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The PARK2/Parkin receptor on damaged mitochondria revisited—uncovering the role of phosphorylated ubiquitin chains

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Phosphorylated ubiquitin produced by PINK1 kinase functions as a PARK2/Parkin activator by derepressing intramolecular autoinhibition of PARK2 E3 activity. Unexpectedly, we revealed that phosphorylated polyubiquitin chain also functions in the PARK2 recruitment process as a PARK2 receptor. Phosphorylated ubiquitin enables us to comprehensively understand how PINK1 and PARK2 catalyzes (phospho-)ubiquitination of depolarized mitochondria and subsequent mitophagy.

PINK1/PARK6 (*PTEN induced putative kinase 1*) and *PARK2* (*parkin RBR E3 ubiquitin protein ligase*) have been identified as the causal genes responsible for hereditary recessive early-onset parkinsonism. PARK2/Parkin is a ubiquitin ligase (E3) that catalyzes ubiquitination of depolarized mitochondria to facilitate mitophagy-based removal of damaged mitochondria from cells. PARK2 is broadly distributed in the cytosol under normal conditions and is spatially separated from mitochondrial substrates. However, when the mitochondrial membrane potential ($\Delta\Psi$m) decreases, PARK2 is activated and promptly recruited to depolarized mitochondria, which allows ubiquitination of mitochondrial substrates on the outer mitochondrial membrane (OMM). PINK1, a serine/threonine kinase, is essential in PARK2 activation and recruitment, both of which are dependent on PINK1 kinase activity. In 2014, we and 2 other research groups reported that PINK1 phosphorylates ubiquitin, and that phosphorylated ubiquitin functions as a PARK2 activator by derepressing the autoinhibition of PARK2 E3 activity.

While significant progress has been made in elucidating the mechanism of PARK2 activation described above, the molecular basis for PINK1-mediated recruitment of PARK2 to depolarized mitochondria has remained obscure. Despite the various mechanisms proposed for this recruitment process none adequately explained the accumulated data. For example, the simplest model suggested that PINK1 interacted physically with PARK2 and thus functioned as the PARK2 receptor. This model easily explains why PINK1 is essential for mitochondrial PARK2 localization. However, although PINK1 forms a super-molecular weight complex composed of a PINK1 dimer and the translocase of the outer mitochondrial membrane machineries in response to a decrease in $\Delta\Psi$m, PARK2 is not associated with this complex. It is thus unlikely that PINK1 and PARK2 stably associate on depolarized mitochondria. Because PINK1 phosphorylates PARK2 in addition to ubiquitin, the second model proposed that the PINK1 phosphorylation event converts PARK2 to a membrane bound form. This model is sufficient to explain why the kinase activity of PINK1 is required for PARK2 mitochondrial localization without the need for PINK1 to associate with PARK2. This hypothesis, however, inadequately explains selective recruitment of PARK2 to depolarized mitochondria when both depolarized and polarized mitochondria are present in the cell. A third model suggested that phosphorylation of PINK1 mitochondrial substrates increases the affinity of mitochondria for PARK2, such that PARK2 is recruited to those mitochondria. This model is compatible with

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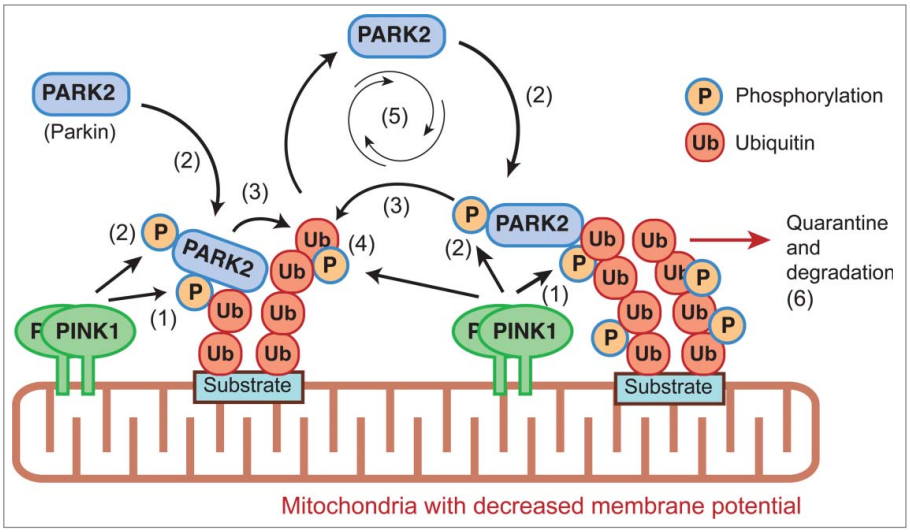

