Pink1, the first ubiquitin kinase

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Pink1 and Parkin, identified through studies of hereditary early onset Parkinson's disease, are involved in mitochondria quality control. Parkin E3 ubiquitin ligase activity is activated by Pink1 kinase activity, although the mechanism is still elusive. Three recent reports uncover a surprising mechanism in which Pink1 directly phosphorylates ubiquitin to boost Parkin activity.

See also: **LA Kane *et al*** (April 2014),
A Kazlauskaite *et al* (May 2014) and
F Koyano *et al* (June 2014)

Pink1, a mitochondrial kinase, and Parkin, a cytosolic E3 ubiquitin ligase, function in mitochondrial maintenance. Pink1 is normally imported into mitochondria, cleaved by PARL protease, and degraded by the proteasome. When mitochondria lose their membrane potential, Pink1 starts to accumulate on the mitochondrial outer surface, where it recruits and activates Parkin to trigger protein ubiquitination, eventually leading to mitochondrial autophagy (reviewed in Narendra *et al*, 2012). Parkin E3 ubiquitin ligase activity is inhibited by its N-terminal ubiquitin-like (UBL) domain through interaction with C-terminal RING-between-RING (RBR) E3 catalytic domain (Chaugule *et al*, 2011). Recombinant Pink1 can phosphorylate the UBL domain at Ser65 *in vitro*, and Parkin Ser65 phosphorylation is readily detected when mitochondria are depolarized (Kondapalli *et al*, 2012; Shiba-Fukushima *et al*, 2012). A simplified model is that Pink1 phosphorylation of Ser65 in the UBL domain releases Parkin autoinhibition. However, phospho-mimetic substitution (Ser65Glu) or even UBL domain deletion cannot bypass the requirement of Pink1 for Parkin activation (Sarraf *et al*, 2013). Are there any other Pink1 substrate(s) or Parkin phosphorylation

sites involved in Parkin activation? Three recent reports provide an unexpected answer: Pink1 directly phosphorylates ubiquitin, and this is critical for Parkin activation (Kane *et al*, 2014; Kazlauskaite *et al*, 2014; Koyano *et al*, 2014). This discovery represents the first example of a functional phosphorylation on ubiquitin.

To identify the full spectrum of Pink1 substrates, proteomic approaches were used to compare the profile of mitochondria isolated from wild-type or Pink1-deficient cells after treatment by CCCP, a mitochondria-depolarizing drug (Kane *et al*, 2014; Kazlauskaite *et al*, 2014). Surprisingly, a phosphopeptide identified in mitochondria-associated proteins only in wild-type cells derives from ubiquitin, and phosphorylation occurs at the conserved Ser65 residue, which corresponds to Ser65 in the Parkin UBL. Based on structural similarity between ubiquitin and the UBL domain, Koyano *et al* (2014) hypothesized that Ser65 on ubiquitin may be phosphorylated by Pink1, and indeed ubiquitin in CCCP-treated lysates exhibited a phosphorylation-dependent electrophoresis band-shift, and the same phospho-Ser65 peptide could be identified by mass spectrometry. Quantification showed that phospho-ubiquitin represents 0.05% of total endogenous ubiquitin, although it is not known whether all the pSer65 ubiquitin molecules are associated with mitochondria. The kinase–substrate relationship was further established by *in vitro* kinase assays, in which recombinant Pink1 could phosphorylate wild-type but not S65A ubiquitin (Kane *et al*, 2014; Kazlauskaite *et al*, 2014; Koyano *et al*, 2014).

Does phospho-S65 ubiquitin play any role in Parkin regulation, and is there any interplay between Ser65 ubiquitin and Parkin UBL domain Ser65 phosphorylation? Using *in vitro* Parkin E3 activity assays, all three groups provide compelling evidence that

both phosphorylations are involved in and required for full Parkin activation. Kazlauskaite *et al* found that substituting wild-type ubiquitin with S65A ubiquitin greatly diminished Parkin E3 activity, but Parkin with a UBL S65A mutation also had much lower activity compared to wild-type Parkin, indicating that both phosphorylations are required. Kane *et al* found that prephosphorylation of ubiquitin by recombinant Pink1 could significantly enhance Parkin-mediated protein ubiquitination *in vitro*. Using GFP-Parkin and purified depolarized mitochondria as a source of Pink1, Koyano *et al* found that phospho-mimetic S65D ubiquitin, but not WT or S65A ubiquitin, could stimulate autoubiquitination of GFP-S65E-Parkin bearing an additional phospho-mimetic substitution in the UBL domain, but observed no enhancement with GFP-WT-Parkin.

How does the phospho-Ser65 ubiquitin activate Parkin? Koyano *et al* showed that phospho-mimetic S65D ubiquitin with a C-terminal diglycine mutation, which abolishes its conjugation to target proteins, could still activate the E3 activity of Parkin, excluding the possibility that phospho-S65 ubiquitin is preferred by Parkin for conjugation. They further showed that phospho-mimetic S65D ubiquitin binds to S65E Parkin, but not WT Parkin. The activation mechanism appears to be allosteric and allows functional access of Parkin to its cognate ubiquitin-conjugating E2 enzyme, since phospho-S65 ubiquitin accelerates Parkin discharge of UBCH7~ubiquitin (Kazlauskaite *et al*, 2014; Koyano *et al*, 2014). In summary, these studies uncover a surprising missing link in Parkin activation (Fig 1).

