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Parkin-mediated selective mitochondrial autophagy, mitophagy: Parkin purges damaged organelles from the vital mitochondrial network

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Abstract

Cellular homeostasis is linked tightly to mitochondrial functions. Some damage to mitochondrial proteins and nucleic acids can lead to the depolarization of the inner mitochondrial membrane, thereby sensitizing impaired mitochondria for selective elimination by autophagy. Mitochondrial dysfunction is one of the key aspects of the pathobiology of neurodegenerative disease. Parkin, an E3 ligase located in the cytosol and originally discovered as mutated in monogenic forms of Parkinson's disease (PD), was found recently to translocate specifically to uncoupled mitochondria and to induce their autophagy.

Keywords

Parkin; Parkinson's disease; mitophagy; mitochondrial quality control; PINK1

Mitochondria and mitochondrial autophagy, mitophagy

One of the cellular organelles, mitochondria, can be paraphrased as a master regulator for the life of cell, due to mitochondrial functions in energy consumption and promotion of cell death, apoptosis [1]. Recent work on the biology of the autophagy has demonstrated that autophagy is one of the regulating mechanisms for mitochondrial quality control, especially as an active mechanism for elimination of damaged or excess mitochondria from the cell [2] [3] [4].

Recent studies on mammalian systems have suggested that mitochondrial elimination by autophagy is essential for variety cellular events, including the maturation of erythroid cells [5] [6] [7] and the maintenance of neuronal tissues, which may be targeted in neurodegenerative diseases [8]. Mitochondria also have a risk of causing normal cell dysfunction at any time due to problems, such as oxidative stress [9] or mutations in the mitochondrial genome [10], occurring along the metabolic pathway mediated by mitochondrial respiratory chain complexes. Thus, mitochondria need to remain functional by several mechanisms since the

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function is tightly linked to the homeostasis of cells (Figure 1). Cells may compensate for mitochondrial defects by the function of antioxidant enzymes, DNA repair, or complementing the damage through the fusion of a healthy mitochondrion with a damaged mitochondrion. Alternatively, proteins on the damaged mitochondria may be selectively degraded [11]. Furthermore, in order to prevent the problem of unsalvageable damaged mitochondria spreading within the cell, there is a mechanism to eliminate the dysfunctional mitochondria by autophagy, called mitophagy [2] [3] [4].

Mitophagy was originally found under starvation conditions, which are a trigger for the bulk autophagy process. Bulk autophagy captures cytoplasmic components simultaneously, and then degrades them to recycle amino acids resources. Organelle specific mitophagy can be seen in the maturation process of erythroid cells, which requires mitochondrial elimination. Although some observations suggest the presence of the elimination system for this dynamic organelle, the molecular mechanisms are poorly understood [12].

Recent works [4,13–16] explore how the maintenance of a functional mitochondrial network, i.e. mitochondria quality control [17], is mediated by Parkin, which is an E3 ligase originally discovered as mutated in monogenic forms of Parkinson’s disease (PD) [18]. Parkin is working to selectively recognize and eliminate damaged mitochondria from the cell by autophagy [4]. These findings illuminate the possibility of clinical application by providing a working model for the pathological state and pathogenic mechanism of PD.

1. Mitochondrial dysfunctions and Parkinson’s disease

PD is one of the neurodegenerative diseases characterized by degeneration of dopaminergic neurons of the substantia nigra in the midbrain which leads to the principal symptoms: progressive movement dysfunctions [19]. A relationship between mitochondrial dysfunction and neurodegenerative disorders has been suggested in numerous studies, especially for PD [20]. The dysfunction of mitochondria in PD patients or PD animal models is due to at least one of the following: deletion of mitochondrial DNA, accumulation of mitochondrial DNA mutations [10] or the increase of oxidative stress from reactive oxygen species (ROS) which is generated through the mitochondria-mediated metabolic pathways [9]. Further demonstrating the relationship between mitochondrial function and PD, some of the chemical toxins that induce parkinsonism [21] are inhibitors of the mitochondrial respiratory chain complexes. Additional evidence for a link between PD and mitochondria is supported by molecular studies using PD-associated genes (*PARK* genes) products. Some of the PD-associated gene products are located on or linked to mitochondria [22,23], especially PINK1 (*PARK6*) and Parkin (*PARK2*). A *Drosophila* model using *pink1* and *parkin* strains has been thoroughly characterized [24–30]. Mutant fly strains of pink1 and parkin phenocopy one another with phenotypes including the disruption of dopaminergic neurons and swollen mitochondrial morphology. Recent work suggests that PINK1 and Parkin function in the same pathway for the maintenance of mitochondrial integrity [24–26]. From genetic approaches, it was revealed that PINK1 functions upstream of Parkin in the regulation of mitochondrial dynamics [27–30].

