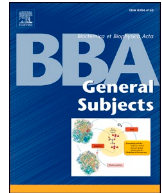
BBA - General Subjects 1865 (2021) 129894

Contents lists available at ScienceDirect

BBA - General Subjects

journal homepage: www.elsevier.com/locate/bbagen



Review

A mechanistic review of Parkin activation

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A R T I C L E I N F O

Keywords:
Parkin
RBR
E3
Autoinhibition
Ubiquitylation

A B S T R A C T

Parkin and phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) constitute a feed-forward signalling pathway that mediates autophagic removal of damaged mitochondria (mitophagy). With over 130 mutations identified to date in over 1000 patients with early onset parkinsonism, Parkin is considered a hot spot of signalling pathways involved in PD aetiology. Parkin is an E3 ligase and how its activity is regulated has been extensively studied: inter-domain interactions exert a tight inhibition on Parkin activity; binding to phosphoubiquitin relieves this auto-inhibition; and phosphorylation of Parkin shifts the equilibrium towards maximal Parkin activation. This review focusses on recent, structural findings on the regulation of Parkin activity. What follows is a mechanistic introduction to the family of E3 ligases that includes Parkin, followed by a brief description of structural elements unique to Parkin that lock the enzyme in an autoinhibited state, contrasted with emerging models that have shed light on possible mechanisms of Parkin activation.

1. Introduction

Parkinson's disease (PD) was first characterised in 1817 based on a triad of motor symptoms: tremor, rigidity and bradykinesia. Today, PD constitutes the most common movement disorder, affecting 0.1% of the global population, with the prevalence increasing to 4% with advancing age [1,2]. Motor symptoms in PD stem from loss of dopaminergic neurons in the substantia nigra pars compacta, yet despite this understanding of the pathophysiology there is a lack of available biomarkers for clinical use. Thus, accurate diagnosis of patients often occurs only once more than 30% of all nerve cells in the pars compacta have been lost, after which point the motor symptoms typically appear [3]. There is no cure for PD, with available treatments limited to symptomatic management options that may only provide benefit briefly, before losing their efficacy [4].

A breakthrough in understanding of PD molecular mechanisms came with an earlier observation that exposure to a mitochondrial toxin can cause a rapid-onset PD, implicating mitochondrial dysfunction in PD pathology [5]. Subsequently, numerous studies have associated a combination of genetic mutations on 11 genes with heritable forms of PD [6]. However, mutations at so-called "PARK" loci are estimated to underly the pathogenesis of <5% of all PD cases. Remarkably, the evidence on genetic predispositions and environmental factors both highlighted that the aetiology of PD involves proteins implicated in maintenance of mitochondrial function or degradation of dysfunctional mitochondria [7].

Parkin (*PARK2*) and phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1; *PARK6*) may harbour mutations that cause an autosomal recessive form of early onset PD, where the age at onset can be below 20 [8–10]. With over 130 distinct mutations identified in over 1000 patients, and observed in 20–71% of all cases, biallelic Parkin mutations are the most common cause of early onset PD [11–13]. Thus, Parkin has been under major investigation with the aim of identifying cell signalling pathways involved in PD aetiology.

Parkin is a cytosolic member of the Really Interesting New Gene (RING)-in-between-RING (IBR)-RING (RBR) ubiquitin (UB) ligase family with an N-terminal UB-like (UBL) domain, while PINK1 is a protein kinase with mitochondrial targeting sequence [10,11]. The protein ubiquitylation cascade and Parkin topology are discussed in greater detail in Sections 2 and 3, respectively. The functions of these proteins are linked in a feed-forward signalling pathway that mediates autophagic removal of damaged mitochondria (mitophagy). In this pathway, depolarisation of mitochondria prevents constitutive, presenilin-associated rhomboid-like protein (PARL)-protease-dependent degradation of PINK1, allowing PINK1 to accumulate specifically on dysfunctional mitochondria [14,15]. PINK1 then phosphorylates UB chains linked to outer mitochondrial membrane (OMM) proteins [16–18]. This Ser65-phosphorylated UB (pUB) recruits Parkin to mitochondria, where

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https://doi.org/10.1016/j.bbagen.2021.129894
Received 17 January 2021; Received in revised form 5 March 2021; Accepted 18 March 2021
Available online 20 March 2021
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PINK1 can subsequently phosphorylate Parkin's UBL domain at Ser65, at a site homologous with the PINK1 UB phosphorylation site [19–21]. While cytosolic Parkin displays attenuated activity, once bound to pUB and phosphorylated by PINK1, the auto-inhibition of Parkin is fully relieved [22,23]. Upon activation, Parkin modifies various OMM proteins, predominantly with K11- and K63-linked UB chains [24]. Parkin-dependent ubiquitylation of OMM proteins generates more substrate for PINK1, thus increasing the pool of pUB on damaged mitochondria and closing the feed-forward loop. Ultimately, (p)UB-chains on mitochondria recruit autophagy receptors and promote autophagosome assembly at the damaged mitochondria [25–27].

PINK1-Parkin signalling is well understood and has recently been reviewed in greater detail elsewhere [28,29]. Aiming to avoid redundancy with recent, extensive reviews on Parkin signalling, the focus will be on recent, structural findings on the regulation of Parkin activity. What follows is a short, mechanistic introduction to the RBR type E3 ligase family that includes Parkin (Section 2), followed by a brief review of the structural elements unique to Parkin that lock Parkin in an autoinhibited state (Section 3), contrasted with emerging models that have shed light on possible mechanisms of Parkin activation (Section 4).

2. Hybrid mechanism of ubiquitin transfer

Ubiquitylation is a post-translational modification involving the concerted actions of UB-activating (E1), UB-conjugating (E2) and UB-ligating (E3) enzymes. The human genome harbours two E1, approximately 40 E2 and over 600 validated E3 enzymes [30]. The E1 activates the C-terminus of UB, triggering transfer of UB onto the E2 catalytic cysteine [31,32]. Once "loaded" with UB, the E2 ~ UB conjugate (where ~ denotes thioester linkage) functions as a UB transporter [33]. Transfer of UB onto substrates, however, requires E2 ~ UB conjugates to pair with E3s, which ensure substrate selection and catalyse transfer of UB onto the substrate lysine. Substrate proteins can be modified with a single UB on a single or multiple lysine residues, termed mono, or multi-mono ubiquitylation. Moreover, the UB moiety on the substrate protein can stimulate the formation of UB chains through one of its seven internal lysine residues, termed poly-ubiquitylation [34]. Protein ubiquitylation can thereby generate myriad structurally distinct signals that trigger various cell signalling events ranging from proteasomal degradation [35] and DNA repair [36–39] through to lysosomal targeting [40].