To understand how phospho-ubiquitin activates Parkin, the next step is to identify the exact binding site for phospho-Ser65-ubiquitin on Parkin, which was not
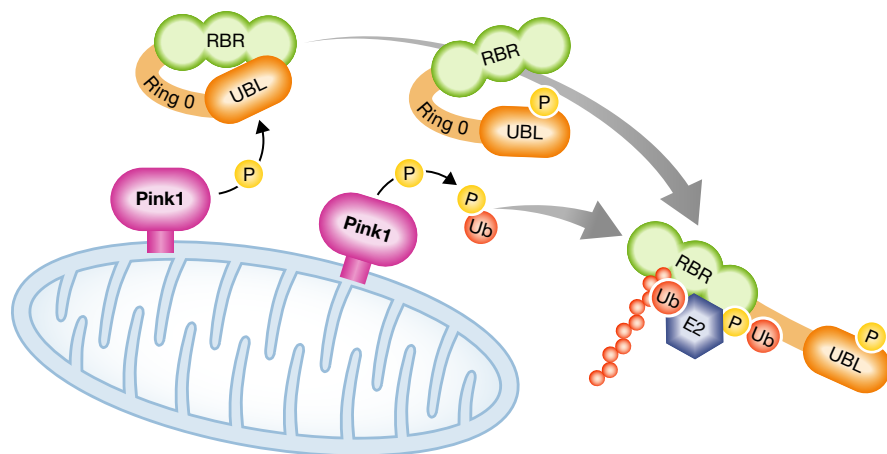
Figure 1. Model of how Pink1-mediated ubiquitin and UBL phosphorylation may activate Parkin.
Pink1 phosphorylates both ubiquitin and the Parkin UBL domain at the Ser65 residue. Parkin UBL domain phosphorylation exposes a phospho-Ser65 ubiquitin binding site, and phospho-ubiquitin binding in turn activates Parkin E3 activity.

characterized in the three recent studies. Interestingly, Wauer and Komander (2013) structurally identified a potential phospho-peptide binding site in Parkin's 'Ring0' domain, a highly conserved region between the UBL and the catalytic RBR domains. By determining crystal structures of Parkin, both Wauer and Komander (2013) and Trempe *et al* (2013) found a novel mechanism of Parkin regulation, in which the Ring0 domain inhibits Parkin E3 activity by blocking off the catalytic Cys431 in the RBR domain, such that activation of latent E3 activity of Parkin RBR domain in *in vitro* assays required removal of the Ring0 domain. Therefore, it is tempting to speculate that Ring0 represents a phospho-ubiquitin binding region involved in switching on Parkin ubiquitin ligase activity, although ultimately crystal structures of S65D ubiquitin bound to S65E Parkin will be necessary to validate such a molecular mechanism of activation.

Another major remaining question is whether UBL domain phosphorylation serves simply to relieve Parkin auto-inhibition. Kazlauskaite *et al* showed that like phospho-Ser65-ubiquitin, the isolated phospho-Ser65-UBL domain can activate *in vitro* Parkin E3 activity in *trans*. ΔUBL-Parkin, though still able to translocate to depolarized mitochondria, is defective in mitochondria ubiquitination. Deleting the UBL domain greatly diminished ubiquitin trapping by Parkin bearing a serine substitution of its catalytic cysteine 431, also indicating a positive role for the UBL domain

(Zheng & Hunter, 2013). Pink1 can stimulate Parkin self-association (Lazarou *et al*, 2013), and therefore, it is possible that the phospho-UBL domain may play a role similar to phospho-ubiquitin in activating additional Parkin molecules in *trans*.

Phosphorylation is generally counteracted by dephosphorylation. A mitochondrial phosphatase PGAM5 is a known binding partner of Pink1 (Imai *et al*, 2010), and in contrast to Pink1 being stabilized, PGAM5 cleavage is triggered by loss of mitochondrial membrane potential (Sekine *et al*, 2012). It will be interesting to test if PGAM5 can dephosphorylate p-Ser65-ubiquitin and p-Ser65-Parkin, and whether inhibition of PGAM5 could increase the efficiency of mitochondria clearance by Pink1/Parkin.

These findings also call attention to the function of phosphorylation of ubiquitin and other ubiquitin-like modifiers in general. Ubiquitin phosphorylation has been documented on S65, S57, T7, T12, Y59 in previous phospho-proteomic studies (referenced in Kane *et al*, 2014; Kazlauskaite *et al*, 2014), although the responsible kinases and especially their functional importance had remained elusive. Presumably, phosphorylation can change the binding properties of ubiquitin and ubiquitin chains, and phosphorylation occurring before conjugation may also affect linkage type or chain elongation.

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Conflict of interest

The authors declare that they have no conflict of interest.

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