Structural Biology: Parkin's Serpentine Shape Revealed in the Year of the Snake

Parkin is an E3 ubiquitin ligase, mutations in which are responsible for autosomal recessive juvenile parkinsonism. Recently, several structures of Parkin have been solved, revealing its serpentine shape and modes of auto-inhibition.

Katja K. Dove and Rachel E. Klevit*

E3 ubiquitin ligases play key roles in subcellular signaling pathways and malfunctions in these proteins can have dramatic effects on human health. Since the discovery of the E3 ligase Parkin in 1998, intense efforts to define its biological function and its role in the development of Parkinson's disease have led to an emerging picture in which Parkin exists in an inactive state that must be activated to localize it to mitochondria and to unleash its enzymatic activity. Parkin belongs to a small but important class of E3 ubiquitin ligases — RING-between-RING (RBR) E3 ligases — that combine elements of both RING and HECT E3 ligases to catalyze substrate ubiquitination [1]. The defining feature and minimal catalytic unit of RBR E3 ligases is the RBR domain, which consists of two Zn^{2+}-binding RING domains (R1 and R2) and an intervening in-between-ring (IBR) domain. The mechanism characteristic of RBR ligases combines interactions of the R1 domain with ubiquitin-conjugating enzymes (E2s), reminiscent of RING-type E3 ligases, with a subsequent transfer of ubiquitin onto an active-site cysteine (Cys) contained within the R2 domain to form an E3~Ub thioester intermediate (similar to HECT-type E3 ligases). In addition to its carboxy-terminal RBR domain, Parkin contains an amino-terminal ubiquitin-like domain (UbID) followed by a RING0 (R0) domain that directly precedes the R1 domain (Figure 1A) [2]. Given the intense biomedical interest, it is remarkable that Parkin has eluded structural biologists for so long. This may be due in part to Parkin's unusually high Cys content — a feature that makes generation of homogenous, recombinant Parkin challenging. Almost 10% of its amino acids are Cys, compared with the proteomic average frequency of 2%. Of the 35 Cys residues in human Parkin, 28 coordinate eight Zn^{2+} ions, Cys431 is the ligase active site, and the remaining six have unknown function. The almost simultaneous report of Parkin structures by four groups [3–6] is therefore a significant milestone in the field.

In these new papers, four crystal structures of Parkin R0–RBR constructs have been solved (pdb 4K7D [3], pdb 4I1H and 4I1F [4], pdb 4BM9 [5]), together with a crystal structure of full-length Parkin at lower resolution (Figure 1B, pdb 4K95 [3]), and an NMR structure of the R2 domain (pdb 2LWR [6]). Despite different crystallization conditions, the structures are highly similar. The five structured domains are well defined, while some of the intervening sequences between domains lack density in the crystals, indicative of disordered protein regions. Several remarkable features are revealed. First, the RBR domain does not appear as a single structural unit that can be viewed in isolation. Rather than contacting one another, the R1, IBR, and R2 domains are intertwined with the UbID and R0 domains to create a Parkin molecule that is folded back onto itself, loosely resembling a coiled snake (Figure 1C,D). Of particular note, R0 (cyan) is inserted between R1 (dark blue) and R2 (orange) and occludes the active site on R2 or, in the coiled snake analogy, Parkin has its 'fangs' (i.e., the active site, red spheres in Figure 1) tucked away under R0 (cyan). This arrangement suggests an auto-inhibited state, an emerging theme among RBR E3 ligases [7]. Yet, the strategies utilized by different RBR E3 ligases to achieve auto-inhibition vary. The RBR E3 HHARI has its own domain (the Ariadne domain) that is also positioned to occlude the active site. The Ariadne domain is structurally distinct from R0 and is positioned carboxy terminal to the RBR domain [8]. Another example, the RBR E3 HOIP — a member of the linear ubiquitin chain assembly complex known as LUBAC — is auto-inhibited by an amino-terminal UBA domain, although the structural details of this interaction have yet to be elucidated [9,10]. Clearly, there are numerous strategies by which the active sites of RBR E3 ligases are auto-inhibited.

A second remarkable feature is the linker between the IBR and R2 domains (amino acids 378–414), most of which is not visible in the crystal structures, consistent with it being disordered. Solution NMR studies confirm its flexibility [6], emphasizing that the IBR and R2 domains are able to assume a range of positions relative to each other. Intriguingly, a central portion of the linker (amino acids 391–403; yellow region in Figure 1) adopts a helical structure that lies across the putative E2-binding site on R1. This element is dubbed a 'tether' [4] or 'REP' (repressor element of Parkin) [3], as its position suggests that it prevents E2 binding. Indeed, E2 binding is enhanced when contacts between the REP and R1 are disrupted by mutations, but a construct consisting of R1–IBR–REP–R2 is highly active in auto-ubiquitination assays. This suggests that the contact between R1 and REP may serve to modulate ligase activity rather than inhibit it completely. Consistent with this notion, Trempe *et al.* [3] show by solution NMR that the R0–RBR Parkin construct as was crystallized is able to bind to the E2 UbcH7 with moderate affinity — a reminder that these crystal structures are snapshots of a single conformation of a presumably dynamic molecule.

