

# The ubiquitin signal and autophagy: an orchestrated dance leading to mitochondrial degradation

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## Abstract

The quality of mitochondria, essential organelles that produce ATP and regulate numerous metabolic pathways, must be strictly monitored to maintain cell homeostasis. The loss of mitochondrial quality control systems is acknowledged as a determinant for many types of neurodegenerative diseases including Parkinson's disease (PD). The two gene products mutated in the autosomal recessive forms of familial early-onset PD, Parkin and PINK1, have been identified as essential proteins in the clearance of damaged mitochondria via an autophagic pathway termed mitophagy. Recently, significant progress has been made in understanding how the mitochondrial serine/threonine kinase PINK1 and the E3 ligase Parkin work together through a novel stepwise cascade to identify and eliminate damaged mitochondria, a process that relies on the orchestrated crosstalk between ubiquitin/phosphorylation signaling and autophagy. In this review, we highlight our current understanding of the detailed molecular mechanisms governing Parkin-/PINK1-mediated mitophagy and the evidences connecting Parkin/PINK1 function and mitochondrial clearance in neurons.

**Keywords** mitochondria; mitophagy; Parkin; Parkinson's disease; PINK1  
**DOI** 10.15252/embr.201541486 | Received 2 October 2015 | Revised 22 December 2015 | Accepted 5 January 2016 | Published online 8 February 2016  
**EMBO Reports (2016) 17: 300–316**

See the Glossary for abbreviations used in this article.

## Mitochondria and Parkinson's disease

After symbiosis of  $\alpha$ -proteobacteria in pre-eukaryotic cells, mitochondria became essential organelles in eukaryotic cells. They not only generate ATP through an electron transport chain system, but also function as scaffolds for many cellular metabolic pathways such as iron-sulfur cluster biogenesis, amino acid synthesis and lipid metabolism, and regulation of apoptosis. However, in compensation for the cellular energy production and the control of cell homeostasis, mitochondria are presented with a number of

obstacles that must be overcome. One of the obstacles is the generation of ROS as a byproduct of the oxidative phosphorylation process, which damages proteins, lipids, and mitochondrial DNAs. Although minor amounts of damage to the mitochondria can be nullified by the redistribution of recycled contents via fusion/fission cycles [1] and/or by intraorganellar quality control such as proteolysis [2], excessive damage will disrupt the membrane potential across the inner membrane, eventually leading to cell death. For these reasons, dysfunctional mitochondria (which sporadically appear with some frequency) with significantly impaired membrane potential must be properly eliminated; otherwise, the condition can lead to a deterioration in cell homeostasis and potentially to the development of neurodegenerative disorders. Of note, post-mitotic neuronal cells in particular require robust surveillance systems for assessing mitochondrial quality due to their high energy demand.

PD is a highly prevalent neurodegenerative disorder (affects ~2% of those 65 years of age and older) that is clinically characterized by movement-related symptoms including rigidity, tremor, postural instability, and gait disturbance. The PD motor symptoms result from the massive degeneration of dopaminergic neurons in the substantia nigra, causing a 70–80% depletion in dopamine levels [3]. In the remaining neuronal cells, cytosolic protein aggregates called Lewy bodies, the primary structural component of which is  $\alpha$ -synuclein, can be observed [4]. Since the majority of PD is sporadic, it is quite difficult to ascertain a clear pathogenic mechanism. Despite occurring with much less frequency, however, autosomal dominant and recessive genetic forms of PD have been identified among early-onset parkinsonism patients. Over the past 15 years, genetic researchers have identified a diversity of genes that either contribute to monogenic forms of PD or contribute as risk factors to the development of the disorder [5]. For example, the genes *SNCA* ( $\alpha$ -synuclein) and *LRRK2* function in autosomal dominant PD, while the genes *PARKIN*, *PINK1*, and *DJ-1* are causal for recessive PD. To date, nearly 30 distinct chromosomal regions implicated in the complex etiology mechanism have been identified even in the familial cases of PD; however, recent molecular studies have unequivocally shown the critical roles played by Parkin and PINK1 [6].

Parkin was first identified in 1998 as a gene product mutated in recessive forms of familial parkinsonism in Japanese patients [7].

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## Glossary

AAA	ATPases associated with various cellular activities
APC/C	anaphase-promoting complex/cyclosome
ATG	autophagy related
ATP	adenosine triphosphate
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
CHCHD2	coiled-coil-helix-coiled-coil-helix domain-containing 2
CRISPR	clustered regularly interspaced short palindromic repeat
DFCP1	double FYVE domain-containing protein 1
DUB	deubiquitinating enzyme
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
GABARAP	GABA(A) receptor-associated protein
GAP	GTPase-activating protein
GTP	guanosine triphosphate
HECT	homologous to the E6AP carboxyl terminus
IBR	in-between-RING
ITC	isothermal titration calorimetry
KO	knockout
LIR	LC3-interacting region
LRRK2	leucine-rich repeat kinase 2
MDV	mitochondrial-derived vesicle
Mfn1/2	mitofusin-1/2
MIA	mitochondrial intermembrane space assembly
Miro1	mitochondrial Rho 1
Mit/TFE	microphthalmia/transcription factor E
MPP	mitochondrial processing peptidase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	mass spectrometry
mTOR	mammalian target of rapamycin
NBR1	neighbor of BRCA1 gene 1
NDP52	nuclear dot protein 52
NDUFA10	NADH:ubiquinone oxidoreductase subunit A10
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PARL	Presenilin-associated rhomboid-like
PD	Parkinson's disease
PE	phosphatidylethanolamine
PI3K	phosphatidylinositol 3-kinase
PI3P	phosphatidylinositol 3-phosphate
PINK1	PTEN-induced putative kinase 1
RBR	RING-in-between-RING
REP	repressor element of Parkin
RING	really interesting new gene
ROS	reactive oxygen species
shRNA	small hairpin RNA
TAX1BP1	Tax1 (human T-cell leukemia virus type I) binding protein 1
TBK1	TANK1-binding kinase1
TFEB	transcription factor EB
TIM	translocase of the inner mitochondrial membrane
TOM (TOMM)	translocase of outer mitochondrial membrane
UBD	ubiquitin binding
Ubl	ubiquitin-like
UBR	ubiquitin protein ligase E3 component n-recognin
ULK1	unc-51 like autophagy activating kinase 1
UPR <sup>mt</sup>	mitochondrial unfolded protein response
USP	ubiquitin-specific protease

*PARKIN* encodes a 465-aa protein that belongs to the E3 ubiquitin ligase enzyme family [8–10]. To date, more than 100 loss-of-function mutations have been identified in the *PARKIN* gene with many mutations prevalent among familial cases in which onset of the disorder occurs in those younger than 30 years old.

The second most common gene product associated with autosomal recessive juvenile parkinsonism is PINK1, which was initially identified in 2001 from an Italian family [11] and then later described in several European families [12]. PINK1 is a 581-aa protein expressed ubiquitously [13]. According to the amino acid sequence, PINK1 harbors a mitochondrial targeting sequence followed by a hydrophobic segment at the N-terminus and a large kinase domain at the C-terminus.

Potential linkage between mitochondrial dysfunction and PD emerged from multiple lines of evidence [14]. For example, uptake of MPTP (a byproduct of “synthetic heroin”) [15], paraquat [16], and rotenone [17] (chemical herbicide and pesticide), all of which cause a deficiency in mitochondrial respiratory chain function, led to parkinsonian symptoms in humans and animal models. Recently, another causative gene of the autosomal dominant form of familial PD, *CHCHD2*, was identified from a genome-wide linkage analysis [18]. The gene encodes a 151-aa protein with twin CX<sub>9</sub>C motifs that localize the protein in the mitochondrial intermembrane space. Interestingly, the mitochondrial intermembrane space assembly (MIA) pathway involved in import of the precursor and subsequent maturation of Mix17 (the yeast homologue of CHCHD2) is related to electron transfer to the respiratory chain complex [19,20]. Although further functional studies are required, CHCHD2 is the first mitochondrial causal gene product, mutation of which can affect mitochondrial integrity through functional depression of the respiratory complexes.

In addition, *Drosophila* studies have shown that the loss of either *parkin* or *pink1* function results in phenotypes similar to mitochondrial impairments such as muscle degeneration and male sterility [21–24]. Overexpressed Parkin can partially compensate for some *pink1* loss-of-function (i.e. mitochondrial abnormality). This suppression is not derived from the simple protection of apoptotic cell death. Mutant flies that have lost both *parkin* and *pink1* do not exhibit stronger phenotypes than those seen with either mutant alone. These *in vivo* studies strongly suggest that Parkin and PINK1 function in a common pathway that maintains mitochondrial integrity.