Taken together, these lines of evidence demonstrate that the relationship between mitochondrial dysfunction and PD-pathogenic mechanism strongly suggests that the accumulation of mitochondrial damage might cause PD pathogenesis; however, the detailed mechanisms are not completely understood.

2. Cellular localization and function of Parkin

PARK2, an autosomal recessive-juvenile PD (AR-JP)-causing gene, was identified from Japanese patients with PD [18], and it is now known to be the causative gene in 10 to 15% of

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juvenile Parkinson's disease. *PARK2* gene encodes Parkin, which is an E3 ubiquitin ligase [31]. Since many substrate proteins in various subcellular locations are ubiquitinated by Parkin [32], there was no unified theory on the cellular localization of Parkin. Based on the idea that Parkin functions in the maintenance of mitochondrial integrity, we compared mitochondria in a healthy state to those in a damaged state induced by a chemical reagent, CCCP (carbonyl cyanide m-chlorophenyl hydrazone), which causes depolarization of mitochondria. Surprisingly, Parkin had drastic accumulation on the depolarized, fragmented mitochondria [4]. Parkin can be selectively recruited to individual electrochemically compromised mitochondria, which display greater Parkin accumulation than electrochemically active mitochondria (figure 2). This finding led to the hypothesis that impaired mitochondria are selectively targeted by Parkin [4].

It was unclear whether mitochondrial depolarization or the fragmentation that results from the depolarization was the signal for the translocation of Parkin to the mitochondria. Since these occur very close in time, we addressed the role of fragmentation alone. To cause fragmentation without mitochondrial depolarization in HeLa cells stably expressing YFP-Parkin, we overexpressed vMIA (viral mitochondrial inhibitor of apoptosis), a human cytomegalovirus structural protein that fragments mitochondria without inducing mitochondrial depolarization [33]. YFP-Parkin in these cells did not show mitochondrial localization, suggesting that mitochondrial fragmentation alone does not induce Parkin translocation. Next, we addressed if mitochondrial depolarization without fragmentation could target Parkin to the mitochondria. We used overexpression of Drp1Lys38Ala [34] (a mutation in dynamin-related protein 1, Drp1, which causes a inhibition of mitochondrial division) to block mitochondrial division and then induced mitochondrial depolarization with CCCP (Figure 2). Even in the absence of mitochondrial fragmentation, YFP-Parkin accumulated on the elongated mitochondria treated with CCCP. Taken together these results indicate that mitochondrial depolarization, but not fragmentation, is a signal for Parkin translocation to the mitochondria. Since CCCP also induces the disruption of a chemical potential (ΔpH) of mitochondria, we further examined the condition of Parkin recruitment. The treatment with valinomycin, which dissipates membrane potential loss, but not ΔpH, recruits Parkin onto mitochondria, whereas nigericin, which lowers pH but not membrane potential, does not recruit Parkin onto mitochondria [13]. These evidences also indicate that the translocation of Parkin is strictly regulated in a response to the electrochemical conditions of the mitochondria.

