PINK1-Parkin Signalling in Parkinson's Disease and Beyond

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Activation of the E3 ubiquitin ligase Parkin

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Abstract

The PINK1 (phosphatase and tensin homologue-induced putative kinase 1)/Parkin-dependent mitochondrial quality control pathway mediates the clearance of damaged organelles, but appears to be disrupted in Parkinson's disease (PD) [Springer and Kahle (2011) Autophagy 7, 266–278]. Upon mitochondrial stress, PINK1 activates the E3 ubiquitin (Ub) ligase Parkin through phosphorylation of the Ub-like (UBL) domain of Parkin and of the small modifier Ub itself at a conserved residue [Sauvé and Gehring (2014) Cell Res. 24, 1025–1026]. Recently resolved partial crystal structures of Parkin showed a 'closed', auto-inhibited conformation, consistent with its notoriously weak enzymatic activity at steady state [Wauer and Komander (2013) EMBO J. 32, 2099–2112; Riley et al. (2013) Nat. Commun. 4, 1982; Trempe et al. (2013) Science 340, 1451–1455; Spratt et al. (2013) Nat. Commun. 4, 1983]. It has thus become clear that Parkin must undergo major structural rearrangements in order to unleash its catalytic functions. Recent published findings derived from X-ray structures and molecular modelling present a complete structural model of human Parkin at an all-atom resolution [Caulfield et al. (2014) PLoS Comput. Biol. 10, e1003935]. The results of the combined *in silico* simulations-based and experimental assay-based study indicates that PINK1-dependent Ser^{65} phosphorylation of Parkin is required for its activation and triggering of 'opening' conformations. Indeed, the obtained structures showed a sequential release of Parkin's intertwined domains and allowed docking of an Ub-charged E2 coenzyme, which could enable its enzymatic activity. In addition, using cell-based screening, select E2 enzymes that redundantly, cooperatively or antagonistically regulate Parkin's activation and/or enzymatic functions at different stages of the mitochondrial autophagy (mitophagy) process were identified [Fiesel et al. (2014) J. Cell Sci. 127, 3488–3504]. Other work that aims to pin-point the particular pathogenic dysfunctions of Parkin mis-sense mutations have been recently disseminated (Fabienne C. Fiesel, Thomas R. Caulfield, Elisabeth L. Moussaud-Lamodiere, Daniel F.A.R. Dourado, Kotaro Ogaki, Owen A. Ross, Samuel C. Flores, and Wolfdieter Springer, submitted). Such a structure–function approach provides the basis for the dissection of Parkin's regulation and a targeted drug design to identify small-molecule activators of this neuroprotective E3 Ub ligase.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease and the result of the degeneration of dopaminergic neurons within the *substantia nigra pars compacta*. Besides several other genetic causes, autosomal recessive PD can be caused by mutations in the phosphatase and tensin homologue-induced putative kinase 1 (*PINK1*) and *PARKIN* genes [1]. Interestingly, PINK1 and Parkin protein are cooperatively functioning within the mitochondrial quality control (mitophagy) pathway [2]. The serine/threonine phosphorylating activity of PINK1 is pivotal for the recruitment of the E3 ubiquitin (Ub) ligase Parkin from the cytosol to depolarized mitochondria [3–6]. Parkin catalyses the addition of Ub to various protein targets localized at the mitochondrial surface [7,8]. Interestingly, both Ub and Parkin are phosphorylated by PINK1 at Ser^{65}

Key words: molecular dynamics, Parkin, Parkinson's disease (PD), phosphatase and tensin homologue-induced putative kinase 1 (PINK1), simulations, ubiquitin (Ub).

Abbreviations: ER, endoplasmic reticulum; HCI, high content imaging; HECT, homologous-to-the-E6-AP-carboxyl-terminus; IBR, in-between-RING; MDS, molecular dynamics simulation; PD, Parkinson's disease; PINK1, phosphatase and tensin homologue-induced putative kinase 1; pSer^{65}, phospho-Ser^{65}; REP, repressor element of Parkin; RING, really-interesting-new-gene; Ub, ubiquitin; UBL, Ub-like.