### Cytosolic E3 ligase Parkin is recruited to damaged mitochondria for autophagic degradation

Ubiquitin plays pivotal roles in many different cellular functions including protein degradation, signaling, endocytosis, and the immune system. While E1s (ubiquitin-activating enzymes) and E2s (ubiquitin-conjugating enzymes) activate ubiquitin via thioester intermediates, E3 ubiquitin ligases, the final enzymes in the ubiquitination cascade, transfer the ubiquitin moiety from the E2 to a lysine residue on protein substrates [25–27]. While at least 1 E1 and about 40 E2s are encoded in the human genome, the abundance and diversity of E3 ligases (roughly 500–1,000, but we cannot determine the exact number because of subset diversity) is striking with an even greater number of proteins thought to undergo ubiquitination. This abundance indicates that E3 ligases are the key factors in providing the substrate specificity essential to the ubiquitin network. Because ubiquitin itself can also serve as a ubiquitination site to form polymeric ubiquitin chains, different chain linkages can be formed. MS-based proteomic and biochemical approaches showed that all lysine residues (K6, K11, K27, K29, K33, K48, and K63) [28] and an N-terminal methionine residue [29] can serve as

ubiquitination sites. In fact, the K48-linked chain, the most common and abundant ubiquitin chain, has been identified as a signal for proteasome-mediated degradation, while the K63-linked chain induces clearance of the substrate protein via the autophagy-lysosome pathway [30–32] and activates the DNA damage response [33]. The E3 ligases are classified into at least four types: HECT (homologous to the E6AP carboxyl terminus), RING (really interesting new gene), U-box, and RBR (RING-in-between-RING). Although RBR-type ligases contain two RING domains (RING1 and RING2), they receive ubiquitin on a cysteine in the RING2 domain via a thioester intermediate like HECT, functioning as HECT/RING hybrids [34]. Another feature of RBR ligases is that their ligase activities are normally autoinhibited.

Parkin is an RBR-type E3 ligase that normally localizes in the cytosol as an autoinhibited form. Parkin was first proposed to ubiquitinate misfolded proteins for proteasome-dependent degradation. While this hypothesis may have explained the accumulation of protein aggregates in neuronal cells of PD patients, it could not explain the relationship between Parkin and mitochondrial integrity observed in the *Drosophila* studies.

In 2008, Richard Youle's group reported that cytosolic Parkin is recruited to damaged mitochondria for its degradation through an autophagy pathway, which undoubtedly opened a new research field termed Parkin-mediated mitophagy [35]. When mitochondria lose their membrane potential following the addition of a chemical compound like CCCP (see Box 1), cytosolic Parkin is recruited to

### Box 1

We would like to highlight the various tools that have been utilized so far in elucidating the mechanism of Parkin-/PINK1-mediated mitophagy.

#### 1 Chemical compounds triggering Parkin translocation:

##### a. CCCP

Carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) is an ionophore that disrupts the mitochondrial proton gradient by allowing protons to cross lipid bilayers. CCCP is the chemical compound most frequently used to trigger PINK1 accumulation following Parkin translocation. However, CCCP affects lysosomal and Golgi pH and LC3 lipidation in a Parkin-independent manner [158–161]. Therefore, interpretation of data on autophagy function during CCCP-induced mitophagy should be carefully considered.

##### b. Valinomycin

Valinomycin is a potassium-selective ionophore that accelerates the transport of potassium ion across the membrane. In the presence of valinomycin, mitochondria take up potassium at the expense of the proton gradient, resulting in dissipation of the membrane potential. Similar to CCCP, valinomycin induces extensive PINK1 accumulation and Parkin translocation.

##### c. Antimycin A

Antimycin A binds to the Qi site of cytochrome c reductase in complex III and inhibits electron transfer from cytochrome b to cytochrome c, which leads to a collapse in the membrane potential [162]. Antimycin A alone or in combination with oligomycin (an inhibitor of the F<sub>0</sub>F<sub>1</sub> ATP synthase) also induces Parkin translocation.

##### d. Paraquat

Paraquat is a chemical herbicide that generates ROS. Exposure to paraquat increases the risk for Parkinson's disease. Cultured cell studies showed that 2 mM paraquat treatment for 24 h [35] or 10 mM paraquat treatment for 6 h [163] induces Parkin translocation.

##### e. Rotenone

Rotenone is a complex I-specific inhibitor that shuts off the supply of electrons to complex II. Rotenone competes with MPP<sup>+</sup> (the oxidized product of MPTP that causes parkinsonism) for complex I activity [164]. Rotenone induces PINK1 accumulation on the mitochondria [62].

#### 2 Mito-KillerRed

A genetically encoded photo-sensitizer named KillerRed is a dimeric red fluorescent protein developed from the hydrozoan chromoprotein anm2CP [165]. Photo-activation of KillerRed with light at 540–580 nm can generate ROS. Therefore, mitochondria-targeted KillerRed (mito-KillerRed) can induce ROS-mediated damage in the matrix in a spatiotemporally controlled manner that more closely mimics physiological conditions than CCCP treatment. Photo-activating mito-KillerRed in a specific area was reported to induce local Parkin recruitment [156,166–168].

#### 3 ΔOTC

ΔOTC is an ornithine transcarbamylase mutant lacking 85 aa (residues 30–114) from the N-terminus and is a model substrate for activating the mitochondrial unfolded protein response (UPR<sup>mt</sup>) in mammalian cells [169] and in flies [170]. It can be targeted to the mitochondria as well as wild-type OTC, but forms insoluble aggregates in the matrix. When expressing ΔOTC in cultured cells, PINK1 accumulation and subsequent Parkin translocation were observed without the loss of membrane potential, probably due to clogging of the TIM23 translocation channel [171].

#### 4 Mito-Keima

Keima is a novel fluorescent protein probe used to detect autolysosome formation. While Keima has an emission peak at 620 nm, the excitation spectrum varies under different pH conditions (Ex 440 nm in a neutral state and Ex 586 nm in an acidic state). Therefore, Keima fused with a mitochondrial matrix targeting signal (mito-Keima) in conjunction with fluorescent microscopy can function as a reporter for delivery of damaged mitochondria to the lysosome [172]. Furthermore, when combined with FACS, a small amount of mitophagy can be monitored quantitatively in an unbiased way [131,147].

#### 5 Phos-tag PAGE

Phos-tag is a molecule that selectively binds to phosphorylated ions [173]. SDS-PAGE containing an acrylamide-pendant Phos-tag ligand (Phos-tag PAGE) can separate phosphorylated and non-phosphorylated forms of proteins such as PINK1, Parkin, and ubiquitin [62,68].

#### 6 Recombinant Ser65-phosphorylated ubiquitin

Using the amber suppression technique, it is possible to incorporate phosphoserine (as well as its non-hydrolyzable analog) at a desired position in the target protein in bacterial cells, for example, Ser65-phosphorylated ubiquitin [174–176]. Thus, milligram quantities of the recombinant protein free from contamination of the non-phosphorylated form can be prepared, as previously used to demonstrate Parkin activation [79].

#### 7 Phosphorylated ubiquitin antibody

Novel antibodies recognizing Ser65-phosphorylated ubiquitin, but not non-phosphorylated ubiquitin, confirm that the phosphorylated ubiquitin signal increases with mitochondrial stress in a PINK1-dependent manner under endogenous conditions. Importantly, the signal is detected in human post-mortem brain sections of the substantia nigra and increases with age and disease. Therefore, this can be used as a potential biomarker for PD [177].

the damaged mitochondria. Once Parkin reaches the mitochondrial outer membrane, its E3 activity is fully activated [36] and various mitochondrial outer membrane proteins are ubiquitinated [37] (the detailed mechanism is discussed later). Because of the robust ubiquitination, p97 and proteasomes are also recruited to mitochondria and a portion of the outer membrane proteins, such as Mfn1/Mfn2, is thought to be degraded via the proteasome [38]. Mfn1/Mfn2 are integrated in the outer membrane exposing the large GTPase domain to the cytosol for involvement in the mitochondrial fusion [39]. While the necessity of proteasomal degradation for downstream autophagy activity and clearance of damaged mitochondria remains a matter of debate [40,41], rapid poly-ubiquitination of Mfn1/Mfn2, which occurs within 1 h of CCCP treatment, is important to segregate damaged mitochondria from the healthy mitochondrial network [42]. Hexameric AAA+ ATPase p97 (also known as VCP) is a multifunctional protein primarily involved in ubiquitin-dependent proteolysis [43]. While different cofactors that bind to p97 specify the distinct localization and function [44], the Npl4/Ufd1 heterodimer contributes to some degree to the degradation of outer membrane proteins during mitophagy [45,46]. In yeast, another cofactor termed Vms1 was reported to recruit Cdc48 (yeast homologue of p97) to mitochondria following mitochondrial oxidative stress [47]. Vms1 is highly conserved from yeast to humans, but mammalian Vms1 function especially in Parkin-mediated mitophagy remains unclear. In addition to Mfn1/Mfn2, Miro1 (involved in mitochondrial transport along microtubules), MitoNEET/CISD1 (a 2Fe-2S containing protein), and TOMM70 (an import receptor of mitochondrial precursor proteins), all of which are integral mitochondrial proteins, were identified in quantitative proteomic approaches as proteins that are significantly reduced in response to Parkin recruitment [41,48]. Miro1 contains GTPase and EF-hand domains that are crucial for connecting mitochondria to the microtubule network through associations with its binding partners, Milton and kinesin heavy chain. Studies using primary neurons revealed that rapid Miro1 degradation arrests microtubule-dependent mitochondrial trafficking, thereby preventing damaged mitochondria from moving, especially to the axon terminal [49]. Although Mfn1/Mfn2 and Miro1 were reported to be poly-ubiquitinated by endogenous Parkin [38,49–52], the physiological importance underlying degradation of the other Parkin substrates is unknown. Because a broad group of outer membrane proteins are ubiquitinated by both endogenous and exogenous Parkin following CCCP treatment [37], it is clear that Parkin does not possess rigorous substrate specificity. This characteristic is compatible with a positive feedback ubiquitination amplification model (discussed later). Prolonged CCCP treatment (for 24–48 h) selectively degrades the mitochondria in an autophagy-dependent manner. Since the first report by Richard Youle's group, many other groups have confirmed Parkin recruitment to damaged mitochondria and have elucidated the molecular mechanism leading to Parkin recruitment and the subsequent clearance of mitochondria.