E3 ligases play a key role in directing substrate specificity and triggering transfer of UB from E2 ~ UB conjugates onto specific substrate lysine residues. The large number of E3 ligases can be categorized into three groups on the basis of the reaction mechanism they employ: RING type, HECT (Homologous to E6-AP C-Terminus) type, and RBR type [41]. The RING type E3 ligases act as a scaffold, binding to E2 ~ UB and catalysing direct transfer of UB from E2 ~ UB to a lysine residue in the substrate [42–44]. HECT type E3 ligases, on the other hand, follow a different mechanism, where the N-terminal lobe of the HECT domain recruits the E2 ~ UB conjugate and mediates transfer of the UB cargo onto the catalytic cysteine residue of HECT C-terminal lobe, thereby forming an E3 ~ UB thioester linkage prior to substate ubiquitylation [41,45–47]. The RBR E3 ligase family, which Parkin belongs to, were originally defined based on their three Zn^{2+}-coordinating RBR subdomains: (1) RING1 domain, which shares homology with canonical RING domains and engages the E2 ~ UB; (2) IBR domain, which regulates activity by binding allosteric UB molecules; and (3) RING2, which contains a catalytic cysteine to form an E3 ~ UB [48,49]. RBR type E3 ligases display a RING/HECT hybrid mechanism that mediates substrate ubiquitylation, whereby RBR RING1 interacts with the E2 ~ UB conjugate in a RING E3-like fashion, but with a requirement for the donor UB to be transferred onto the RBR RING2 catalytic cysteine prior to substrate ubiquitylation in a manner similar to HECT-type E3s [50]. Of note, IBR and RING2 domains display a conserved fold (2.6 Å RMSD between 40 overlapping C_a atoms between IBR and RING2) [51] that is distinct from that of canonical RING domains (8.5 Å RMSD between 32 overlapping C_a atoms between Parkin RING2 and RING finger protein 4 [RNF4] RING domain [PDB: 4AP4]) [25,52]. Reflecting this, alternative nomenclature highlighting the function of these domains has been proposed, with the IBR and RING2 domains termed the benign-catalytic (BRcat) and required-for-catalysis (Rcat) domains, respectively [53].

Although a common feature of all E3 ligases is the ability to interact with an E2 ~ UB conjugate, E2 ~ UB conjugates are highly dynamic in solution and can adopt an array of conformations. Often, the conformation of the E2 ~ UB conjugate is influenced by the presence of an E3 ligase [54–56]. For instance, whilst in solution UBE2D2 ~ UB forms a highly dynamic and extended "open" conformation with only transient interaction surfaces observed between UB and E2 [55,57]. Alternatively, UBE2L3 ~ UB, which discharges UB preferentially to cysteine [50], prefer a "closed" arrangement [58] where the hydrophobic patch formed around UB Ile44 interacts directly with the E2 crossover helix, first identified for yeast Ubc1 ~ UB [59]. In contrast, E2 ~ UB conjugates in the presence of a RING-type E3 enzymes such as baculoviral IAP repeat containing protein 7 (BIRC7) or RNF4 are typically stabilized in the "closed" conformation [25,57,60], while UBE2L3 and UBE2D conjugates switch to an "open" state in the presence of the RBR E3 ligases Heme-oxidized IRP2 UB ligase 1 (HOIL-1)-interacting protein (HOIP), Human Homolog of Ariadne (HHARI) and Parkin.

Induction of a closed E2 ~ UB conformation is a key feature of the RING E3 ubiquitylation mechanism. In the RING-type E3:E2 ~ UB complex, the contact surface between the E2 and RING E3 proteins is approximately 15 Å away from the E2 active site and includes Zn^{2+}-coordinating loops and the RING central helix (Fig. 1A). A conserved residue at the C-terminal end of the RING core, typically arginine, lysine, or asparagine, interacts with the E2 carbonyl backbone as well as the UB tail, serving as a so-called "linchpin" in RING E3:E2 ~ UB complexes. The linchpin residue contributes to stabilisation of E2 ~ UB in the "closed" conformation, allowing the substrate lysine to initiate a nucleophilic attack on the E2 ~ UB thioester and thus receive the UB [50,57,61].

RING1 domains in RBR E3 ligase family members such as Parkin, HOIP, HHARI are structurally similar to canonical RING domains and encompass a classical cross-brace arrangement and Zn^{2+}-coordination topology [44,62]. Nevertheless, RBR RING1 domains lack the linchpin mechanism required to stabilise the "closed" conformation of E2 ~ UB [63]. Moreover, the crystal structure of HHARI in complex with UBE2L3 ~ UB (PDB 5UDH) [63] reveals that the E3:E2 interface involves a loop on RING1 that is two residues longer than its canonical RING E3 counterparts, thereby preventing formation of the "closed" E2 ~ UB conformation by obstruction of the donor UB [63]. The crystal structure of HOIP:UBE2D2 ~ UB further supports this stabilisation of the E2 ~ UB in the "open" state [64,65]. While there is no structure of Parkin in complex with a E2 ~ UB conjugate, the aforementioned loop extension is conserved across all RBR E3 ligases [53], including Parkin, suggesting that promotion of an "open" E2 ~ UB conformation may be a common feature of all RBR E3 ligases (Fig. 1B) [58,60]. It is generally understood that by promoting "open" E2 ~ UB states, RBR E3s prevent direct UB transfer onto substrate lysine residues, thereby forcing the reaction mechanism to go through the Rcat catalytic cysteine (Fig. 1C) [64]. In line with this, substitution of HHARI RBR RING1 with a canonical RING imparts the ability to promote the "closed" E2 ~ UB conformation, conferring the synthetic RING/RBR hybrid the ability to catalyse direct substate ubiquitylation, without the need to utilise Rcat catalytic cysteine [64].