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[9–18]. Phosphorylation of Parkin is necessary to activate Parkin whereas phosphorylation of Ub that is attached to mitochondrial substrate proteins might help to efficiently recruit Parkin to damaged mitochondria. The addition of poly-Ub chains to mitochondria and the subsequent recruitment of adapter proteins cluster mitochondria at perinuclear regions and initiate degradation of individual substrates proteins and of entire organelles via the proteasome or autophagy/lysosome systems respectively [3,7,19,20]. The neuroprotective effect of the *PINK1* and *PARKIN* genes is lost with mutations in those genes which disrupt mitophagy at different steps during the sequential process [3–6].

PINK1 is known to phosphorylate Ser^{65} of Parkin within the Ub-like (UBL) domain and it has been shown that activation of Parkin's enzymatic function and mitochondrial translocation are linked [14–16,21,22]. Based on the presence of characteristic Zn^{2+} coordinating domains, Parkin has long been assumed a classical really-interesting-new-gene (RING)-type E3 Ub ligase. However, a novel hybrid mechanism for Ub transfer for Parkin and other members of the RING-between-RING (RBR) family was previously identified [23]. Although Parkin binds the E2 coenzyme via its RING domain, it physically receives the Ub moiety on its active centre Cys^{431} similar to homologous-to-the-E6-AP-carboxyl-terminus (HECT)-type E3 Ub ligases. Thus, E2 coenzymes that charge Parkin with Ub play a prominent role in its activation and enzymatic functions [24].

Several recent crystal structures for Parkin indicate a 'closed' and inactive conformation coming from several intramolecular interactions among the individual domains [25–27], consistent with an earlier report that suggested an auto-inhibitory interaction between the UBL domain and the C-terminal region [28]. Other structural data has been critical in developing hypotheses for the mechanism of Parkin, translocation and activation, such as the 2D-NMR HSQC spectrum based studies for individual domains, MS and SAXS experiments [29]. These X-ray data sources were combined with the computational modelling to establish a complete structure for human Parkin at an all-atom resolution [30]. In combination with additional molecular dynamics simulations (MDSs), a conformational pathway of Parkin activation could be developed. Thereby, PINK1-dependent phosphorylation at Ser^{65} initiated structural changes in a series of propagating motions that released Parkin from its auto-inhibitory state, allowing activation of its enzymatic activity [30].

This review is focused on selected aspects of Parkin activation that arise as a consequence of phosphorylation of the UBL domain and lays out structural and functional findings as a basis for future drug discovery efforts to stimulate the neuroprotective E3 Ub ligase Parkin.