### PINK1 as a mitochondrial stress sensor

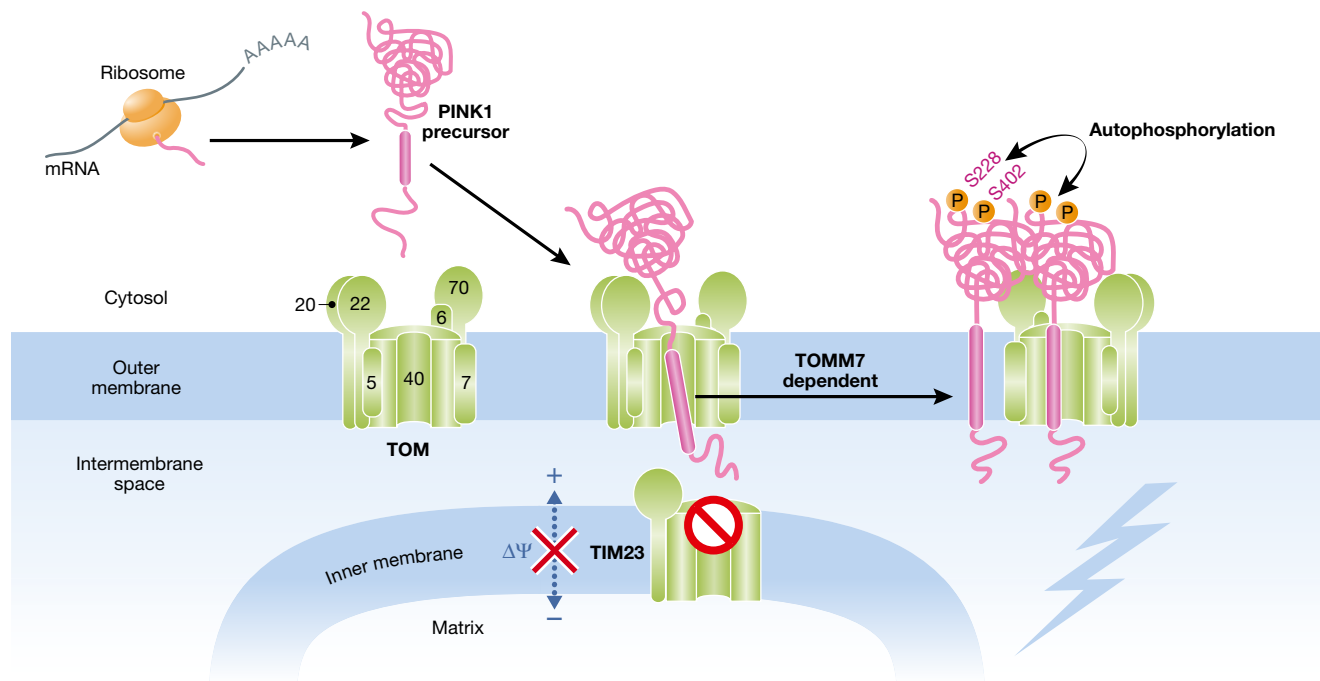
PINK1 functions upstream of Parkin recruitment [36,53–56]. Following disruption of the mitochondrial membrane potential, the

serine/threonine kinase PINK1 switches the import pathway from the inner membrane to the outer membrane where associations with the TOM complex stabilize PINK1 on the outer membrane [57] (Fig 1). The TOM complex is a major protein translocator complex consisting of multi-subunits [58,59]. Knockout (KO) studies revealed that one of the subunits of the TOM complex, TOMM7, is essential for inserting PINK1 into the outer membrane via the TOM complex (Fig 1). TOMM7-KO cells though retain normal protein import efficiencies into the matrix or inner membrane from the cytosol [60]. When PINK1 is stabilized on the outer membrane, it forms a large complex (~700 kDa on blue-native PAGE) comprising the TOM machinery and at least two PINK1 molecules [57,61]. PINK1 dimers contribute to the intermolecular autophosphorylation of residues S228 and S402 that will be conformationally close to one another [62,63] (Fig 1). Inhibiting autophosphorylation prevents Parkin translocation despite accumulation on the outer membrane, suggesting that PINK1 is not only quantitatively but also qualitatively regulated. Unexpectedly, residues 34–90, rather than the PINK1 transmembrane segment, are required for outer membrane localization in damaged mitochondria. This unique signal presumably interacts with the TOM complex when the primary targeting signal is blocked [64]. The “association” of PINK1 with the TOM complex though is dispensable for Parkin recruitment to the mitochondria since ectopically targeted PINK1 can recruit cytosolic Parkin to other organelle membranes, such as the peroxisome or lysosome, that lack the TOM machinery [57]. Furthermore, this also suggests that mitochondrial proteins (if they are specifically on mitochondria) are dispensable; the exceptions are PINK1 and the import machinery that is essential for PINK1 outer membrane localization for Parkin translocation.

### Molecular mechanism of Parkin recruitment

What molecular mechanism drives Parkin recruitment to the damaged mitochondria? The answer of this key question has been solved incrementally. The first clue was that PINK1 kinase activity is essential for Parkin recruitment. Kinase-inactivated PINK1 cannot recruit Parkin even when it accumulates on the mitochondria. Therefore, Parkin stable recruitment is mediated by the enzymatic (phosphorylation) activity of PINK1. The second clue came from the fact that Parkin E3 ligase activity itself is also essential for Parkin stable recruitment. As Parkin is a member of the RBR-type E3 ligases, a ubiquitin molecule is transferred from the E2 to the conserved and catalytic Cys431 of Parkin via thioester formation before loading ubiquitin onto the substrate. A Parkin C431A mutation inhibits stable translocation to the damaged mitochondria [65–67]. Furthermore, monitoring ubiquitination of N-terminally fused GFP as a pseudosubstrate revealed that pathogenic mutations such as K161N, K211N, and T240R that impede Parkin translocation to mitochondria also disable the E3 ligase activity [36,67].

In 2012, the Hattori and Muqit groups independently reported that PINK1 phosphorylates Ser65 in the Ubl (ubiquitin-like) domain of Parkin. Shiba-Fukushima *et al* [68] monitored the phosphorylation status of Parkin by phos-tag PAGE (see Box 1) following disruption of the membrane potential and found that the Parkin Ser65 residue was phosphorylated in a PINK1-dependent manner.