3. Regulation of Parkin by auto-inhibition and allosteric activation

Parkin-mediated ubiquitylation can only take place once Parkin RING1 engages E2 ~ UB and the donor UB is transferred from the E2 catalytic cysteine to Cys431 on Parkin Rcat, termed transthiolation

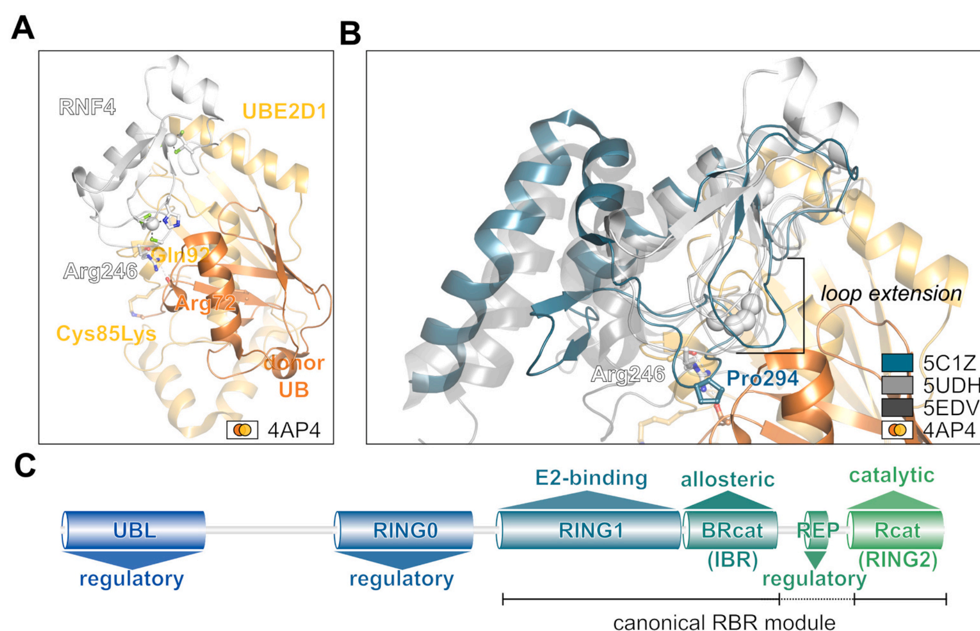

Fig. 1. Differing mechanism of E2 ~ UB recruitment by canonical RING E3s and RBR RING1.
(A) Ribbon representation of RNF4 RING domain bound to UBE2D1 ~ UB (PDB: 4AP4 [25]). Zn^{2+} ions are shown as white spheres and residues involved in their coordination are shown as sticks. Side chain of RNF4 linchpin Arg246 and backbone carbonyls of UBE2D2 Gln92 and UB Arg72 are also shown as sticks, with hydrogen bonding interaction between them depicted as white dashed lines. (B) Same as in (A), but with RING1 domains of Parkin (PDB: 5C1Z [73]), HHARI (PDB: 5UDH [63]) and HOIP (PDB: 5EDV [64]) superimposed on RNF4 RING domain. Parkin Pro294, corresponding to RNF4 linchpin Arg246 is shown as sticks. RING1 loop extension relative to RNF4 RING is also demarcated. (C) Parkin domain organisation diagram drawn to scale, illustrating Parkin UBL, RING0, RING1, BRcat (IBR), REP and catalytic Rcat (RING2) domains coloured from blue to green, respectively.

(Section 2) [67]. It has been extensively shown, however, that Parkin exists in a compact, autoinhibited conformation, where the UBL domain exerts an inhibitory effect on both E2 binding and transthiolation [68,69]. Auto-inhibition of Parkin transthiolation, with its allosteric release is discussed further in Section 4 in the context of prominent Parkin activation models. The focus of this section will be on how the

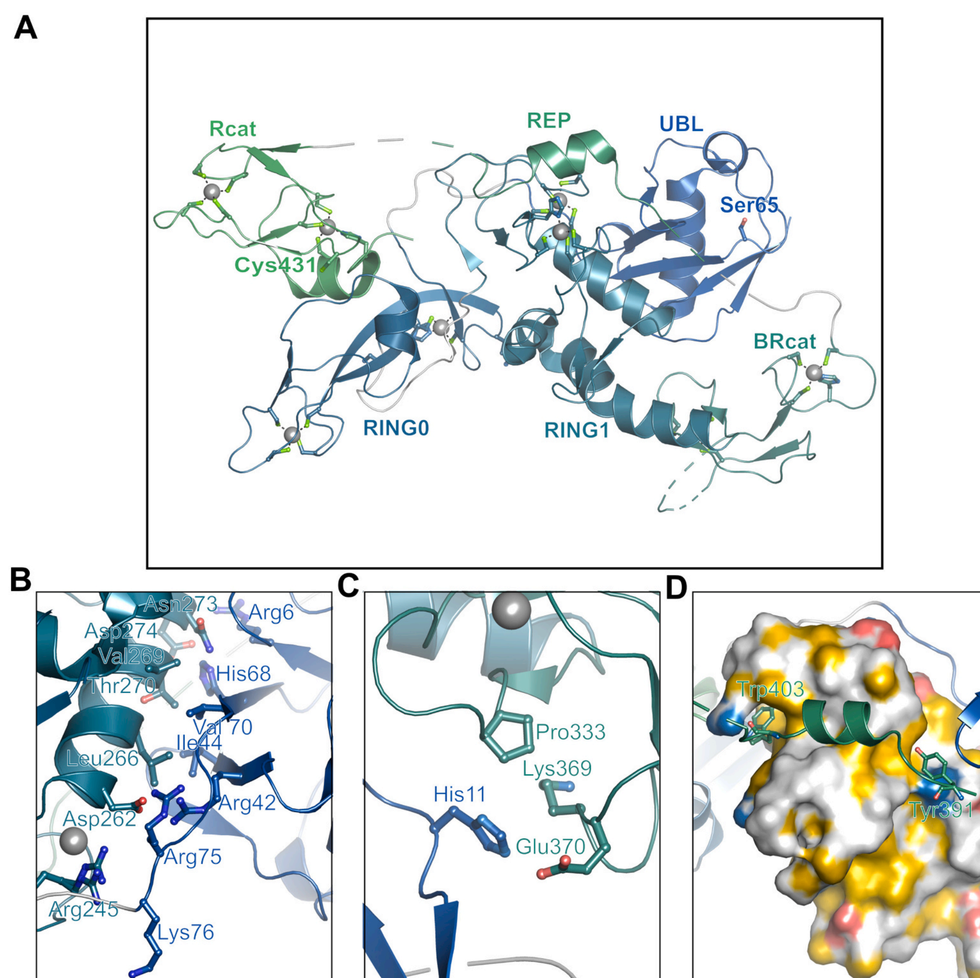

Fig. 2. Structure of Parkin in the inactive state.
(A) Ribbon representation of inactive Parkin (PDB: 5C1Z [93]) showing UBL, RING0, RING1, BRcat, REP and Rcat domains coloured from blue to green, respectively. Zn^{2+} ions are shown as white spheres and residues involved in their coordination are shown as sticks. UBL Ser65 and catalytic Rcat Cys431 are also shown as sticks. (B) Close-up of interface between UBL and RING1 domains. (C) Close-up of interface between UBL and BRcat domains. (D) Close-up of interface between REP and RING1 domains, with the RING1 surface shown and coloured to indicate hydrophobic, negatively charged and positively charged regions yellow, red and blue, respectively.