Full-length model for human Parkin protein

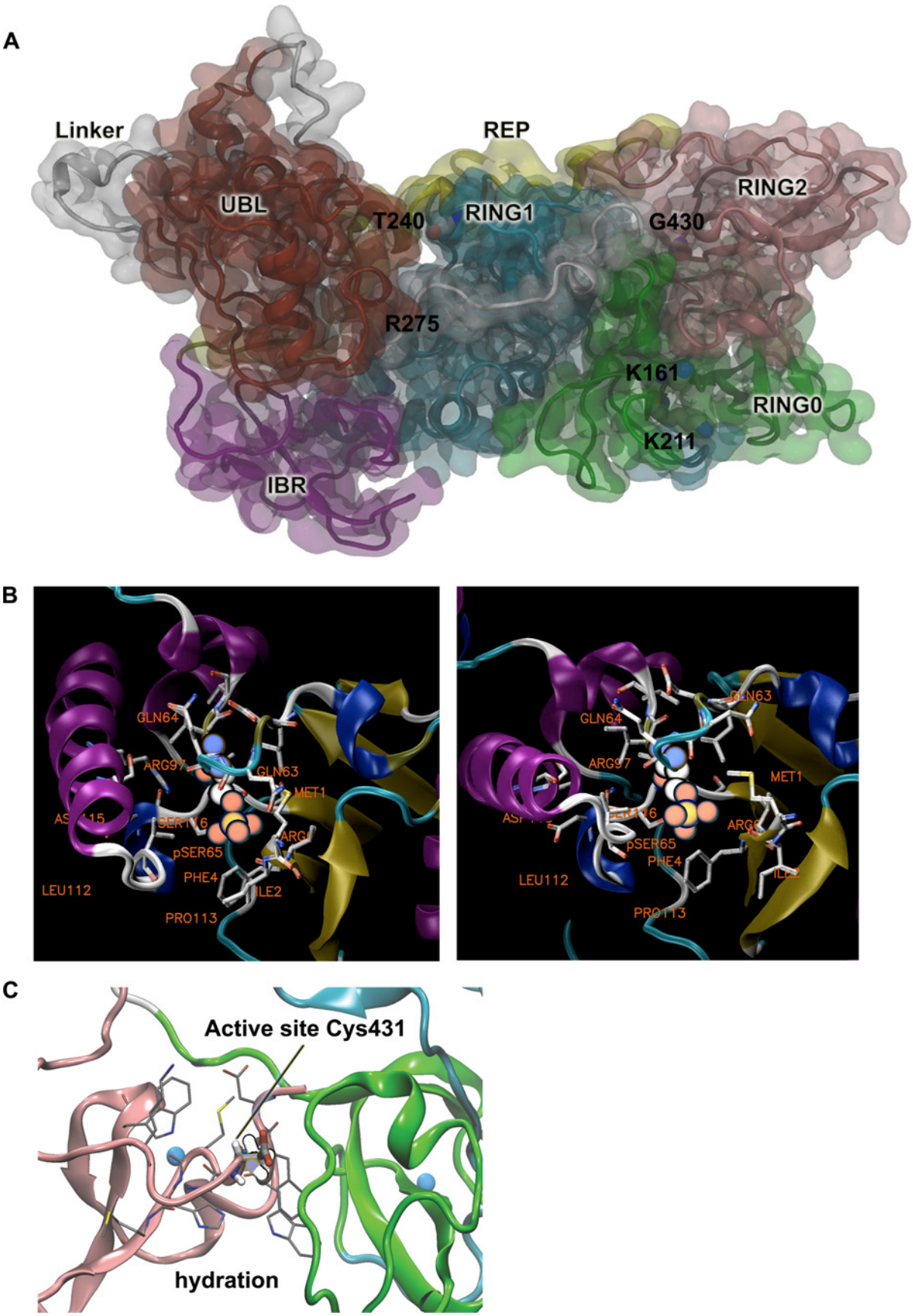
Using established molecular modelling methods [31–33], recent partial X-ray structures (human N-terminal truncated Parkin (PDB IDs: 4BM9 [27] and 4I1H [25]) and of its rat homologue (PDB IDs: 4K7D and 4K95 [26])] were incorporated into the generation of a first full-length structural model for human Parkin protein [30] (Figure 1A). Briefly, the complete N-term structural model including residues 1 through 140 had not been previously determined. Modelling of the N-terminus revealed that the linker region is comprised of two sub-domains: (1) a semi-globular domain from residue 77 to 125 that appears highly dynamic and (2) a tethering loop region from residue 126 to 140 that connects to the RING-0 domain [30]. Examination of the linker's secondary structural features designates the following regions: (1) α-helical regions from Gly^{77} to Gly^{85} and from Arg^{89} to Ser^{108}, (2) a helix loop turn from Leu^{112} to Ser^{116}, (3) minor β-roll (antiparallel) from Val^{117} to Leu^{123} paired to the β-strands from the UBL domain residues F^4-N^8 and L^{41}-F^{45} and (4) random coil from Leu^{123} through Arg^{140} [30].

Based on this full-length model of human Parkin, numerous variants carrying PD-associated mutations or post-translational modifications were created to investigate structural conformers in subsequent simulation studies. At least three intramolecular interactions contribute to the auto-inhibition of Parkin: (1) the UBL-linker region appears to act as a spring/clamp that holds Parkin in its 'closed', intertwined conformation; (2) the repressor element of Parkin (REP) blocks the E2 coenzyme-binding site in RING-1; and (3) RING-0 occludes the catalytic centre Cys^{431} in RING-2, resulting in a spatial separation between the active sites of E2 and Parkin by more than 50 Å (1 Å = 0.1 nm). It is thus evident that Parkin must undergo major conformational rearrangements in order to gain enzymatic activity. In general, Ub ligases have the capacity to go through large-scale conformer switching in the course of their catalytic cycles that may remodel the domain interfaces [34]. The exposure of the RING domain [35,36] as a phosphorylation-dependent process and release of the auto-inhibition for RING and HECT E3 ligases have been shown [35,37].