The structures confirm that the R0, R1, IBR, and R2 domains each coordinate two Zn^{2+} ions [2] and identify the eight residues in each domain that serve as ligands. As predicted, the R1 domain assumes a fold similar to a canonical RING domain. The R2 domain, however, does not resemble a canonical RING fold, which consists of a characteristic cross-brace pattern of Zn^{2+} ligands. Rather, the R2 domain is similar in structure to IBR domains, which sequentially arrange Zn^{2+}-liganding residues. The R0 domain coordinates two Zn^{2+} ions in a hairpin arrangement, adopting a unique fold that has previously been dubbed the unique Parkin domain (UPD) [11]. Although

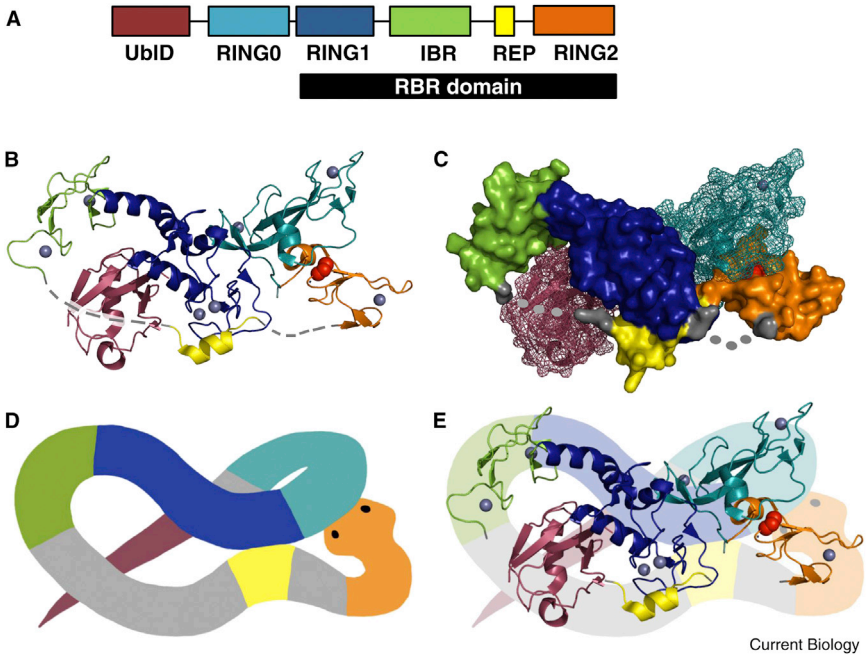

Figure 1. The structure of Parkin reveals a serpentine shape.

(A) Domain architecture depicting the linear arrangement of the five Parkin domains. The RBR domain consists of R1–IBR–REP–R2 as indicated by the black bar. (B) A ribbon and (C) surface/mesh representation of the full-length crystal structure of rat Parkin (pdb 4K95, [3]). Each domain is colored according to the diagram above. In (C), the three domains that make up the RBR domain (R1, dark blue; IBR, green; and R2, orange; shown as solid surfaces) are intertwined with the UbID (maroon, mesh) and R0 (cyan, mesh). The active site Cys431 (indicated by the red spheres) is occluded by R0 (cyan). Unstructured linker regions on either side of the REP (yellow) are not visible in the crystal structure. The connection between IBR and R2, grey lines (B) or dots (C) were drawn freehand to represent the unstructured linker regions. (D) The structure of Parkin folds back onto itself, loosely resembling a coiled snake. Each colored segment represents the matching domains using the colors above. The snake model illustrates that: the active site (mouth of the snake, orange) is hidden behind R0 (cyan); the REP (yellow) contacts R1 (dark blue) at the proposed E2-binding site (not shown); and the UbID (maroon) binds to the backside of R1. (E) Overlay of the full-length crystal structure of rat Parkin and the snake model. (We thank Jill M. Hoyt for the drawing of the snake.)

the R1 domain resembles a canonical RING domain, its function is different in at least one important way: the R1 domain lacks a critical E3/E2 hydrogen-bonding residue that typically follows the last Zn^{2+}-coordinating residue. This interaction is required for allosteric activation of E2∼Ub to transfer ubiquitin to a lysine residue [12]. Parkin contains a proline at this position, consistent with the report that RBR E3 ligases, and particularly the R1 domain, do not activate E2∼Ub conjugates [1,12].