Parkin UBL domain and the linker connecting the BRcat and Rcat domains, termed as the repressor element (REP), occlude the E2-binding site on Parkin RING1 (Fig. 2A), and how dual-phosphorylation by PINK1 can relieve this autoinhibition [62,70,71].

3.1. Inhibition of E2 ~ UB binding

Several crystal structures have shown that the compact autoinhibited structure is primarily driven by large interfaces between the UBL domain and the rest of the structure [72,73]. The most extensive interface is defined by several UBL-RING1 contacts and includes hydrophobic interactions between UBL Ile44 and Val70 and RING1 Leu266, Val269 and Thr270 (Fig. 2B). Also, UBL Arg42 and RING1 Asp262 form a salt bridge, while UBL Arg6 and His68 tether RING1 Asn273 and Asp274. The N-terminus of the UBL domain is further stabilized by UBL Arg75 and Lys76 interacting with RING1 Arg245 (Fig. 2B). A further interface is formed between the UBL and BRcat domains, with UBL His11 interacting with BRcat Pro333, Lys369 and Glu370 (Fig. 2C). The compact packing of the Parkin UBL domain onto the RBR module also explains the earlier observation that N-terminal tags on Parkin may relieve autoinhibition [74–76]. N-terminally tagged Parkin displays *in vitro* activity due to interference with these inhibitory intramolecular interactions, with cleavage of the tag restoring auto-inhibition [77]. In line with this, while a fragment of Parkin composed of the RING0 and RBR domains (hereafter R0RBR) displays higher residual activity than wild-type Parkin, titration of UBL restores auto-inhibition of this fragment [73]. Point mutations that disrupt these inhibitory interactions activate Parkin both *in vitro* and in cells [72,78]. Moreover, presence of pathological PD mutations in the UBL domain, which abolish the autoinhibited conformation of Parkin, further confirm the importance of compact UBL-R0RBR packing for auto-inhibition of Parkin activity.

The other auto-inhibitory element blocking E2 recruitment on Parkin RING1 is the REP region. The REP helix is tethered between the two Zn^{2+}-coordination centres in Parkin RING1, with Tyr391 and Trp403 on either end of the REP helix protruding into hydrophobic patches found on RING1 surface and cementing this interaction (Fig. 2D). Mutation of either of these hydrophobic REP anchors disrupts the REP-RING1 interface and leads to Parkin activation [72].

3.2. Allosteric release by pUB-binding

As described above, Parkin activity is tightly regulated by inter-domain interactions. Recruitment of Parkin on pUB or direct phosphorylation of Parkin at the UBL domain can relieve the auto-inhibition of E2 ~ UB binding and transthiolation independently [18,20,21,23,79]. Maximal E3 ligase activity, however, requires synergistic effect of both events. [78,80] With over 10 crystal structures investigating different aspects of Parkin inhibition in the Protein Data Bank (PDB), the allosteric mechanisms behind Parkin activation are being revealed [44,62,68,69,72,73,81–83].

Central to the allostery behind the release of Parkin activity is the RING1 domain. RING1 is comprised of two lobes: the canonical cross-brace fold consisting of one central helix flanked by two zinc-coordinating β-hairpin loops; and the C-terminal extension unique to RBRs that fold into one short and one longer hairpin and kinked α-helix

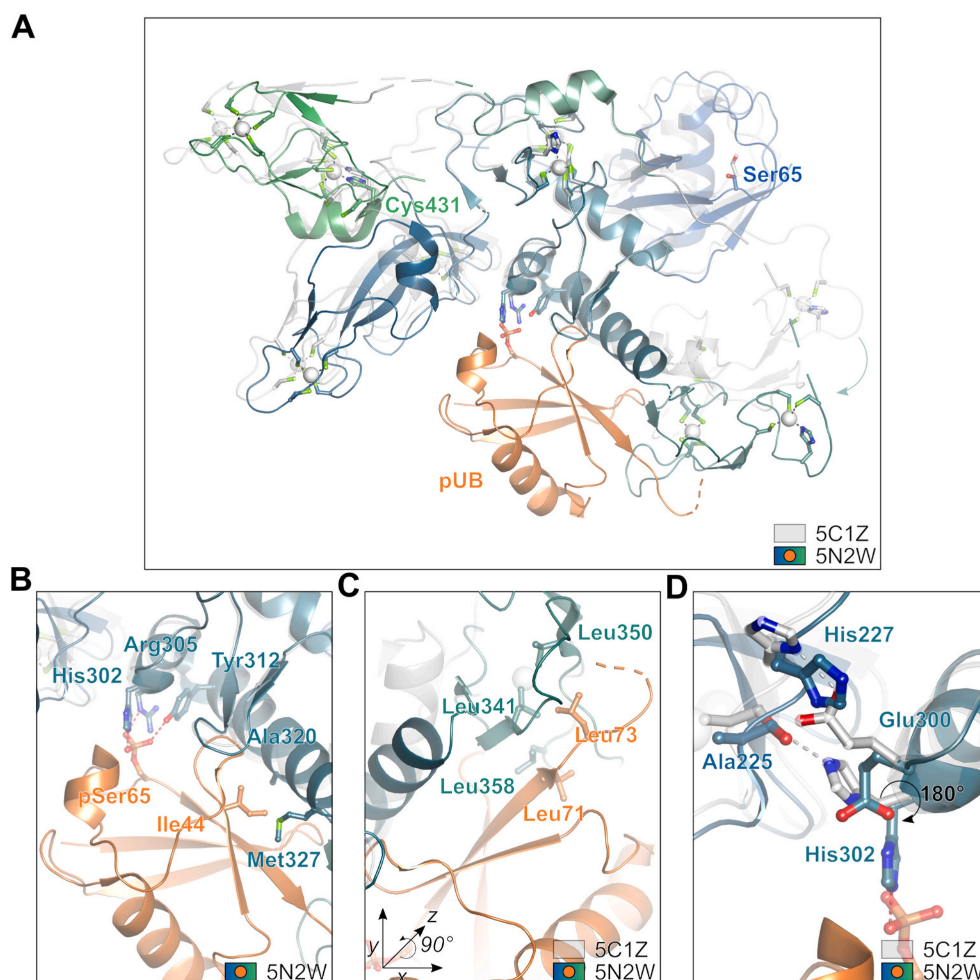

Fig. 3. Allostery behind Parkin activation by pUB-binding.
(A) Ribbon representation of Parkin:pUB complex (PDB: 5N2W [69]) coloured and viewed as in Fig. 2A, with pUB also shown as ribbons and coloured orange. Superimposition of inactive Parkin (PDB: 5C1Z [73]) is shown as white ribbons for comparison between inactive and pUB-bound states. Zn^{2+} ions are shown as white spheres and residues involved in their coordination are shown as sticks. UBL Ser65, Rcat catalytic Cys431, pUB pSer65, and RING1 residues involved in phosphate-coordination are also shown as sticks. (B) Close-up of the pUB coordination by Parkin RING1 helical extension region. (C) Close-up pUB C-terminal tail and Parkin BRcat interaction. Spatial relation between panels A and C is illustrated. (D) Close-up of the network of hydrogen bonds between Parkin RING0 and Rcat domains that is disrupted by pUB binding. Colouring of Parkin domains and pUB is as described in Fig. 2A. Spatial relations of panels A, B and C relative to Fig. 2A is illustrated where different.