Phosphorylation of Parkin at Ser^{65} induces structural changes

Ser^{65} of Parkin within its UBL domain localizes to a cleft formed by the UBL-linker interface. Phospho-modification of Parkin at this residue triggered an initial structural change within the vicinity (i.e. 'opening' of the cleft) that eventually resulted in release of the N-terminus from its close contacts with RING-1 and IBR (in-between-RING) domains and further propagated into greater structural changes [30]. In addition, several mutations of Ser^{65} were tested to imitate/block the effect of the phosphorylation, which resulted in various cleft-widening gaps that allowed increasing (phospho-mimic, glutamic acid and aspartic acid) or decreasing (phospho-dead, alanine) aqueous solvation of the interface during MDS sampled conformational paths [30] (Figure 1B). Pocket hydration and SASA (solvent accessible surface area) calculations of the internal pocket were determined demonstrating the pronounced effect from phosphorylation at Ser^{65} (Supplementary Movie S1) that were confirmed by cell-based analysis [30].

Figure 1 | Structural modelling of full-length human Parkin protein and MDS studies



(**A**) Full-length structural model for human Parkin protein [30]. The 3D structural domains are colour matched to with UBL in red, linker in gray, RING-0 in green, RING-1 in cyan, IBR in purple, REP in yellow and RING-2 in pink. Structure is rendered in ribbon-cartoon with semi-transparent solvent-accessible surface area overlaid to illustrate protein bulk structure. Each domain and discussed mutant residue is labelled. (**B**) Critical effect of phosphorylation at Ser^{65} is shown for Parkin structure. Left panel is early in MDS sampling and right panel is after > 120 ns of MDS. The effect on the cleft between the linker and UBL domains is shown. (**C**) Mutation in the active site region for Parkin is shown. Nearby residues (< 4Å) that may alter binding are shown in licorice rendering. The zinc finger ions are shown as cyan spheres. Mutation of $G^{430}D$ alters electrostatic distribution for the adjacent catalytic centre Cys^{431}.

Subsequent large-scale MDS led to the generation of > 30000 conformers for a pathway describing the complete dissociation of the UBL domain from the auto-inhibited conformation through an 'open' and activated form of Parkin [30]. Various inter-domain parameters on the structure [RMSD, RMSF (root mean square fluctuation), distance based metrics] indicate that several 'safety belts' which hold Parkin in its 'closed' conformation are released prior to triggering E3 ligase activity [30]. For activation of Parkin, the following key regions are released: UBL-linker region dissociates from the RING-1 and IBR domains; the REP region is loosened of its RING-1 contacts (Tyr^{391} to Cys^{238} distance increases) making the E2-binding site available for association of an Ub-loaded coenzyme and the RING-0 domain is released resulting in increased hydration around the active site residue Cys^{431} (Figure 1C). It is assumable that these three 'safety belts' are triggered sequentially [30].

Various MD simulations were conducted using phospho–Ser^{65} ($pSer^{65}$) and Ser^{65} to study the motion of the auto-inhibited structure that leads to an active conformation of the E3 enzyme. The molecular motion required for release of the auto-inhibitory structure was furthered using accelerated molecular dynamics and sampling algorithms, like Maxwell's demon molecular dynamics [32,33], which allows for speeded sampling along conformation space. In the cases where the structure is between two known conformers, sampling algorithms are quite efficient and useful. A large set of conformers was generated for analysis that revealed critical interactions at the 'safety belts' to be triggered step-wise during release of the UBL domain and motion away from IBR toward RING-2. Particularly of note, the generated data indicated that the REP region becomes unbound from E2 region, thus allowing an incoming E2 enzyme greater access to negotiate binding at the E2 site.