The new studies further validate that mutations of Cys431 unequivocally abrogate Parkin activity both *in vitro* and in cell-based assays, and they provide an atomic-level glimpse at Parkin's active site, albeit in the context of an inhibited conformation that displays low activity *in vitro*. Several authors note that His433 and Glu444 are in close proximity to Cys431, identifying a potential catalytic triad in which the pKa of Cys431 is suppressed, rendering it highly reactive [4,5]. Such enhanced intrinsic reactivity explains the observation that some minimal R2 constructs of HHARI and HOIP exhibit some latent activity *in vitro* [9,13]. Moreover, it may also explain why RBR E3 ligases have evolved to adopt an auto-inhibited conformation, while their HECT counterparts are not usually found to be auto-inhibited. Although mutations of His433 and Glu444 decrease intrinsic reactivity of Cys431 *in vitro* [3–5], only His433 mutants significantly impair Parkin function in cell-based assays [4].

Such disparities raise the specter of conflicting reports of Parkin activity and function in the literature. There are likely several reasons that contribute to these discrepancies. Different Parkin constructs exhibit variations in activity levels *in vitro* and the presence of large amino-terminal tags can also alter the level of observed activity [3,5,14]. Furthermore, differences in *in vitro* and cell-based assays arise from the dearth of understanding regarding the process by which Parkin is activated in cells. Lazarou *et al.* [15] have shown that a ligase-active RBR-only construct can localize to mitochondria to which an active-site-dead Parkin is tethered. However, this process still requires treatment with the mitochondrial uncoupler CCCP. This suggests that simply unleashing the ligase activity of Parkin is not sufficient for mitochondrial recruitment, and that other, still unknown factors aid in this process. In sum, the previous experience of the field suggests that care should be used in the design and interpretation of experiments using forms of Parkin other than the full-length native protein.

Mutations in PARK2 (Parkin) account for a large fraction of the identified Parkinson's disease (PD) mutations, making them an important target for drug development. Parkin presents unique challenges as a drug target as it is highly complicated from a structural and functional point-of-view. For example, PINK1, which is also mutated at high frequency in PD, encodes a much larger protein, but the majority of mutations are harbored within a single protein domain, the kinase domain. In contrast, PD-associated mutations in Parkin are distributed throughout its five domains, highlighting the multi-faceted functions of Parkin. The structure of Parkin in an auto-inhibited state with its intertwined domains makes clear that large rearrangements are required for its catalysis of ubiquitin transfer. Thus, a given mutation may affect each Parkin state in a different way, making it difficult to predict its consequences.

There are likely four non-mutually exclusive outcomes of mutations in Parkin: destabilization or a decrease in Parkin solubility; decrease in ligase activity; increase in ligase activity; and alteration of Parkin's recruitment to mitochondria. Destabilizing/solubility mutations include Zn^{2+}-binding residues and others that destabilize the folded Parkin structure [4,6]. Such mutations can be detrimental by effectively decreasing the pool of functional Parkin or may be toxic due to an increased tendency to aggregate.

The defining ligase-dead mutation is a substitution of active site Cys431. Other known PD-associated mutations that decrease Parkin's ligase activity *in vitro* include the putative catalytic triad R2 residue Glu444 and residues in R1 that are predicted to affect E2 binding such as Thr240. Mutations that affect recognition and/or binding of substrate to Parkin may also manifest as decreased ligase activity mutants, although identification of a putative substrate-binding domain remains elusive. A PD-associated mutation that enhances Parkin ligase activity *in vitro* occurs at Ala398, located at the interface between R1 and REP [5]. Other synthetic, structure-based mutations that disrupt inter-domain contacts and thereby release auto-inhibition are Trp403Ala, Phe463Ala, and Trp183Ala [3–5]. The cellular consequences of Parkin-activating mutations remain to be seen. To date, no PD-associated mutations have been demonstrated to affect Parkin recruitment to mitochondria without affecting ligase activity. Recent advances in the structural biology of Parkin afford the ability to understand PD-associated mutations structurally and confirm structural outcomes of a variety of PD-related mutations.

Serendipitously, in the year of the snake, several structures of Parkin have been solved, revealing its serpentine shape. These structures provide a long overdue foundation on which to build a sound understanding of Parkin function both in the interpretation of current literature and as a guide for the design of future studies. The structure suggests that, for Parkin to become an active ligase, all five of its domains must undergo substantial rearrangements: R0 must dissociate from R2 to expose the active site; R2 must move towards R1; the REP must dissociate from R1 to enhance E2 binding; and the UbID may have to move away from R1. What molecular process(es) precipitate(s) these events (i.e., the uncoiling and possibly, recoiling of the snake) and the precise role of each domain remain questions of fundamental importance to our understanding of Parkin function. As the nature of these key processes becomes clear, future milestones in the structural biology of Parkin can hopefully be achieved in short order.

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Department of Biochemistry, University of Washington, Seattle, WA 98195, USA.
*E-mail: klevit@uw.edu

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