What is the function of Parkin after translocation to damaged mitochondria? If the idea that Parkin mediates the maintenance of mitochondrial integrity is true, Parkin may translocate to the damaged mitochondria to eliminate problems. Healthy mitochondria create ATP by the respiratory chain complexes during which they generate the membrane potential. Membrane potential is indispensable to the membrane structure and the functional maintenance of mitochondria, disruption of the membrane potential results in mitochondrial fragmentation [35]. Recent work by Twig et al. demonstrated that a subpopulation of depolarized and fragmented mitochondria that are removed from the mitochondrial network, are captured by autophagosomal structures which then undergo the autophagosomal/lysosomal cellular digestion system [3]. Indeed, after Parkin translocation to the damaged mitochondria, Parkin-labelled mitochondria could be eliminated from cells through an autophagy/lysosome dependent manner [4]. Co-localization of mitochondria and autophagosomes was found under mitochondrial depolarization conditions in cells expressing Parkin. In cells not expressing Parkin, most cells had little co-localization between mitochondria and autophagosomes (Figure 3). Parkin may promote autophagosome recruitment to Parkin-labelled mitochondria, which are depolarized and likely have accumulated damages. These autophagosomes, which include Parkin-labelled mitochondria, proceed to the lysosomes to be degraded. This sequential process was significantly blocked by the addition of autophagosome or lysosome inhibitors. Furthermore, Parkin failed to eliminate depolarized mitochondria [4] in mouse embryonic

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fibroblasts derived from *ATG5* gene knockout mice [36], which due to the elimination of the key component, Atg5, in the autophagy process cannot form autophagosomes. From all of these results, it was clear that the mechanism for the selective, Parkin-mediated elimination of damaged mitochondria is autophagy-dependent.

3. Remaining questions and perspective

Others and we have confirmed experiments identifying the mechanisms of Parkin-mediated selective mitophagy in mammalian system [4,13–16]; 1) Parkin has the ability to specifically recognize and localize to the damaged mitochondria, and 2) Parkin localized on damaged mitochondria induces the elimination of mitochondria using autophagy (mitophagy). The molecular mechanism of mitochondrial quality maintenance mediated by Parkin is intriguing, especially when considering the perspective of the relationship between neurodegenerative disorders and mitochondria.

The point to emphasize here is not the mitochondrial elimination by means of bulk autophagy previously observed in the starvation-state in which cytoplasmic components were simultaneously taken up (bulk mitophagy), but rather the key point is that Parkin induces the specific elimination of damaged mitochondria (selective mitophagy). This likely explains the signification of mitochondrial dysfunction and the increase in intracellular oxidative stress in PD patients and animal model. What is the significance of Parkin-mediated elimination of damaged mitochondria from the cell? We propose that Parkin naturally contributes to protection of the cell from the adverse effects of the intracellular spread of damaged mitochondria by eliminating severely damaged mitochondria from within the cell (Figure 4). Further supporting a protective function for Parkin, there are also reports that increasing the intracellular overexpression of Parkin suppresses cell death [37–39]. Parkin could protect preemptively against cell death, the worst scenario for a cell, by keeping the cell healthy through mitochondrial quality control. However, further studies described as followed for the insight of mitophagy are still on going and required.

1) Translocation mechanism of Parkin—For the mechanism of Parkin translocation to mitochondria, it has been shown that PINK1 overexpression induces Parkin translocation [30]. As mentioned above, PINK1 and Parkin function together in a same pathway to maintain mitochondrial integrity. It is logical that if PINK1, which localizes to mitochondria and functions upstream of Parkin, might be required for the Parkin function, specifically translocation to damaged mitochondria. Very recent interesting works are predicting this point [14–16,30]. These groups found independently that PINK1 is required for the Parkin recruitment to mitochondria. Moreover, PINK1 overexpression suffices to recruit Parkin to mitochondria with normal membrane potential. These observations are suggesting that the physical interaction of PINK1 and Parkin promotes the redistribution of Parkin from the cytosol to the mitochondria.

2) Parkin as an E3 ubiquitin ligase—Many patient mutations within the *PARK2* (Parkin) gene cause a decrease or complete loss of E3 ubiquitin ligase activity of Parkin protein [40]. In the animal model for PD deletion of *PARK2* causes mitochondrial dysfunction and the increase of oxidative stress.