E2 coenzyme usage of Parkin

By scoring Parkin translocation upon siRNA-mediated knockdown of specific E2 enzyme using high content imaging (HCI), several coenzymes were identified that either positively or negatively regulate Parkin activation, translocation and enzymatic functions during mitochondrial quality control [24]. Ub-conjugating enzyme E2 (UBE2D) family members and UBE2L3 act redundantly and are necessary for charging of Parkin with Ub and thus its translocation to damaged mitochondria. Using the HCI scoring system, also knockdown of UBE2N was found to exhibit a minor effect on Parkin translocation to mitochondria, although it primarily affected clustering of mitochondria around perinuclear regions as determined in follow-up assays. UBE2N was not required for charging of Parkin with Ub, but rather cooperatively acted together, but downstream of UBE2D and UBE2L3. Another recent study has come to the same overall conclusion that UBE2N, UBE2D and UBE2L3 are required for efficient execution of Parkin-dependent mitophagy, although the authors do not attribute this effect to a delay in translocation but to the inhibition of autophagy [38]. At least for UBE2N this has been questioned recently [39]. Interestingly, knockdown of UBE2R1 enhanced Parkin translocation and increased mitochondrial clustering [24]. Although the exact molecular mechanism remained elusive, the existence of such negative regulation of Parkin by Ub signalling may open up further avenues for a targeted activation of Parkin.

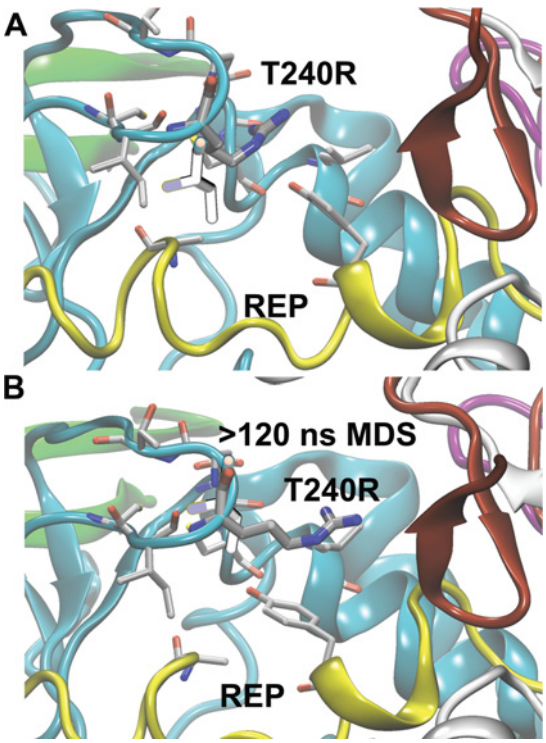
A role for UBE2D and UBE2L3 for Parkin auto-ubiquitination had already been established [23]. Kazlauskaite et al. [17] performed a comprehensive *in vitro* ubiquitination assay with mitochondrial Rho GTPase 1 (Miro1) as a substrate. In addition to UBE2D and UBE2L3 several other E2 enzymes (UBE2E1/2/3, UBE2H, UBE2J1/2, UBE2K and UBE2W) showed a much higher activity in the presence of functional PINK1. In contrast with another study, a prominent role of UBE2A for Parkin-dependent mitophagy [40] could not be established. The genome-wide siRNA screen by Hasson et al. [41] that monitored Parkin translocation, revealed several E2 enzymes. In addition to UBE2D and UBE2L3, the endoplasmic reticulum (ER)-anchored UBE2J2 that has been implicated in ER-associated degradation (ERAD) pathway and the orphan E2 UBE2O were identified. It is thus possible that additional E2 enzymes play important roles during Parkin-mediated mitophagy. In fact, it seems that specific E2 enzymes operate at distinct steps during this process and probably with different purposes.