Figure 1. Model for PARK2/Parkin recruitment to damaged mitochondria by phosphorylated polyubiquitin chains. When ΔΨm decreases, PINK1 accumulation on depolarized mitochondria results in phosphorylation of already-existing ubiquitin chains (1). Cytosolic PARK2/Parkin is then recruited to phosphorylated polyubiquitin chains on depolarized mitochondria (2), and mitochondrial PARK2 undergoes PINK1-catalyzed phosphorylation (2). Active PARK2 produces more polyubiquitin chains on the depolarized mitochondria (3), with the resulting ubiquitin chains phosphorylated by PINK1 (4). Using this positive feed-forward cycle of reactions (5), depolarized mitochondria are rapidly decorated with phosphorylated ubiquitin chains, which enhance quarantine and degradation of low-quality mitochondria (6).

the requirement for PINK1 kinase activity in PARK2 recruitment, and that PARK2 is selectively directed to a subset of mitochondria when energized and depolarized mitochondria coexist. However, if some mitochondria-localized PINK1-substrate (s) function as a PARK2 receptor, it is difficult to explain results showing that PINK1 targeted to peroxisomes or lysosomes, which lack a genuine PINK1 substrate, are still able to recruit PARK2 to the respective organelles. An even more fundamental deficiency in all of the models is their inability to incorporate the critical importance of the PARK2 E3 activity in depolarized-mitochondrial localization. Thus, until recently, the largest unresolved issue in this field was elucidation of the mechanism by which PARK2 is recruited to damaged mitochondria.

In our paper we argue that phosphorylated ubiquitin chains are the genuine PARK2 receptor. First, we found that phosphorylated polyubiquitin chains exist in cells when ΔΨm is decreased, and PINK1 phosphorylates polyubiquitin chains in vitro. Second, a linear ubiquitin chain (tetra-ubiquitin) of phosphomimetic ubiquitin (S65D) recruits PARK2 to energized mitochondria even in the absence of PINK1, whereas a linear ubiquitin chain (tetra-ubiquitin) of a phosphorylation-deficient mutant does not. Third, under more physiological conditions, a lysosomal polyubiquitin-chain containing phosphorylated ubiquitin recruits phosphomimetic PARK2 to the lysosome. Fourth, PARK2 translocation to depolarized mitochondria is abrogated in U2OS-shUb cells in which the endogenous ubiquitin is replaced with S65A phosphorylation-deficient mutants. In addition, physical interactions between phosphomimetic PARK2 and phosphorylated ubiquitin are detected by immunoprecipitation from cells and in vitro affinity isolation experiments using recombinant phospho-polyubiquitin chain and phosphomimetic PARK2. We thus propose a completely novel model for PARK2 recruitment in which the phosphorylated ubiquitin chains on depolarized mitochondria function as the genuine PARK2 receptor.

Importantly, this model can explain the unanswered, seemingly strange, questions described above. If PARK2 exhibits a broad range of substrate specificity, it is likely that ubiquitination of diverse OMM proteins would result. Consequently, we can easily imagine that these ubiquitin chains function as substrates for the next-round of PINK1 phosphorylation, which then recruit and activate more PARK2 molecules. Indeed, using a specific anti-phospho-ubiquitin antibody prepared in house, we observed PARK2-dependent accumulation of phosphorylated ubiquitin on depolarized mitochondria. This interdependent relationship between PARK2 E3 activity and ubiquitin phosphorylation suggests a feed-forward cycle. If they constitute a positive feed-forward mechanism, then the dependence on PARK2 E3 activity for efficient recruitment to depolarized mitochondria becomes clear—PARK2 promotes the amplification and accumulation of phosphorylated polyubiquitin chains on damaged mitochondria. This system enables a small amount of PINK1 to recruit PARK2 dramatically. Moreover, if phosphorylated polyubiquitin chains are the PARK2 receptor, it becomes much easier to explain the confounding data that show targeting of PINK1 to peroxisomes or lysosomes, where genuine PINK1 substrates do not exist, also results in recruitment of PARK2 to the respective organelles. Ubiquitination is commonly used as a signal throughout the cell; thus, all organelles contain many ubiquitinated proteins. The ubiquitinated proteins on peroxisomes or lysosomes could easily be phosphorylated by ectopically-targeted PINK1, which would then recruit PARK2. Similarly, for PARK2 recruitment on depolarized mitochondria, the initiating signal for PARK2 recruitment appears to be mitochondrial proteins previously ubiquitinated by other E3s. However, once these pre-ubiquitinated proteins are phosphorylated by PINK1 on depolarized mitochondria, they act as beacons for PARK2 activation and recruitment, which then triggers local phosphorylated-ubiquitin signal amplification via a positive-feedback loop (**Fig. 1**). Consequently, phosphorylated ubiquitin chains function as a signal for PINK1-PARK2-mediated mitophagy of depolarized mitochondria.