**Figure 1. PINK1 accumulation in the outer membrane of the damaged mitochondria.**

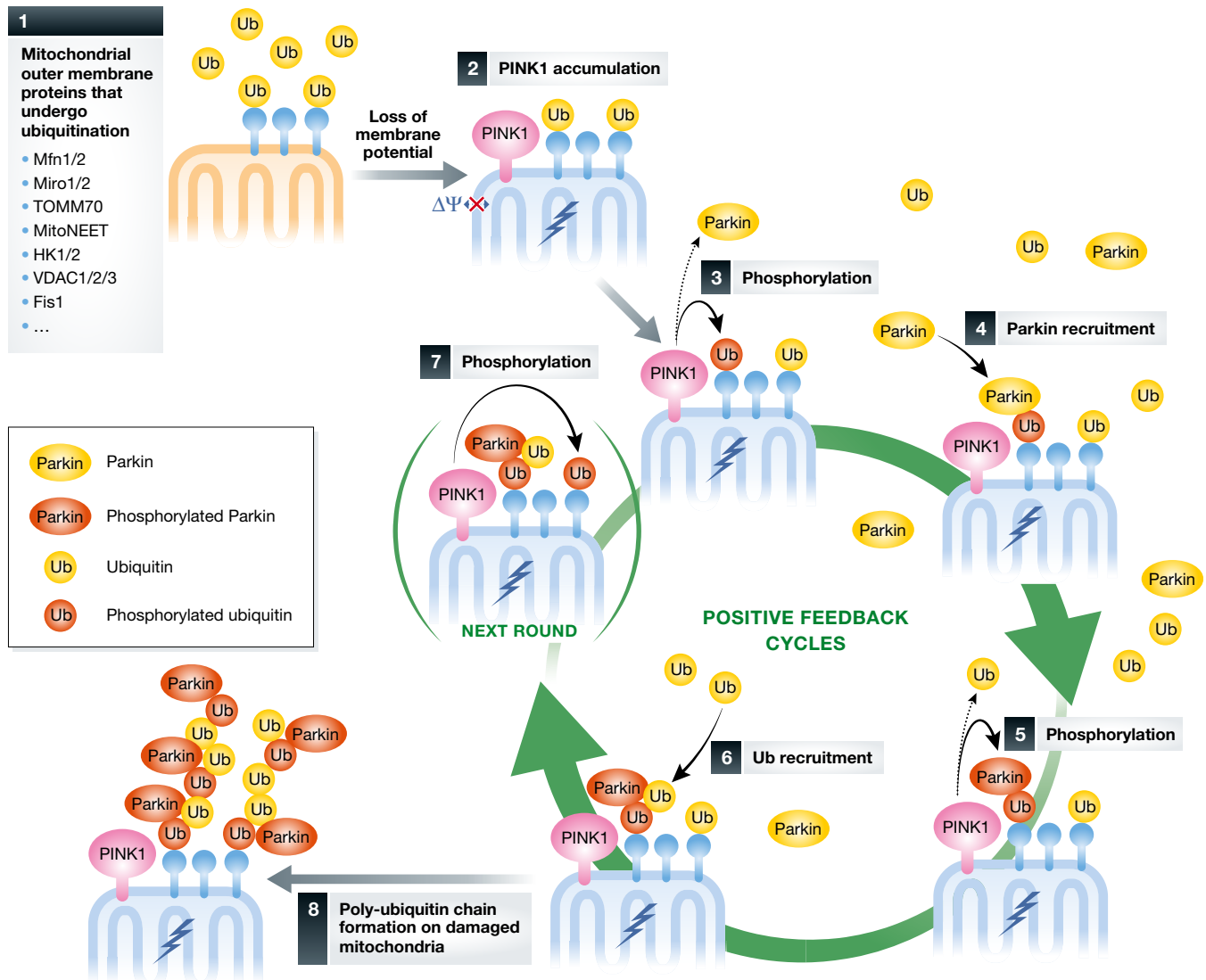
The newly synthesized PINK1 precursor is targeted to the damaged mitochondria. Because of the loss of membrane potential, the PINK1 precursor is not allowed to enter the inner membrane via the TIM23 complex. Instead, the PINK1 precursor is inserted into the outer membrane through the TOM complex in a TOMM7-dependent manner. PINK1 stabilized on the outer membrane then forms a large complex with the TOM complex and undergoes intermolecular autophosphorylation at residues S228 and S402. 5, 6, 7, 20, 22, 40, and 70 denote TOMM5, TOMM6, TOMM7, TOMM20, TOMM22, TOMM40, and TOMM70, respectively.

Kondapalli *et al* tested whether catalytically active PINK1 [69] directly phosphorylates various PD-associated proteins *in vitro* and identified Parkin Ser65 as a PINK1 phosphorylation site. Muqit and our groups also observed that phosphorylated Parkin enhances E3 ligase activity, consistent with the idea that Parkin ligase activation and recruitment are coupled [70,71]. However, observations that Parkin translocation was not completely inhibited by a phosphorylation-deficient S65A mutation raised the possibility that another PINK1 substrate was needed for Parkin translocation.

Early in 2014, three groups independently reported that PINK1-mediated phosphorylation of ubiquitin at Ser65 plays an important role in Parkin activation [72–74]. Similar findings were reported later [67,75]. Phosphorylation and ubiquitination are two major post-translational modifications, and this finding represented the first example in which ubiquitin, a post-translational modifier itself, also underwent phosphorylation [76]. Kane *et al* first found that when overexpressed, all Ser/Thr residues conserved in human and *Drosophila* Parkin and even the whole Ubl domain where the Ser65 residue resides are not essential for the recruitment, and then identified a PINK1-dependent phosphorylated ubiquitin peptide by MS. Kazlauskaitė *et al* and Koyano *et al* also utilized MS approaches. The latter group built on structural and sequence similarities between ubiquitin and the Parkin Ubl domain to facilitate MS-based confirmation of ubiquitin Ser65 phosphorylation. These groups reached the following conclusions: Endogenous PINK1 phosphorylates ubiquitin at Ser65 and phosphorylated ubiquitin activates Parkin E3 ligase activity [67,75,77]. Both overexpression of a

phosphorylation-deficient S65A ubiquitin mutant [74] and replacement of endogenous ubiquitin with a S65A mutant [78,79] inhibited Parkin translocation, indicating that ubiquitin phosphorylation by PINK1 is essential for Parkin stable recruitment. Furthermore, since PINK1 phosphorylates poly-ubiquitin chains [67,75,80] and phosphorylated Parkin tightly binds phosphorylated poly-ubiquitin chains [67,78,79], a positive feedback ubiquitination cycle that accelerates both Parkin translocation and poly-ubiquitin chain formation on the surface of the damaged mitochondria was proposed [67,79] (Fig 2). A few hours of depletion of the mitochondrial membrane potential can recruit most of the cytosolic Parkin onto the mitochondria even when overexpressed. Only a positive feedback amplification process can explain this robust translocation mechanism.

Several deubiquitinating enzymes (DUBs) that counteract Parkin E3 ubiquitin ligases by catalyzing the removal of ubiquitin from substrates have been reported to regulate mitophagy. USP30 is a mitochondria-anchored DUB that specifically cleaves Parkin-generated K6- and K11- and K63-linked ubiquitin chains on mitochondria [67,81] and *in vitro* [75]. USP30 overexpression inhibits mitophagy, whereas USP30 shRNA or overexpression of enzymatically inactive USP30 enhances mitophagy, indicating that USP30 directly opposes Parkin function. Moreover, knockdown of *Drosophila* USP30 largely rescued mitochondrial morphology defects in the flight muscles caused by *parkin* or *pink1* mutants and reversed defects in dopamine levels against paraquat treatment *in vivo* [82]. USP8, which normally regulates endosomal trafficking [83], has also been proposed as a regulator of Parkin recruitment to mitochondria



**Figure 2. Positive feedback ubiquitination cycles induced by Parkin and ubiquitin chain formation on damaged mitochondria.**

Although most of the ubiquitin diffuses in the cytosol, a fraction should reside on the outer membrane of healthy mitochondria since the ubiquitin system also contributes to the turnover of mitochondrial proteins under normal conditions [178] (Step 1). Following dissipation of the membrane potential, PINK1 is stabilized on the damaged mitochondria (Step 2). PINK1 can then phosphorylate the ubiquitin that is conjugated to the mitochondrial proteins, or PINK1 may also phosphorylate Ser65 of cytosolic Parkin (Step 3). Of note, phosphorylated ubiquitin stably stays on the mitochondria because hydrolysis of phosphorylated ubiquitin chain by DUBs is impaired [75]. Through higher affinity with phosphorylated ubiquitin, cytosolic Parkin is recruited to and retained on the mitochondria (Step 4). PINK1 further phosphorylates Parkin on the mitochondria. PINK1 may also phosphorylate Ser65 of cytosolic ubiquitin (Step 5). The fully activated, phosphorylated Parkin can then elongate ubiquitin chains or generate a new ubiquitinated substrate from cytosolic-free ubiquitin. In other words, cytosolic ubiquitin is recruited to the mitochondria through a ubiquitination reaction by activated Parkin (Step 6). The ubiquitin on the mitochondrial substrate is phosphorylated by PINK1 (Step 7 is the next round of the Step 3). Positive feedback amplification cycles (Steps 3–7) result in both Parkin and ubiquitin recruitment to and poly-ubiquitin chain formation on the damaged mitochondria (Step 8).

[84]. siRNA knockdown of USP8 impaired the recruitment of Parkin to damaged mitochondria and up-regulated Parkin levels [84]. While USP8 deubiquitinates the K6-linked ubiquitin chain on Parkin, it does not hydrolyze ubiquitinated chains on mitochondrial substrates. Overexpression of USP15 was reported to reduce K48- and K63-linked ubiquitin chains on damaged mitochondria in response to mitophagy [85]. The antagonistic relationship between Parkin and USP15 was further investigated in a fly model *in vivo*. The physiological roles of DUB, in particular how Parkin and DUBs

coordinately regulate mitochondrial fidelity, however, remain to be fully investigated.