Effect of PD mutations on Parkin structure and function

Recent work from Springer and co-workers (Fabienne C. Fiesel, Thomas R. Caulfield, Elisabeth L. Moussaud-Lamodiere, Daniel F.A.R. Dourado, Kotaro Ogaki, Owen A. Ross, Samuel C. Flores, and Wolfdieter Springer, submitted) examined 21 *PARKIN* mis-sense mutations distributed across all individual domains of Parkin, including $K^{161}N$, $K^{211}N$, $T^{240}R$, $R^{275}W$ and $G^{430}D$ that were modelled on

Figure 2 | Molecular dynamics effect on Parkin REP region from $T^{240}R$ mutation

(**A**) Initial orientation of Arg^{240} is shown pointed away from REP into aqueous. Rendering is shown in licorice (stick) with colouring in standard (carbon-gray, oxygen-red, nitrogen-blue, sulfur-yellow). For reference, original position for Thr^{240} is shown in Goodsell style. (**B**) After 120 ns of MDS, the Arg^{240} residue engages in repeated interactions with residues from REP. Rendering same as in (**A**).



pSer^{65} Parkin protein structure (Figure 1A). In order to pinpoint the exact dysfunctions of individual Parkin mutations during the sequential process of Parkin activation and mitophagy, local and global conformational changes that occur in the newly published structural model using MDS [30] were examined in addition to functional defects. Findings from this study indicate that $K^{161}N$ and $K^{211}N$ only minimally induce conformational change. Pathogenic effect of these mutations is likely to be the result of mutating the positively charged residues to uncharged asparagine that may lower the affinity of a presumed phospho-binding site in RING-0 to incoming phospho–Ub moieties. In contrast, $T^{240}R$ located in the E2-binding site showed major local conformational changes that probably result in stabilization of the REP region with the adjacent domains via an arginine-to-REP region interaction (Fabienne C. Fiesel, Thomas R. Caulfield, Elisabeth L. Moussaud-Lamodiere, Daniel F.A.R. Dourado, Kotaro Ogaki, Owen A. Ross, Samuel C. Flores, and Wolfdieter Springer, submitted), rather than directly disrupting the E2-binding site (Figures 2A and 2B). The $R^{275}W$ mutation created a swatch of local and global conformational changes that propagate throughout the entire structure (Fabienne C. Fiesel, Thomas R. Caulfield, Elisabeth L. Moussaud-Lamodiere, Daniel F.A.R. Dourado, Kotaro Ogaki, Owen A. Ross, Samuel C. Flores, and Wolfdieter Springer, submitted). The $G^{430}D$ mutation only seemed to alter the active site by delocalizing the attractive charge for Cys^{431}, which would consequently lower the catalytic efficiency for the reaction with substrates. HCI analysis to measure Parkin translocation to damaged mitochondria and complementary enzymatic activity measurements (specifically Ub-charging experiments with and without carbonyl cyanide m-chlorophenylhydrazone (CCCP) treatment) were used to demonstrate agreement within the dataset (Fabienne C. Fiesel, Thomas R. Caulfield, Elisabeth L. Moussaud-Lamodiere, Daniel F.A.R. Dourado, Kotaro Ogaki, Owen A. Ross, Samuel C. Flores, and Wolfdieter Springer, submitted).

Concluding remarks

Parkin is an auto-inhibited Ub E3 ligase with roles in the regulation of mitochondrial quality control mediated by both proteasome and autophagy/lysosome degradation systems. Although Parkin acts broadly neuroprotective, inactivation/inhibition through mutations or post-translational modifications results in PD. Given the complex and sequential activation process of Parkin that involves phosphorylation and consequent structural changes, several distinct dysfunctions can result in its loss of function. Structure–function studies now provide the chance not only to dissect the regulation and enzymatic activity of Parkin but also to identify chemical and genetic modifiers that may be useful to activate Parkin and/or to maintain an active conformation. Current work is underway to identify, discover and design small molecules that opportunistically take advantage of the 'safety belts' critical for Parkin's auto-inhibition. Moving forward with a complete understanding of Parkin's activation, and the ability to develop efficacy, ADME (adsorption, digestion, metabolism and excretion) and improved target specificity with cell-based and *in vivo* experiments, will be a key determinant in future drug discovery efforts.

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