To link this evidence to ubiquitin ligase activity of Parkin and Parkin-mediated mitophagy, we also can expect that some substrates for Parkin exist on the mitochondria. One possible candidate for mitochondrial substrate comes from *Drosophila* studies [27–29]. The mutant strains of *parkin* or *pink1* can be partially complemented by the suppression of mitochondrial fusion proteins, such as Opa1 and Mitofusin/Marf. This suggests that the pro-fission state of mitochondria is required for mitophagy and that Parkin can ubiquitinate and degrades these

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mitochondrial fusion proteins. Thus, a PINK1/Parkin pathway may regulate mitophagy process by changing mitochondrial dynamics [17], especially forcing mitochondria to an excessive fission state, which allows mitochondria to be captured by autophagosomes. Moreover, recent work supports this idea by demonstrating that the fission of mitochondria is required for the autophagic degradation of mitochondria [3]. Conflicting with above, it was shown that the suppression of *PINK1* results in the fragmentation of mitochondria [41] and induces mitochondrial autophagy, however we suggest based on an our working model that loss of PINK1 or Parkin function exacerbates accumulation of mitochondrial damage, due to defective removal of damaged mitochondria, and the ensuing excessive damage results in mitochondrial fragmentation.

3) Autophagosomes recruitment to the damaged mitochondria—Although we clearly showed that autophagic structures are recruited to Parkin-labelled, damaged mitochondria, the molecular mechanism of this is still unknown. Targeting of ubiquitin onto organelle or inclusion bodies surface is sufficient to recruit autophagic structures [42–44], and it has been suggested that p62 acts as a bridging protein between the ubiquitinated proteins/structures and autophagosomes. A recent work suggests that p62 may involve with Parkin-mediated clearance of the depolarized mitochondria; cells silenced p62 expression decrease the mitochondrial clearance upon depolarization [15]. Further prediction proposed by Vives-Bauza et al. suggests that PINK1/Parkin mediated damaged mitochondria clearance is regulated by transportation of damaged organelles to the lysosomes in a microtubule dependent manner [14]. Since many evidences are suggesting the sequential steps of mitochondrial clearance by PINK1/Parkin pathway followed by autophagic pathway, we still poorly understand the molecular mechanisms. Parkin ubiquitinates various substrates not only for the degradation by ubiquitin-proteasome pathway, but also for signal transduction [32]. Most ubiquitinated proteins that are degraded by the proteasome system are tagged by Lys48 type ubiquitin chains, whereas ubiquitinated proteins that have a Lys63 linked ubiquitin function in signal transduction [45]. Based on our findings, Parkin may function simultaneously to ubiquitinate mitochondrial dynamics proteins to induce pro-fission state by Lys48 ubiquitination and degradation, and tagging for the autophagosomes recruitment by Lys63 ubiquitination of unidentified mitochondrial proteins. More experiments are required to distinguish what role the Parkin-mediated ubiquitination plays in mitophagy.

4) Lessons from mitochondrial elimination by other pathways—Studies in yeast identified autophagy-related genes [*ATG* genes) and uncovered the mechanisms of several types of autophagy process; macroautophagy for non-selective autophagy, cytoplasm to vacuole targeting (Cvt) pathway, pexophagy, and mitophagy for selective autophagy of several proteins or organelles [46]. Very recent studies with excellent genetic screens by Okamoto et al. and Kanki et al. identified independently a mitophagy-related gene in yeast, named *ATG32* [47,48]. Atg32 protein is identified as an outer mitochondrial membrane protein, and a receptor molecule for Atg11 proteins, which is a key component for the recruitment of pre autophagic structures (PAS) to mitochondria. Atg32 also contains a conserved WXXI/L/V motif for the interaction with Atg8, which is a yeast homologue of a mammalian autophagic initiator, LC3 protein. Mammalian p62 protein also contains this motif to interact with LC3. Thus, these lines of evidence suggest that yeast and mammal may share common components for the selective mitophagy, although any mammalian homologue of yeast Atg-proteins for the selective mitophagy have not yet been identified. Recent studies reported that an outer mitochondrial membrane protein Nix/BNIP3 is required for the selective mitochondrial elimination in an autophagy dependent manner during mammalian reticulocytes maturation [5] [6] [7]. Thus Nix/BNIP3 may represent a functional homologue or a counterpart for Atg32, whereas many details in the process of mitophagy in mammalian system are still open to future studies.