(Fig. 1B) (hereafter RING1 helical extension). The former lobe mediates E2-binding, while the latter serves as an allosteric UB-binding site (Fig. 3A).

During pUB binding, His302, Arg305 and Tyr321 side chains at the N-terminus of the RING1 helical extension, near the RING0-RING1 interface, tether phospho-Ser65 residue from pUB, while at the C-terminus, nonpolar Ala320, Val324 and Met327 provide an interaction surface for pUB's hydrophobic Ile44 patch (Fig. 3B). One of the RING1 β-hairpins interdigitates the two helices near the RING1 helical extension, buttressing the interaction between the pUB Ile44 loop and the RING1 helical extension region. In this arrangement, the UB tail threads into the central hydrophobic grove in the Parkin BRcat domain, where the side chains of UB Leu71 and Leu73 insert between that of BRcat Leu358, Leu341 and Val350 (Fig. 3C). This interaction between the pUB tail and BRcat straightens the long helix in the RING helical extension and swings the BRcat domain approximately 20° away from RING1, thereby creating a deep cleft between RING1 and BRcat (**Fig. 3A** and **C**) [69,81,82]. The mobility of the BRcat domain had previously been noted from NMR dynamics data and comparison of multiple crystal structures [73]. Remarkably, opening of this cleft between RING1 and BRcat exposes a cryptic UB-binding region that is predicted to interact with the donor UB in the E2 ~ UB conjugate. This is discussed in greater detail in Section 3.3.

RING1 also serves as a conduit for hydrogen-bonding interactions that mediate crosstalk between pUB binding and UBL phosphorylation. When Parkin is autoinhibited, the RING1 His302 side chain hydrogen bonds with the RING0 Ala225 backbone carbonyl, with the adjacent RING1 Glu300 tethering RING0 His227. Binding of pUB causes the His302 side chain to swing by ~180°, thereby disrupting this hydrogen-bonding network (Fig. 3D). This weakening of the RING0-RING1 interface has a knock-on effect on RING1-UBL interface at the opposite side, increasing the likelihood of UBL phosphorylation. This is illustrated by the fact that while UBL can bind *in trans* to a fragment of Parkin composed of RING0-RBR with sub-micromolar affinity, no such interaction can be detected when Parkin is bound to pUB [73]. Not surprisingly, therefore, efficiency of Parkin UBL phosphorylation by PINK1 is markedly higher when Parkin is bound to pUB [82]. Reciprocally, introduction of a negative charge on Ser65 of Parkin UBL can disrupt the network of hydrogen bonds tethering the RING0-RING1 interface, increasing availability of the pUB binding site [73]. In line with this observation, Parkin displays sub-micromolar affinity for pUB, but when UBL domain is phosphorylated, this affinity is approximately 20-fold higher [69,81,82].

3.3. E2 ~ UB binding

The two inhibitory interactions that block E2 recruitment by Parkin RING1, involving the UBL and REP, appear intact in a complex of Parkin bound to pUB, its allosteric activator (Fig. 3A). Nevertheless, comparison of the Parkin:pUB and phospho-Parkin (pParkin) mimic Parkin-Ser65Asp:pUB complexes reveal how introduction of a negative charge on UBL Ser65 abolishes a key interaction that tether UBL and REP together and to RING1 [69]. The 17 amino acid linker connecting REP to the BRcat is partly ordered in the Parkin:pUB complex and can be seen threading between UBL and RING1. In this arrangement, the REP-BRcat linker residue Tyr391 interacts with Tyr267 on the central RING1 helix, while the nearby linker residue Gln389 is within van der Waals radius of Ser65 on UBL. Introduction of negative charge on UBL Ser65 disrupts this Gln389-Ser65 interaction, pushing the REP-BRcat linker out and destabilising the UBL-REP-RING1 interface (Fig. 4A) [69]. In line with this, while autoinhibited Parkin does not display detectable binding affinity towards E2s, pParkin:pUB binds UBE2L3 with low-micromolar affinity [69,73].

A recent study reported a crystal structure of phosphorylated *Bactrocera dorsalis* Parkin bound to pUB and in complex with UBE2L3 (hereafter p*Bd*Parkin:pUB:UBE2L3) [83], and R0RBR Parkin bound to pUB and a UBE2L3 ~ UB conjugate [65], confirming that E2 recruitment by activated Parkin follows what is observed in conventional RING E3-E2 interactions (Section 2) [84,85]. Namely, negatively charged residues on the Parkin RING1 central helix and the first Zn^{2+}-coordinating loops tether Arg5 and Arg7 within UBE2L3 N-terminal helix, while hydrophobic residues on the Parkin RING1 central helix and the second Zn^{2+}-coordinating loop interact with UBE2L3 Phe63 and Pro97 on loops 4 and 7, respectively (Fig. 4B). Both structures show that, when in complex with activated Parkin, the UBE2L3 active site points towards the deep UB-binding cleft between RING1 and BRcat (see Section 3.2). The structure of R0RBR:pUB:UBE2L3 ~ UB shows that Parkin stabilises the UB moiety in the "open" state, with the donor UB nestled in the RING1-BRcat cleft [65]. This cleft was first identified in a high resolution crystal structure of human Parkin complexed with pUB that showed this region between Parkin RING1 and BRcat is occupied by a UBL domain of a neighbouring, symmetry related Parkin molecule [69]. Secondly, superposition of the RING1 domain of a HOIP:UBE2D2 ~ UB complex onto Parkin RING1 positions the donor UB in this cleft (Fig. 4C) [86]. In this cryptic UB-binding pocket, Arg275 of the Parkin RING1 central helix and Tyr318 and Glu321 of the RING1 helical extension are proposed to coordinate the donor UB. Detailed mutagenesis and *in vitro* interaction studies have shown that pParkin:pUB with the cryptic UB-binding site mutation Glu321Ala displays wild-type level binding to