### Structural insights into Parkin E3 activity

Parkin structurally consists of Ubl, RING0 (also referred to as unique Parkin domain/UPD), RING1, IBR (in-between-RING), and RING2 domains. Crystal structures including full-length rat Parkin

[86] and Ubl-deleted human Parkin [87,88] revealed an autoinhibited state. The Ubl domain has a similar structural fold to ubiquitin and the Ser65 residue phosphorylated by PINK1 is also conserved. RING0, the zinc finger fold unique to Parkin, is connected to the Ubl domain through a structurally disordered linker, the length and sequence of which diverge across multiple species. Three additional zinc finger fold domains RING1, IBR, and RING2 form a minimal functional unit for RBR-type ligases. A conserved two-turn helix linker termed REP (repressor element of Parkin) is found between the IBR and RING2 domains. Based on the crystal structure, the E2 binding site in the RING1 domain is occluded by REP and the catalytic Cys431 in the RING2 domain where ubiquitin is transferred from E2 enzyme is buried in the RING0 domain. Parkin E3 ubiquitin ligase activity was likewise reported to be normally autoinhibited by the N-terminal Ubl domain [89]. This finding was consistent with the improved human Parkin structure following deletion of the linker between Ubl and RING0 [90,91]. The Ubl domain mainly interacts with the  $\alpha$ -helix (261–274 aa) of the RING1 domain, which spatially blocks E2 enzyme accessibility.

Because complete activation of the Parkin E3 ligase necessitates binding of phosphorylated ubiquitin, it is likely that this binding triggers a conformational change in Parkin. However, the interaction between Parkin and phosphorylated ubiquitin does not utilize the catalytic Cys431 of Parkin or the C-terminal glycine residue in ubiquitin, indicating that phosphorylated ubiquitin is not loaded onto Parkin Cys431 for activation [67,72,75,79]. How does phosphorylated ubiquitin bind to Parkin? Recently, five independent groups concurrently identified Parkin amino acid residues that are crucial for phosphorylated ubiquitin binding [90–94]. A crystal structure of the *Pediculus humanus* (a species of louse that infects humans) Parkin complexed with phosphorylated ubiquitin via disulfide covalent linkages showed that A152, H304, A307, and Y314 residues of *P. humanus* Parkin (corresponding to K151, H302, R305, and Y312 residues in human Parkin, respectively) form a pocket that interacts with the phosphorylated serine in ubiquitin [93]. Site-specific photo-crosslinking methods combined with MS and computational modeling also identified the same binding surface between phosphorylated ubiquitin and full-length rat Parkin [94]. When the phosphate-binding pocket is mutated, the affinity for phosphorylated ubiquitin and Parkin translocation onto the damaged mitochondria were impeded. Moreover, the L283P, G284R, and C352G pathogenic Parkin mutations in the IBR and RING1 domains inhibited interactions with phosphorylated ubiquitin [94]. One of the dynamic conformational changes is that a kinked helix at Gly319 in inactivated Parkin rearranges to form a straight long  $\alpha$ -helix when binding to phosphorylated ubiquitin. This affects the position of the IBR domain, which will in turn stretch the IBR-REP linker and unlock the inhibitory interactions between the E2 binding region and the REP, and the interactions between Cys431 and RING0 [93]. Furthermore, the Parkin conformational change upon phosphorylated ubiquitin binding also enhances Parkin Ser65 phosphorylation by PINK1 [91–93]. Mutational analyses, NMR, and ITC-based experiments showed that binding phosphorylated ubiquitin promotes dissociation of the Ubl domain from the  $\alpha$ -helix (amino acids 261–274, the opposite side of the phosphorylated ubiquitin binding region) in the RING1 domain. This further contributes to enhanced affinity for the E2 enzyme [79,90–93]. In cells,

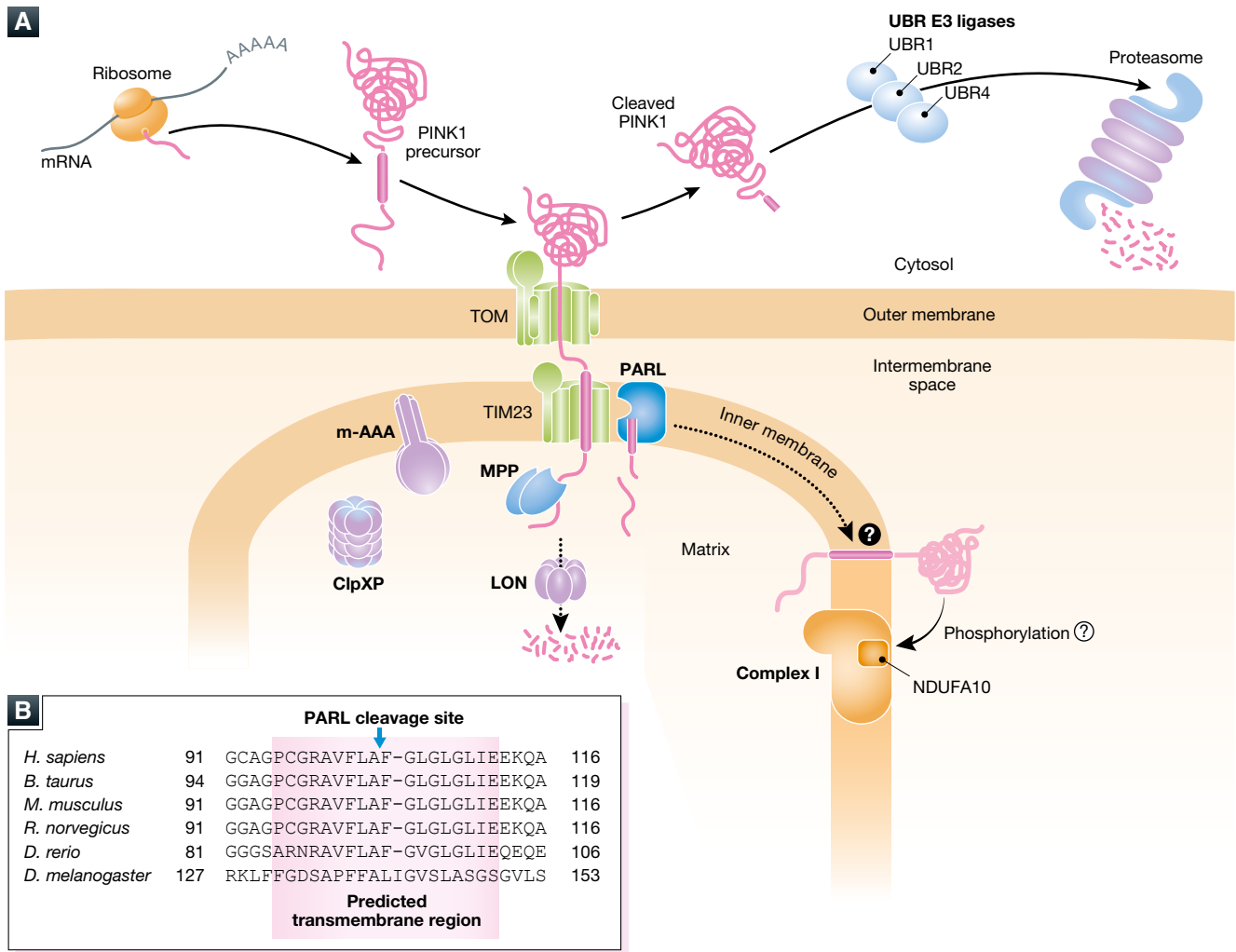
phosphorylation-dependent dissociation of the Ubl domain may have additional roles in mitophagy (e.g. association of the proteasome through Rpn13) [95].

In summary, despite structural and sequence similarities between ubiquitin and the Ubl domain of Parkin, they function antagonistically. Binding phosphorylated ubiquitin to Parkin and dissociation of phosphorylated Ubl from the Parkin core allosterically induce a Parkin conformational change from the intramolecular inhibited state to the maximal E3 active state, which was established and quantitatively measured by proteomics [79].

In sharp contrast to Parkin, there is limited information on the PINK1 structure. PINK1 possesses several motifs conserved among protein kinases. Homology modeling has been used to aid PINK1 structural prediction [96,97], but no NMR or crystal structures of PINK1 from any species have been solved. Because PINK1 efficiently phosphorylates ubiquitin and the Parkin Ubl domain, it should recognize the ubiquitin-fold as a substrate. Furthermore, Ubl or ubiquitin I44A mutations, or replacement of amino acid residues around the I44 patch of ubiquitin with an unnatural amino acid harboring a bulky side chain, inhibited Ser65 phosphorylation, suggesting that this region is functionally important [93,94].