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5) Endogenous mitophagy: *"Does Parkin allow cells to survive by maintaining mitochondrial health? "*—Mitochondria can promote cell death by initiating apoptosis. If cell had a serious problem that could not be resolved by autophagy, they could execute cell suicide by apoptosis to prevent the inflammation of entire tissues. What would be the case for a tissue that cannot choose cell death? One key example would be differentiated nerve cells. In patients with PD the lack of dopaminergic neurons in the substantia nigra is probably due to the relative stress placed on the mitochondria by the generation of reactive oxygen species as a byproduct in the dopaminergic route of these neurons [49] and/or the resulting toxicity of the mutated Parkin protein which may form aggregates in the cytoplasm itself [50]. This increased stress without functional mitophagy, would lead to death of the dopaminergic neurons. Moreover, since dopaminergic neurons appear to require increased Parkin function for mitochondrial quality and functionality more so than other neurons, when the Parkin system for eliminating damaged mitochondria breaks down and no longer functions the neurons probably die quickly. The above explanation does not completely explain the dopaminergic neurons dropout by PD. The ubiquitin ligase activity of Parkin is also required for the clearance of some aggregately proteins, for example alpha synuclein [51], Pael-R [52] and Parkin itself by ubiquitin proteasome system [50]. Disruption of turnover or functions of these proteins by mutated Parkin or PINK1 may also cause the demolition of cell homeostasis. This possibility also clouds studies on PD pathobiology, due to the possibility that dysfunction of protein turnover may also lead to mitochondrial dysfunctions. However these complex possible mechanisms need to be resolved by future investigations, we may have good explanations to tackle them based on our current model of a Parkin-related mitochondrial quality control. As mentioned above, mitochondria need to be maintained as functional in a cell. When cells accumulate damage (i.e. oxidized proteins, aggregates of misfolded proteins, ROS), mitochondria also gain a risk of malfunction caused by this damages. Mitochondria compensate this damage, whereas if damage overwhelms the compensation, cells may have serious problems to keep their functionality. Dysfunction of mitochondrial quality control system, such as impaired Parkin/PINK1 mediated system, also accumulates damaged mitochondria. Then if damaged mitochondria overcome the healthy mitochondrial network, cells also fall into fatal scenario. These proposing models may suggest the pathobiology of various neurodegenerative diseases including PD (Figure 5).

4. Concluding remarks

Recent progress on neurodegenerative disease and mitochondrial dysfunctions are being extended to a variety of scientific fields. Other neurodegenerative disease, such as Alzheimer disease and Huntington disease, also suggest there is a relationship between mitochondrial function and the pathobiology mechanisms. Many unsolved issues remain in this field. Nevertheless, our findings could provide future targets for the therapeutic treatments of PD as well as other neurodegenerative disorders.

