylation of Parkin by PINK1 is controversial, and the previously reported substrates of PINK1, such as the tumor necrosis factor (TNF)-receptor-associated protein 1 (TRAP1) (Pridgeon et al., 2007) and serine protease HTRA2 (also known as OMI) (Plun-

Favreau et al., 2007), which are known to protect cells under stress, are not known to function in mitophagy. Presumably, substrates involved in PINK1-mediated Parkin recruitment would be located either on the OMM or in the cytosol. For Parkin, as suggested above, it has not been fully elucidated how many proteins are subject to ubiquitylation, and subsequently degradation, in a manner mediated by its ubiquitin ligase activity, and whether any of these substrates are crucial for the initiation of mitophagy. In addition, how Parkin mediates autophagy and whether Parkin-induced K48-linked ubiquitin chains, which are typically associated with proteasomal degradation, are essential remains unclear. It is also unknown whether that the K63-linked ubiquitin chains, which Parkin couples onto mitochondria, are important for mitophagy. Another important aspect is, considering that mitochondrial quality control has a crucial role in maintaining cell viability, many tissues could be expected to suffer from loss-of-function mutations in PINK1 and Parkin. Indeed, mutations in *Drosophila* Pink1 and Parkin lead to flight muscles that are particularly defective (Clark et al., 2006; Park et al., 2006), whereas in *Pink1*-knockout mice the heart is abnormal (Billia et al., 2011). Hence, why are substantia nigral neurons in humans so sensitive to loss-of-function mutations in the same genes? And finally, do PINK1 and Parkin at endogenous levels mediate mitophagy in animals? This question in particular remains technically challenging to address, but is of the utmost importance. Answers to these questions will greatly improve our understanding of this intriguing cell biology pathway, as well as yield much-needed insight into the pathogenesis of PD that might help to uncover potential drug targets for treatment or even prevention.

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