### Ubiquitin system for PINK1 degradation

As mentioned above, PINK1 is the key factor in activating the ubiquitin system by phosphorylating both Parkin and ubiquitin in mitophagy. In addition to this, the ubiquitin system is also essential for rapid degradation of PINK1 in healthy mitochondria. PINK1 has a classical N-terminal mitochondrial targeting sequence followed by a hydrophobic transmembrane segment. Once PINK1 is synthesized on cytosolic ribosomes, it is targeted to the mitochondria. The TOM complex recognizes the N-terminal mitochondrial targeting sequence and allows the substrate to cross the outer membrane. N-terminal presequence-containing substrates are then transferred to the TIM23 complex (protein translocator in the inner membrane) for crossing or entering the inner membrane [98]. In the case of the PINK1 precursor, the N-terminal presequence is cleaved by MPP in the matrix, and the following hydrophobic segment is captured by the TIM23 complex, inserted into the inner membrane, and subjected to the second cleavage by PARL between A103 and F104 [99–103]. Interestingly, PARL cleavage releases the cleaved PINK1 back to the cytosol where the newly exposed N-terminal phenylalanine residue is recognized by UBR E3 ligases [104] and rapidly degraded by the proteasome via the N-end rule pathway [105] (Fig 3A). Protein translocation via the TOM/TIM23 pathway is believed to occur at the contact site where the distance from the outer membrane to the matrix is ~50–60 amino acid residues long [106,107]. Consequently, the large C-terminal kinase domain remains outside the mitochondria if the transmembrane segment is arrested for an extended period of time in the TIM23 complex. Rhomboid proteases including PARL preferentially recognize a helix-breaking glycine-/proline-rich segment, which is found in human PINK1 [108]. On the other hand, a proline residue in the transmembrane segment disfavors the lateral release from the TIM23 complex [109]. These features may determine the unique PINK1 retrotranslocation pathway.



**Figure 3. Ephemeral life of PINK1 in the healthy mitochondria.**

(A) The newly synthesized PINK1 precursor on the cytosolic ribosomes is targeted to the mitochondria. After crossing the outer membrane through the TOM complex, the N-terminal mitochondrial targeting sequence is cleaved by MPP in the matrix. The following transmembrane segment is recognized by the TIM23 complex and received second cleavage by PARL between A103 and F104. Most of the cleaved PINK1 is released to the cytosol where the newly N-terminal phenylalanine residue of the cleaved PINK1 is recognized by the N-end rule UBR ligases (UBR1, UBR2, and UBR4 that preferentially recognize type-2 N-degrons) for proteasomal degradation. A matrix ATPase associated with diverse cellular activities (m-AAA) protease is composed of AFG3L2 and paraplegin and has the active site oriented toward the matrix. ClpXP is a matrix ATP-dependent protease composed of hetero-oligomeric ATP-binding subunits and proteolytic subunits. m-AAA and ClpXP also participate in PINK1 degradation. Another ATP-dependent LON protease also contributes, particularly in *Drosophila*, to PINK1 degradation in the matrix. As phosphorylation of NDUFA10 in the respiratory chain complex I is reduced in *Pink1*-KO mice, a small amount of PINK1 retained in the inner membrane might be involved in the maintenance of complex I through phosphorylation. (B) Amino acid sequence alignment of the transmembrane region of PINK1. Amino acid sequences of the predicted transmembrane segments (pink-colored box) from the indicated species are shown [99]. The transmembrane regions are well conserved from zebrafish (*Danio rerio*) and humans (*Homo sapiens*), while the transmembrane segment in fly (*Drosophila melanogaster*) is less conserved with fewer glycine/proline residues. The PARL cleavage site of human PINK1 between A103 and F104 is also shown.

Alternatively, other proteases in the mitochondria such as ClpXP and m-AAA may also function in rapid PINK1 turnover [103] (Fig 3A).

While most of the PINK1 is degraded by the proteasome, the possibility that a small fraction of the PINK1 stays in the healthy mitochondria cannot be excluded. Indeed, phosphorylation of Ser250 in the complex I subunit, NDUFA10, is reduced in the liver and brains of *Pink1*-KO mice [110]. Although whether PINK1 directly phosphorylates NDUFA10 remains unclear, deficiencies in complex I have been found in PD patients

[111,112]. Furthermore, the LON protease in the mitochondrial matrix is reported to degrade PINK1 in *Drosophila* [113]. The transmembrane segment and PARL cleavage site of PINK1 are well conserved from zebrafish to humans, but less so in fly, suggesting that the PINK1 degradation pathway varies depending on the species (Fig 3B).

In summary, a continuous process of mitochondrial import and degradation maintain PINK1 at extremely low levels on healthy mitochondria. In other words, this system functions as a sensitive sensor for the rapid detection of mitochondrial damage.



## Autophagy

Autophagy is essential for Parkin-mediated mitochondrial degradation because *ATG5*-KO cells impede the elimination of the damaged mitochondria even when Parkin is recruited to them [35]. Autophagy is a major intracellular degradation system that sometimes functions in consort with the ubiquitin-proteasome system [114,115]. Both autophagy and proteasomes are well conserved in eukaryotes, but autophagy can encapsulate cytoplasmic materials including bigger protein aggregates and/or unwanted organelles for bulk degradation through the fusion with the lysosome, while proteasomes degrade ubiquitinated protein substrates singly. Although autophagy was traditionally regarded as a non-selective process to keep up with the demand for energy under starvation conditions, evidence is accumulating that indicates many different types of selective autophagy for eliminating specific unwanted organelles, such as peroxisomes (pexophagy) and damaged mitochondria (mitophagy), and infecting pathogens (xenophagy) [116–118]. An astonishing number of *in vivo* studies indicate that autophagy deficiency is associated with many diseases such as neurodegenerative disorders, cancer, microbial infection, and aging [119].

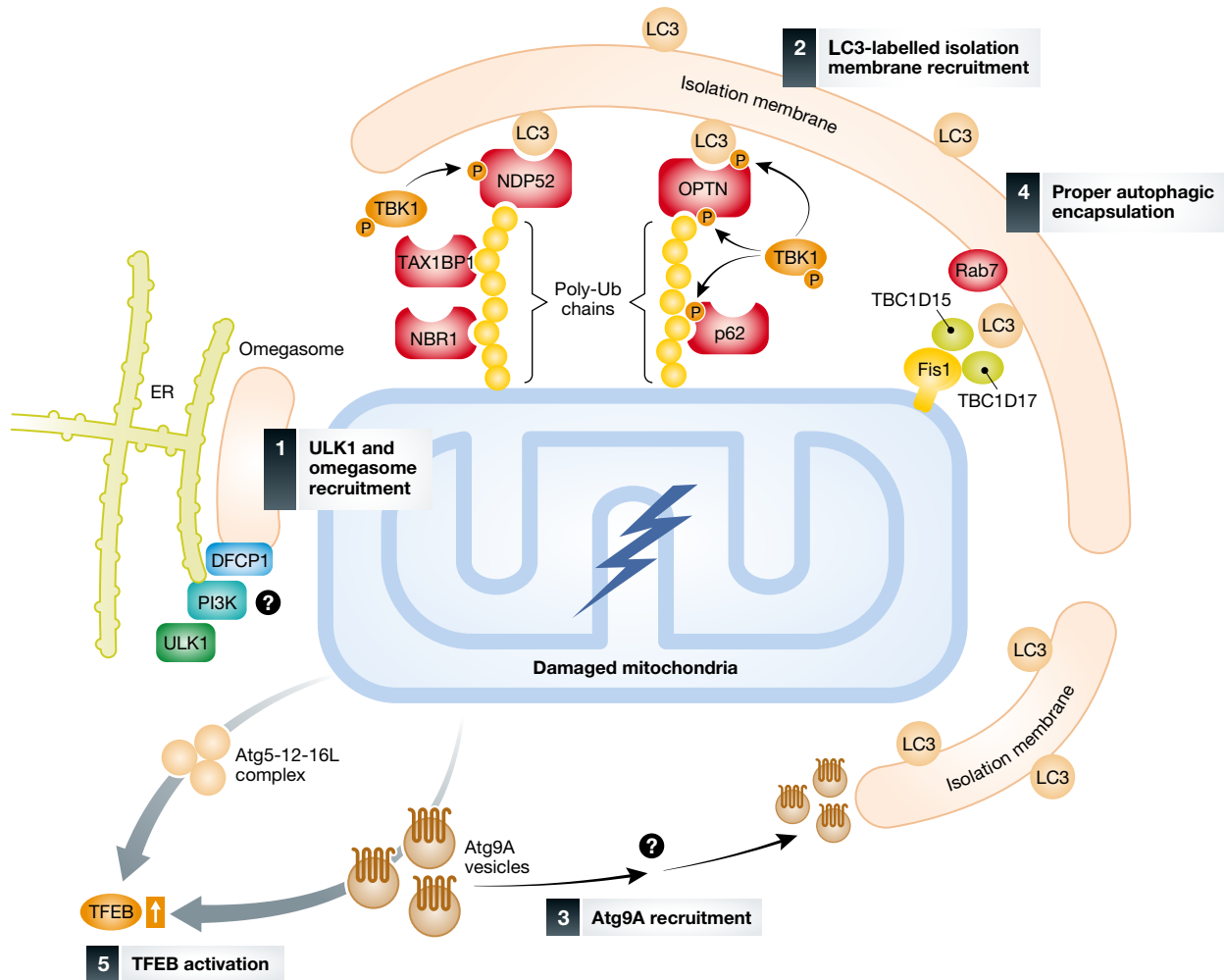
So far, numerous proteins that are essential for autophagy have been identified, many of which are evolutionarily conserved from yeast to humans [120,121]. These Atg proteins form several functional units. The most upstream autophagy regulator is the ULK1 complex, which consists of ULK1, Atg13, FIP200, and Atg101 in mammals. In starvation-induced autophagy, mTOR negatively regulates the ULK1 complex through phosphorylation of ULK1 and Atg13. Following Parkin translocation, the ULK1 complex transiently forms foci on the mitochondria (or contact site between mitochondria and ER) [122] (Fig 4). mTOR1 inhibition was reported to induce mitophagy in consort with the loss of membrane potential [123]. Another autophagy regulator, the PI3K complex, is also transiently recruited to mitochondria in an early stage of mitophagy. The PI3K complex, which generates PI3P, consists of Beclin1, Atg14L, Vps15, and Vps34 in mammals, although Beclin1-Vps15-Vps34 also forms other stable complexes with UVRAG and/or Rubicon for endosomal trafficking [124,125]. DFCP1 diffusely localizes on the ER and Golgi membranes under normal conditions, but upon mitophagy stimulation, it extensively accumulates at a spot where the ER and mitochondria are contacted (Fig 4). Since the FYVE domain in DFCP1 binds to PI3P, DFCP1 may serve as an appropriate reporter of PI3P-enriched isolation membranes at downstream of the ULK1 and PI3K complexes [126,127]. Atg9A is the only known multi-spanning membrane protein among the essential Atg core proteins and behaves uniquely. Atg9A resides on a small vesicular structure that shuttles between the cytosol, trans-Golgi network, and endosomes under normal conditions. The two ubiquitin-like conjugation systems, Atg5-12 and PE (phosphatidylethanolamine)-LC3, are important for elongation and/or complete encapsulation of the isolation membrane. The C-terminal glycine in Atg12 is activated by Atg7 (E1-like) and Atg10 (E2-like) and is finally covalently conjugated to Atg5. In contrast, the C-terminal arginine in LC3 (Atg8 homologue) is first cleaved by Atg4 to expose a glycine residue, and then, LC3 is conjugated to PE through Atg7 (E1-like) and Atg3 (E2-like) activation to become lipidated LC3. Mammals encode at least six Atg8 homologues (LC3A, LC3B, LC3C, GABARAP, GABARAPL1,