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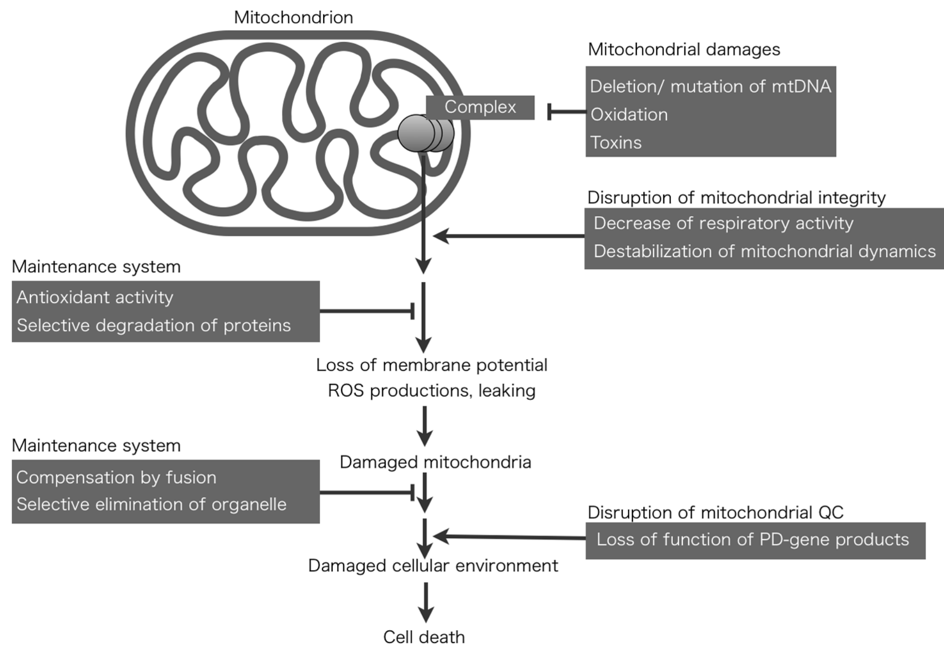

Fig 1. Mitochondria suffer cellular stress and require the maintenance systems

Many stress generated from inner (mtDNA mutations, deletions) or outer (oxidative stress, toxins) of mitochondria damage mitochondrial functions. If mitochondria failed to maintain their functions by their maintenance system, mitochondria proceed to dysfunctional state. Accumulation of dysfunctions, mitochondria should be eliminated by autophagy system, named mitophagy.

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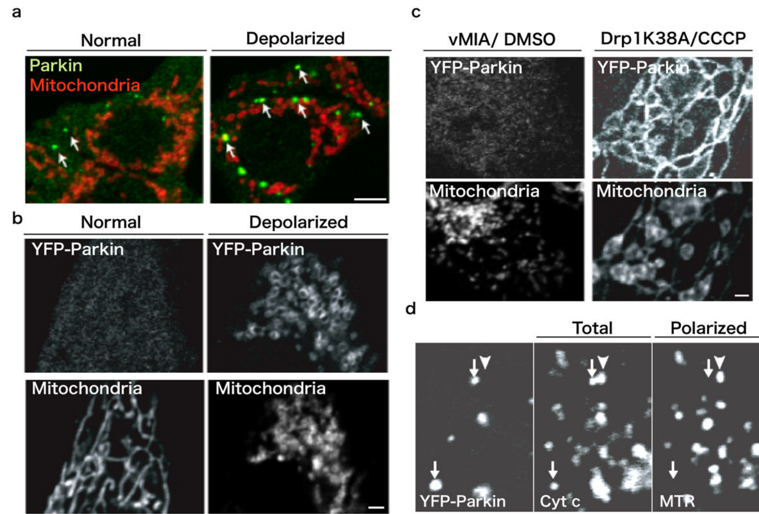

Fig 2. Parkin selectively translocates to the depolarized mitochondria

(a) Under normal conditions (left), most of the Parkin (green) is in cytosol, whereas some (arrows) are found on the fragmented mitochondria (red). When mitochondria were depolarized by CCCP (right), the cytoplasmic Parkin accumulated on the fragmented mitochondria. HEK293T cells, Scale: 5 μm. (b) In HeLa cells expressing YFP-Parkin, Parkin also accumulated on depolarized and fragmented mitochondria after CCCP treatment (right). Scale: 1 μm. (c) Only mitochondrial fragmentation by vMIA expression, whereas mitochondria maintain their membrane potential, does not signal for the Parkin translocation (left). Mitochondria blocked their division by Drp1K38A recruit YFP-Parkin upon depolarization (right). HeLa cells, Scale: 1 μm. (d) Mitochondria in MEF cells derived from Mfn1$^{-/-}$, Mfn2$^{-/-}$[53] mice, which are showing heterogenic mitochondrial membrane potential. Cytochrome c immunostaining indicates total mitochondrial images in a cell, Mitotracker Red indicates polarized (healthy, arrowhead) mitochondria. YFP-parkin accumulates only on depolarized (Mitotracker red-negative, damaged) mitochondria (arrows).