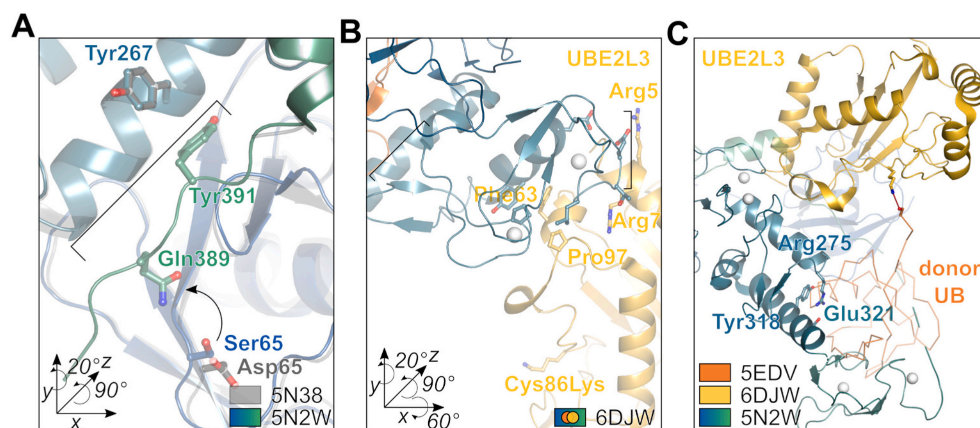

Fig. 4. Disruption of Parkin auto-inhibition and E2 ~ UB recruitment.
(A) Close-up of RING1-UBL interface on Parkin:pUB complex (PDB: 5N2W [69]) overlaid with that of Parkin-Ser65Asp:pUB (PDB: 5N38 [69]), highlighting the destabilisation caused by negative charge on UBL residue 65. (B) Close-up of RING1-UBE2L3 interaction seen in p*Bd*Parkin:pUB:UBE2L3 complex (PDB: 6DJW [83]). (C) Ribbon/line representation of Parkin bound to UBE2L3 ~ UB, showing donor UB nestled in the cryptic donor UB-binding region. This state is modelled by superimposing RING1 of inactive Parkin (PDB: 5C1Z [73]), p*Bd*Parkin:pUB:UBE2L3 complex (PDB: 6DJW [83]) and HOIP:UBE2D2 ~ UB complex (PDB: 5EDV [64]). For clarity, only inactive Parkin is shown, with UBL and REP rendered semi-transparent, with the UBE2L3 and the donor UB shown as yellow ribbons and orange lines, respectively. Spatial relations of panels B, C and D relative to Fig. 2A is illustrated where different.

UBE2L3 alone, while binding to UBE2L3 ~ UB conjugate by a 20-fold reduced affinity [69]. This corroborates the function of the deep RING1-BRcat cleft as the cryptic UB-binding site. Indeed, mutation of UB-binding cleft residues hampers E3-ligase activity [69].

4. Complete Parkin activation: *cis*- and *trans*-activation models

Probably the most enigmatic aspect of Parkin activation has been the observation that even when Parkin is bound to pUB, the catalytic Cys431 in Rcat is poorly accessible at RING0-Rcat interface, and remains ~50 Å away from the E2 binding site on RING1 (Fig. 3A) [44,62,72,73]. Over the last decade two models attempting to explain the mechanism underlying UB-transfer by Parkin have gained attraction and these are the so-called *trans*- and *cis*-activation models.

4.1. Priming for UB-transthiolation

The *trans*-activation model proposes that multiple Parkin molecules cooperate to overcome the distance between Rcat Cys431 and E2 ~ UB thioester and achieve transthiolation. There is no structural model showing directly how Parkin may achieve *trans*-activation, but a domain-swap model exists for the evolutionarily related RBR E3 ligase HOIP, suggesting that trans-activation may exist in other RBR E3 ligases [64]. In the HOIP:UBE2D2 ~ UB model, HOIP can be seen forming a homodimer along its RING-BRcat linker region, which corresponds to REP in Parkin, and coordinating the E2 moiety of UBED2 ~ UB *via* the RING1 domain of one HOIP molecule, while also engaging the UB moiety *via* the Rcat domain of the other HOIP molecule [64].

Several *in vivo* and *in vitro* observations also suggest that this *trans*-activation model is relevant to Parkin. Firstly, overexpression of either catalytically dead Parkin Cys431Ala or E2 ~ UB-binding-deficient Parkin Arg275Trp in HeLa cells, which have low levels of endogenous Parkin, fails to induce mitophagy, yet when the two are co-expressed, this defect is rescued [87,88]. Similarly, pParkin with a cryptic E2 ~ UB binding site mutation, Glu321Ala, displays attenuated binding to UBE2L3 ~ UB and fails to ubiquitylate Miro1 *in vitro*, with titration of a covalently linked Parkin-pUB complex rescuing this Miro1 ubiquitylation defect [69]. This implies that while the mutant Parkin fails to bind to the donor UB, it can still interact with the E2, while the wild-type pUB-Parkin can bind the donor UB, mediating formation of an active complex that involves 2 Parkin assemblies around one E2 ~ UB conjugate. On the contrary, however, a recent report has shown that titration of E2 ~ UB-binding deficient *Rattus norvegicus* Parkin Ala240Arg (Thr240 in human Parkin) on a catalytically dead Parkin does not lead to Parkin auto-modification *in vitro* [83]. It is possible, therefore, that cooperation between multiple Parkin molecules is facilitated by presence of a physiological substrate and does not happen in the minimalist set up of an *in vitro* self-modification assay.

Alternatively, the *cis*-activation model implies that following pUB binding and phosphorylation of the UBL domain, Parkin can unravel, allowing the Rcat domain to traverse across the central RING1 domain to meet the E2 ~ UB conjugate. Several recent reports produced crystallographic and NMR/MD simulation-driven models that illustrate the mechanism behind this *cis*-activation (Fig. 5A) [65,68,83,89]. In this model, once phosphorylated, the UBL domain is released from its RING1 site [90] and rebinds through RING0 Lys161, Arg163 and Lys211 on the