and GABARAPL2) and both the LC3 subfamily and GABARAP subfamily undergo PE conjugation. Although distinct roles in starvation-induced autophagy have been proposed [128], their roles in mitophagy remain unknown.

## Later steps of mitophagy link to autophagy

How are autophagy regulators recruited to the damaged mitochondria? The simple model proposed in 2010 was that p62, which has an ability to bind both LC3 and ubiquitin, serves as the adaptor in recruiting Atg proteins (at least lipidated LC3) to the poly-ubiquitinated surface of the mitochondrial outer membrane [53]. However, *p62*-KO cells can still degrade the damaged mitochondria through the autophagy machinery, raising the possibility that binding between p62 and LC3 is not sufficient for mitophagy [129,130]. In addition to p62, mammals also express at least four additional autophagy adaptors, NDP52, NBR1, optineurin, and TAX1BP1. All possess an LC3-interacting region (LIR), an ubiquitin-associated domain (UBD), and a dimerization (or oligomerization) domain. Therefore, these autophagy adaptors are believed to bridge the LC3-labeled isolation membrane and ubiquitinated cargo. To investigate the individual role of autophagy adaptors in mitophagy, two groups recently used CRISPR/Cas9 genome editing to knockout individually and different combinations of the five autophagy adaptors. Using a detailed mitophagy assay, these two studies found that NDP52 and optineurin primarily (and TAX1BP1 to some extent) function as autophagy adaptors during Parkin-/PINK1-mediated mitophagy, while p62 and NBR1 are not essential [131,132]. Ectopic targeting of the PINK1 kinase domain to the outer mitochondrial membrane in the absence of Parkin and mitochondrial stress recruits optineurin and NDP52, but not p62 [131]. This recruitment may suggest that phosphorylated ubiquitin chains generated locally on the mitochondria by PINK1 serve as a receptor for the selective autophagy machinery. However, Heo *et al* showed that a K63-linked phosphorylated ubiquitin chain binds poorly to recombinant optineurin. This discrepancy can be explained if Parkin-dependent optineurin phosphorylation changes the binding affinity of optineurin to ubiquitin chains [132]. TBK1 participates in this step by phosphorylating optineurin (S177) [133] and p62 (S403) [134], which enhances the interactions with LC3 and ubiquitin, respectively [131,132]. Moreover, TBK1 also phosphorylates other sites of optineurin at S473 and S513 to enhance binding ability to poly-ubiquitinated chains [132,135] (Fig 4). TBK1 itself is activated by Ser172 phosphorylation (PINK1-dependent but not autocatalytic) triggered by binding of optineurin to the ubiquitin chain on damaged mitochondria [132]. Interestingly, these studies imply many functional similarities between mitophagy and xenophagy such as the involvement of NDP52/optineurin and TBK1 phosphorylation. Since the mitochondrion is evolutionary derived from  $\alpha$ -proteobacteria, the autophagy machinery may recognize damaged mitochondria as ROS-producing invaders of the cell [136]. Parkin was also reported to be recruited to *Mycobacterium tuberculosis*-containing phagosomes [137]. The key factors involved in recruiting cytosolic Parkin and unlocking autoinhibited Parkin E3 activity in this process remain to be identified.

Rab GTPase cycles are also important for mitochondrial encapsulation by the LC3-labeled isolation membrane. Two Rab-GAPs,



**Figure 4. Activation and recruitment of autophagy machineries during Parkin-/PINK1-mediated mitophagy.**

Following the generation of poly-ubiquitin chains on damaged mitochondria, the indicated autophagy proteins including adaptors and regulators are recruited to the mitochondria in a multi-independent process. (i) While autophagosomes form at ER–mitochondria contact sites under starvation-induced autophagy [179], how the ULK1 and PI3K complexes and the omegasome marked by DFCP1 recognize mitochondrial damage remains unknown. (ii) Poly-ubiquitin chains on the mitochondria are directly recognized by the autophagy adaptors, in particular NDP52 and optineurin (OPTN), which are phosphorylated by TBK1, and promote the recruitment of the LC3-labelled isolation membrane. (iii) Atg9A vesicles are independently recruited to the mitochondria through an unknown mechanism. (iv) Mitochondria-localized Rab-GAPs, TBC1D15 and TBC1D17, via interaction of Fis1 regulate proper autophagosomal formation by modulating Rab7 activity. (v) Upon mitophagy stimulation, a regulator of autophagy-lysosome biogenesis, TFEB (as well as MITF and TFE3), is activated in an Atg5- and Atg9A-dependent manner.

TBC1D15 and TBC1D17, which associate with the mitochondrial outer membrane via interactions with Fis1, govern autophagosomal morphology by modulating Rab7 activity during Parkin-mediated mitophagy, but not starvation-induced autophagy [138]. Because TBC1D15 (and TBC1D17) can also directly interact with LC3 via the LIR motif, they control proper autophagic encapsulation of damaged mitochondria at the interface between mitochondria and the isolation membrane (Fig 4). Rab7 also localizes to isolation membranes in the early stage of xenophagy [139], suggesting another similarity between mitophagy and xenophagy as both involve a Rab7 activity of isolation membrane expansion.

Hierarchical analysis using several ATG-KO cell lines during Parkin-mediated mitophagy revealed that the recruitment of these autophagy proteins is not a linear cascade, but is rather a multi-independent process [122]. For example, lipidated LC3, the ULK1

complex, and Atg9A vesicles are independently recruited to damaged mitochondria, whose recruitment mechanisms, with the exception of lipidated LC3, are as of yet uncharacterized (Fig 4).

After Parkin translocation, damaged mitochondria are almost completely eliminated within 24–48 h. Therefore, the Parkin/PINK1 activation signal must quantitatively affect not only the ubiquitin system but also autophagy-lysosome function. Under aberrant lysosomal storage or nutrient deprivation conditions, proteins involved in lysosomal and autophagic function are upregulated at the transcriptional levels by TFEB [140–142]. In humans, TFEB together with MITF, TFE3, and TFE3 comprises the MiT/TFE subfamily of basic helix-loop-helix zipper transcription factors and has an ability to bind a specific 10-bp palindromic motif found in the promoter sequence of genes encoding lysosomal and autophagic proteins to modulate their expression. Under normal conditions,

**Sidebar A: In need of answers**

Although many research papers have elucidated detailed molecular mechanisms of Parkin-/PINK1-dependent mitophagy, new discoveries always bring new questions. Here, we would like to outline several open questions that need to be answered in the future to further advance our understanding of mitophagy.