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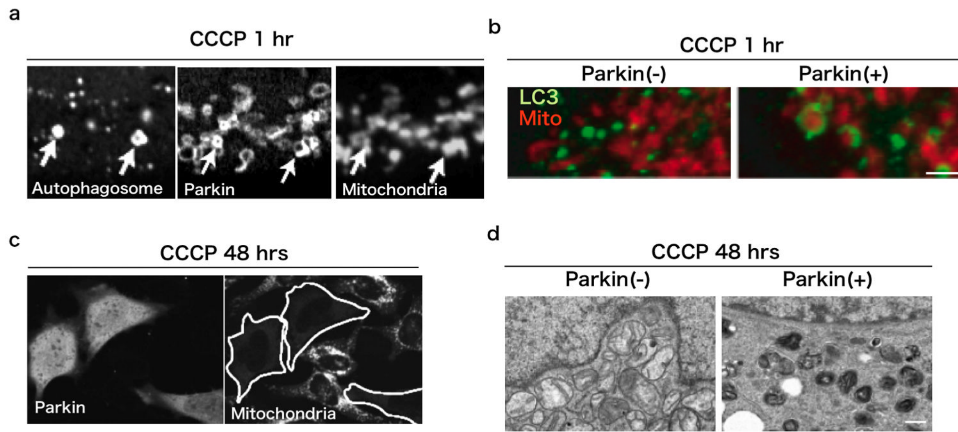

Fig 3. Parkin eliminates damaged mitochondria

(a) 1 hour after mitochondrial depolarization with CCCP, mitochondria (right) are surrounded by Parkin (center) and autophagosomes (LC3, left). (b) The recruitment of autophagosomes to mitochondria is induced by Parkin. In the absence of Parkin expression (left), depolarized mitochondria (red) are not associated with autophagosomes (LC3, green), whereas autophagosomes are more associated with mitochondria in the presence of Parkin (right). Scale: 1 μm. (c) 48 hours after mitochondrial depolarization with CCCP, mitochondria are not detectable with immunostaining. Only cells expressing YFP-Parkin (left), mitochondria (right) are completely eliminated. (d) 48 hours after mitochondrial depolarization with CCCP, mitochondria were taken up by lysosomes only in the HeLa cells expressing YFP-Parkin (right). Scale: 500 nm.

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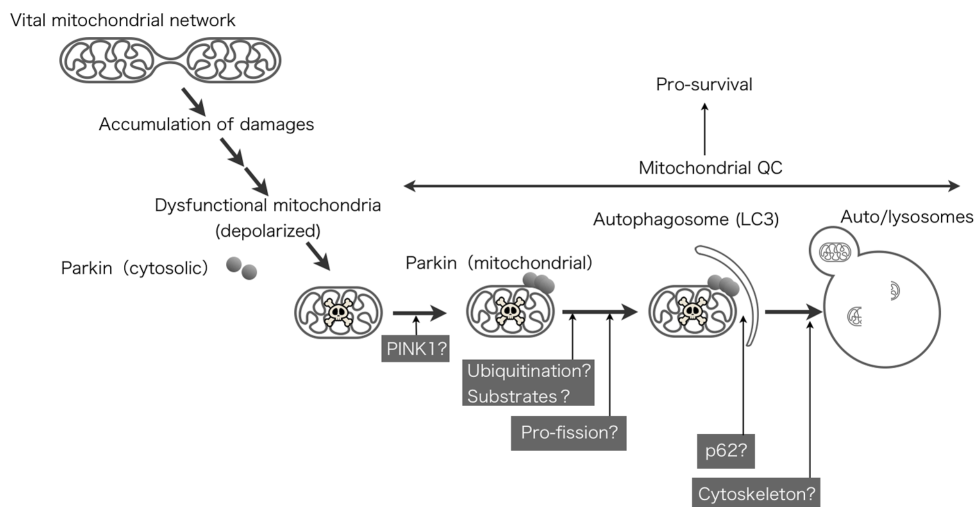

Fig 4. Working model for the Parkin-mediated mitochondrial quality control

Depolarized mitochondria are sensed by Parkin. After Parkin recruitment to the damaged mitochondria (PINK1 dependent), Parkin may ubiquitinate some substrates to degrade or tagging to proceed following process. After translocation, Parkin also recruits autophagosomes to promote mitophagy.