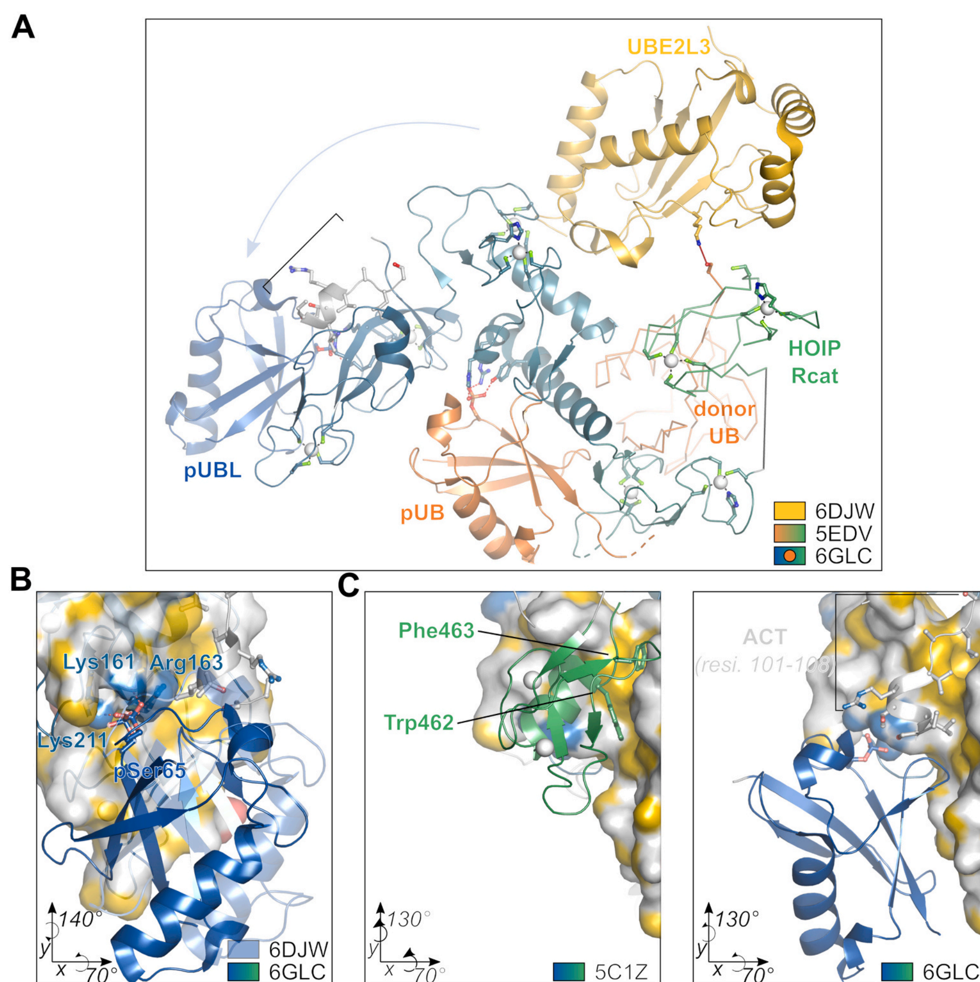

Fig. 5. Parkin in its fully active state and modelled as poised to receive donor UB. (A) Ribbon representation of pParkin$_{\Delta REP\Delta Rcat}$:pUB complex (PDB: 6GLC [68]) coloured and viewed as in Fig. 1A. Fully activated state is modelled by superimposing RING1 of fully-active pParkin$_{\Delta REP\Delta Rcat}$ (PDB: 6GLC [68]), p*Bd*Parkin:pUB:UBE2L3 complex (PDB: 6DJW [83]) and HOIP:UBE2D2 ~ UB complex (PDB: 5EDV [64]). For clarity, only fully active pParkin$_{\Delta REP\Delta Rcat}$:pUB is shown, with the UBE2L3 and the donor UB shown as yellow ribbons and orange lines, respectively. HOIP Rcat is also shown as lines, with the catalytic cysteine poised to receive the donor UB shown as sticks, and E2 ~ UB thioester represented as solid red line. (B) Close-up of pUBL-RING0 interaction as seen in pParkin$_{\Delta REP\Delta Rcat}$:pUB (PDB: 6GLC [68]) and p*Bd*Parkin:pUB:UBE2L3 complex (PDB: 6DJW [83]), where the two Parkin molecules are superimposed over RING0 domain, pParkin is coloured as in Fig. 1A and p*Bd*Parkin is shown as blue ribbons and rendered semi-transparent. RING0 surface is shown and coloured to indicate hydrophobic, negatively charged and positively charged regions yellow, red and blue, respectively. (C) Same as in (B), but highlighting the overlapping RING0-Rcat (left; PDB: 5C1Z [93]) and RING0-pUB/ACT (right; PDB: 6GLC [68]) interfaces.

RING0 domain, with additional contacts mediated by the UBL hydrophobic patch around Ile44 and one of the RING0 β-hairpin loops (Fig. 5B) [68,83]. As the Rcat and pUBL binding sites on RING0 display an overlap (Fig. 5C), this ionic pUBL-RING0 interaction is able to displace Rcat, which is tethered on RING0 predominantly by weak hydrophobic interactions [68,83]. Once Rcat is dislodged from RING0, the hydrophobic groove on RING0 becomes accessible.

The UBL is linked to RING0 *via* a 66-residue linker in human Parkin, which is disordered in autoinhibited Parkin structures [72]. Remarkably, in the pParkin$_{\Delta REP \Delta Rcat}$:pUB complex, a section of the UBL-RING0 linker, from Gln100 through to Ser108, is ordered on the RING0 hydrophobic groove. Critically, linker residues Leu102, Val105 and Leu107 cover the hydrophobic groove on RING0 that is exposed by departure of Rcat, while linker residue Arg104 make a salt bridge with pUBL Asp60, thus stabilising Parkin in the activated state by preventing Rcat from rebinding to RING0 and competing with the pUBL:RING0 interaction (Fig. 5A and C) [68]. Interestingly, while the length of this linker is conserved in most species of Parkin, with the exception of nematodes, sequence conservation is seen only in the middle section corresponding to Ser101 through to Leu123 in the human sequence and exists only among vertebrates.