- 1 How does phosphorylated Parkin accelerate ubiquitin transthiolation from E2s to Cys431?  
Kazlauskaitė *et al* [92] demonstrated that purified recombinant Ser65-phosphorylated Parkin exhibited maximal E3 ligase activity that no longer required Ser65-phosphorylated ubiquitin. This is consistent with the results in which phosphorylated Parkin, but not Parkin bound with phosphorylated ubiquitin, had an “open” structure as evidenced by the accessibility of the catalytic Cys431 to ubiquitin-vinyl sulfone [67,87]. In contrast, K161N and K211N mutations in a different phosphate-binding pocket of the RINGO domain, which are not involved in binding phosphorylated ubiquitin, inhibited full activation of Ser65-phosphorylated Parkin [67]. This raises the possibility of another unknown step in the Parkin conformational change [93]. Structural determination of the Parkin-phosphorylated ubiquitin complex has revealed that the E2 binding region is too spatially removed from the catalytic Cys431 to allow for direct transfer of the ubiquitin molecule. As E2 association with phosphorylated Parkin has been suggested to induce further Parkin conformational changes, the ternary protein complex including phosphorylated Parkin, phosphorylated ubiquitin, and ubiquitin-conjugated E2 enzyme needs to be solved either by biochemical or by structural approaches.
- 2 How are autophagy proteins recruited to the damaged mitochondria following Parkin-/PINK1-dependent ubiquitination?  
Poly-ubiquitinated chains on the surface of the outer membrane of the damaged mitochondria can recruit autophagy adaptors, which further recruit Atg8 homologues via LIR motifs. On the other hand, how essential upstream autophagy proteins such as the ULK1 complex and Atg9A vesicles are recruited to the mitochondria remains unknown. Although ectopic PINK1 targeting to mitochondria suggests that association of the ULK1 complex with the damaged mitochondria is optineurin/NDP52 dependent [131], precise cascades of the steps starting from the poly-ubiquitinated chains for transfer of the signal to the autophagy machinery are largely unknown.
- 3 Do mitophagy defects directly affect the loss of dopaminergic neurons?  
There is no doubt that elimination of the damaged mitochondria by mitophagy machinery is important for maintaining cellular homeostasis via healthy mitochondria. However, there is currently no direct evidence demonstrating how defects in mitophagy affect PD pathologies or degradation of dopaminergic neurons. To clarify these issues, an *in vivo* mitophagy assessment tool for dopaminergic neurons needs to be established.

TFEB transiently associates with the outer surface of the lysosome where it binds the heterodimeric Rag GTPase [143] and is phosphorylated on several residues by active mTORC1 [144–146]. Phosphorylation of Ser211, in particular, serves as a signal for the chaperone 14-3-3 proteins, which bind and sequester TFEB so that the inactivated form of TFEB stays in the cytosol. Upon starvation, TFEB dissociates from 14-3-3 allowing translocation to the nucleus where it induces the transcription of target genes. Recently, Nezich *et al* showed that Parkin-/PINK1-mediated mitophagy stimulation also induces TFEB (as well as MITF and TFE3) translocation to the nucleus and upregulates cathepsin B and p62 mRNAs, known TFEB target genes. Although Atg5 is dispensable for starvation-induced TFEB translocation to the nucleus, Parkin-mediated TFEB translocation requires Atg5 and Atg9A [147] (Fig 4). This strongly suggests that the molecular mechanism for TFEB translocation to the nucleus during mitophagy is different from that during starvation. Furthermore, KO of three MiT/TFE family members (TFEB, MITF, and TFE3) in HeLa cells causes moderate defects in Parkin-mediated clearance of damaged mitochondria as well as p62 expression and lysosome morphology. It will be interesting, in the future, to test the physiological role of mitophagy-dependent TFEB activation *in vivo*.

**Mitophagy-independent Parkin/PINK1 functions**

Many autophagy-independent Parkin functions have been reported. Parkin regulates the level of PARIS (ZNF746), a major transcriptional repressor of PGC-1 $\alpha$  expression, through the ubiquitin-proteasome system [148]. Conditional KO of *Parkin* in adult mice was shown to cause the progressive loss of dopamine neurons in a PARIS-dependent manner via a decline of mitochondrial mass and respiration [149]. Parkin also has been proposed to regulate mitosis

and chromosome segregation [150]. Recently, it was reported that Parkin complexes with Cdc20 and Cdh1, known APC/C co-activators, to mediate the degradation of several mitosis regulators independent of APC/C. This pathway is PINK1 independent; however, Parkin E3 ligase activity is triggered following Plk1 phosphorylation of S378 [151]. Autophagy-independent Parkin/PINK1 regulation of mitochondrial integrity maintenance has also been reported. Local oxidative damage of mitochondria induces small vesicular structures called mitochondrial-derived vesicles (MDVs) that are transported to the late endosome and/or lysosomes. Both Parkin and PINK1 are required for the generation of MDVs, suggesting that Parkin-/PINK1-dependent MDVs are a faster response to mitochondrial damage than autophagic elimination [152,153]. Disruption of the mitochondrial membrane potential has also been reported to alter Rab8 and Rab11 functions following phosphorylation at a conserved Ser111 residue in a PINK1-dependent manner [154]. Although PINK1 does not directly phosphorylate these Rabs, this finding may suggest as of yet-uncharacterized cascades of mitochondrial quality control.

**Conclusion**

Parkin-mediated mitophagy research now spans multiple fields of active research including mitochondria, autophagy, the ubiquitin-proteasome, neurology, and PD. This overlap in research interests and disciplines has in such a short time propelled the many advances in our understanding of Parkin-mediated mitophagy. Based on studies using cultured cells (immortalized and non-neuronal in most cases), we now know the detailed molecular mechanisms underlying Parkin recruitment to damaged mitochondria and how this impacts the subsequent steps leading to their selective engulfment by the autophagosome.

Although initially controversial, recent studies have confirmed Parkin/PINK1 involvement in autophagic clearance in neurons. Cai *et al* [155] showed that the mitochondria in the somatodendritic regions of mature cortical neurons are captured by an LC3-labeled structure in a Parkin-dependent manner. Similarly, Ashrafi *et al* [156] utilized mito-KillerRed (see Box 1) to selectively damage a subset of mitochondria in hippocampal axons and observed autophagosome and axonal lysosome recruitment to the damaged mitochondria that was both Parkin and PINK1 dependent. Furthermore, mitochondrial delivery to the lysosome was observed in mouse primary neurons using mito-Keima (see Box 1). This endogenous Parkin-dependent mitophagy requires a certain period of time, but not any exposure of chemical depolarizing compounds [82]. Despite being independently generated by many research groups, neither simple *Parkin*- nor simple *Pink1*-KO mice exhibit severe PD-like symptoms such as the loss of dopaminergic neurons, thus complicating Parkin/PINK1 mitophagy studies *in vivo*. However, Pickrell *et al* [157] recently created a new PD model mouse by crossing *Parkin*-KO mice and *Mutator* mice that express a defective mitochondrial DNA polymerase. In the resulting *Mutator Parkin*-KO mice, dopaminergic neurons degenerated and L-DOPA (a metabolic precursor of dopamine widely used for PD patients) improved the motor deficit of the mice. They also found the accumulation of phosphorylated ubiquitin in neurons, but not in livers of the *Mutator Parkin*-KO mice, strongly suggesting that PINK1 activation was caused by neuronal mitochondrial dysfunction.

In this review, we shed light on the biochemical and molecular aspects of Parkin/PINK1-mediated mitophagy. Many questions remain to be answered before we can have a completely clear understanding of Parkin/PINK1 functions; however, we will continue to uncover the molecular basis linking the coordinated actions of Parkin/PINK1 and the ubiquitin signal with autophagy for clearing dysfunctional mitochondria.

## Acknowledgements

We thank Catherine L. Nezhich, Michael Lazarou, Richard J. Youle, Lina Herhaus, and Ivan Dikic for valuable comments. We regret that space limitations prevented us from being able to cite the studies of numerous other colleagues, but hope the readers refer to the cited reviews for more information. This work was supported by JSPS PRESTO, KAKENHI Grant Number: 26650042, MEXT KAKENHI Grant Numbers 26111729 and 15H01196, and the Tomizawa Jun-ichi and Keiko Fund for Young Scientist (to N.M.); by JSPS KAKENHI Grant Number 26000014 (to K.T.); and by the Takeda Science Foundation (to K.T. and N.M.).

## Conflict of interest

The authors declare that they have no conflict of interest.

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