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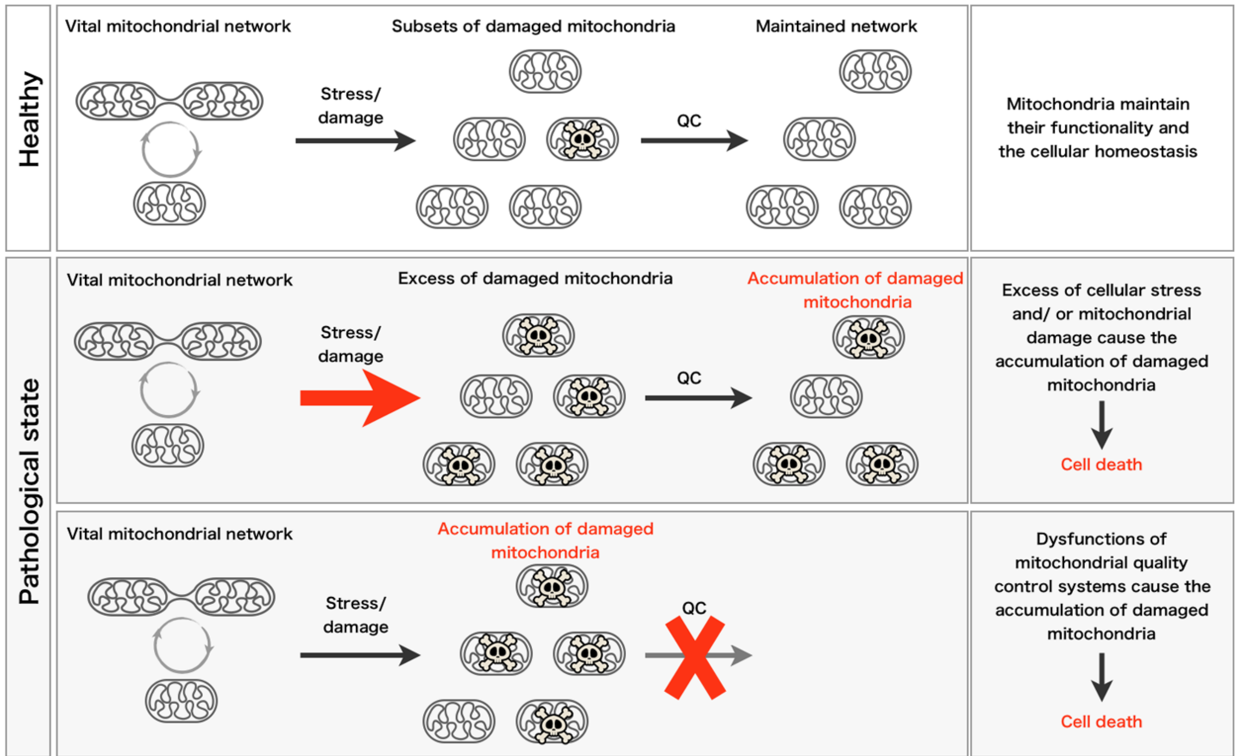

Fig 5. Working model for the Pathogenesis of PD

The vital mitochondrial network is maintained by the quality control (QC) system (top, also see in Figure 1). In pathological states of PD, cellular stress or damage cause the excess of damaged mitochondria, then overwhelm the QC system (middle). Disruption of mitochondrial QC system also accumulates damaged mitochondria (bottom). Both pathological states may cause the collapse of the cellular environment following by the cell death.