Even when autoinhibited, Parkin exists in an equilibrium involving rapid intra-molecular movements [89]. Such domain flexibility often hampers successful crystallisation efforts. Not surprisingly, therefore, crystallisation of pParkin$_{\Delta REP \Delta Rcat}$:pUB and p*Bd*Parkin:pUB:UBE2L3, required some noteworthy adjustments. In the case of pParkin$_{\Delta REP \Delta Rcat}$:pUB, the complex was trapped only with deletion of REP and Rcat domain. In the case of p*Bd*Parkin:pUB:UBE2L3, crystallisation of the complex was facilitated by crosslinking the C-terminus of UBE2L3 to the N-terminus of Parkin UBL domain *via* a ten-residue linker. This linker is not visible in the crystal structure; thus, it is not possible to work out whether the UBE2L3-Parkin interaction or pUBL-RING0 interaction occurs *in trans* or *cis*. While the authors have deposited the crystal structure as a single, fusion polypeptide, they hint that it is more likely that UBE2L3 binds to Parkin RING1 in *cis*, while the pUBL, displaced as a result of either this interaction, or because of the contortion caused by the crosslinking, then interacts *in trans* with RING0 of a neighbouring fusion polypeptide. Nevertheless, the latter structure validates the former, as in spite of inclusion of REP and Rcat domains in the fusion polypeptide, these regions are not visible in the p*Bd*Parkin:pUB:UBE2L3 model, suggesting that pUBL-RING0 interaction indeed releases Rcat, and that in the absence of the donor UB, Rcat is destabilised. Also, consistent with the domain movements shown in these crystal structures relative to those of autoinhibited Parkin, hydrogen-deuterium exchange mass spectrometry (HDX-MS) data from three independent reports confirm that: (1) pUB binding increases UBL solvent-exposure; (2) phosphorylation of UBL leads to re-stabilisation of UBL and reduced solvent-exposure; and (3) concomitant with this, Rcat is released and exposed to solvent [65,68,83].

4.2. *Cis, trans or the whole spectrum*

Importantly, both the *cis*- and *trans*-activating models are based on crystallographic models where the allosteric pUB is monomeric, as is the case with the *in vitro* assays set out to test these hypothetical models (Fig. 6A). In cellular environment, however, the OMM of damaged mitochondria displays UB chains predominantly with Lys6, Lys11 and Lys63 linkage topologies, where the Ser65-phosphorylation status of the UB chains is sub-stoichiometric [24,80]. Thus, Parkin is likely to be recruited on pUB chains, where the pUB is linked to other (p)UB moieties on its distal and/or proximal sites. Inspection of pUB in activated Parkin structures reveal that Lys6 and Lys11 in particular protrude towards the cryptic donor UB binding site [68,69]. This raises the possibility that while a pUB moiety recruits Parkin and triggers the allosteric activation mechanism as described above, a neighbouring (p)UB moiety may occlude recruitment of donor UB, thus preventing that Parkin molecule from forming a fruitful complex with an E2 ~ UB conjugate (Fig. 6B). In line with this, *in vitro* self-ubiquitylation experiments involving Lys6-linked di-pUB, where either distal, proximal or both moieties are phosphorylated (hereafter pUB-UB, UB-pUB and pUB-pUB, respectively) depict complex outcomes. Interestingly, both pUB-pUB and pUB-UB activate Parkin, while UB-pUB fails to do so, with this shortcoming relieved by UBL phosphorylation [91]. Thus, although the *cis*- and *trans*-activation models have often been discussed as being mutually exclusive [83,92], in reality Parkin's cellular environment may necessitate elements from both *cis*- and *trans*-activating models, such as pUBL binding to RING0 to release Rcat, as illustrated by the recent structures, coupled to the ability of two Parkin molecules to dimerise around one E2 ~ UB to mediate UB-transfer to Rcat, similar to that observed for HOIP.

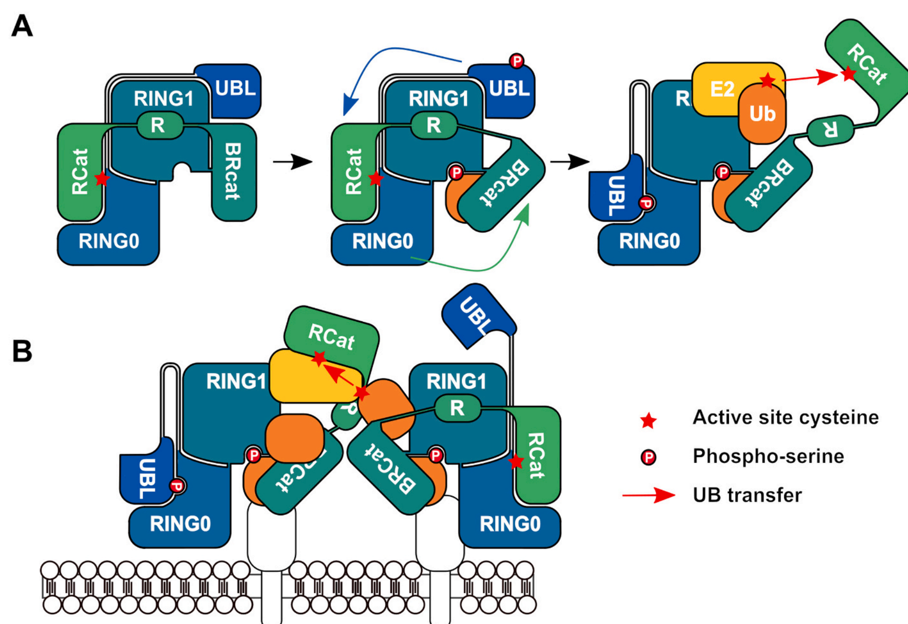

Fig. 6. cis- *vs* trans-activation models explaining mechanism of Parkin activation.
(A) Box-diagrams showing how pUB-binding and phosphorylation of the UBL domain push a single molecule of Parkin into the fully activated state. Parkin is coloured as in Fig. 1C, while E2 and UB are coloured yellow and orange, respectively. Blue-green arrows indicate domain movements triggered by (p) UB binding. (B) Box-diagrams showing how multiple molecules of Parkin may be forced to dimerise around a E2 ~ UB conjugate in order to overcome the steric hindrance caused by poly-pUB chains on OMM proteins of damaged mitochondria. Parkin, E2 and UB and are coloured as in panel A, while OMM phospholipid bi-layer and integral membrane proteins are coloured as while circles. OMM integral membrane proteins are shown as white boxes, with pUB moieties attached on them coloured orange.

5. Concluding remarks

Harbouring over 130 distinct mutations observed in over 1000 patients with early-onset PD, Parkin has been the hot-spot of signalling pathways involved in PD aetiology. Extensive cell-based studies revealed that Parkin functions under PINK1 and generates a feed-forward signalling mechanism that mediates mitophagy. Over the past two decades the question of how Parkin activity is regulated has been extensively studied, demonstrating that: (1) inter-domain interactions exert a tight inhibition on Parkin activity; (2) Parkin-pUB interaction relieves this auto-inhibition; and (3) phosphorylation of Parkin UBL shifts the equilibrium towards maximal Parkin activation. Specifically, data obtained using molecules in solution depict clearly that following pUB binding and phosphorylation of the UBL domain, Parkin can unravel, allowing the Rcat domain to traverse across the central RING1 domain to meet the E2 ~ UB conjugate. While a large body of work elucidates how Parkin behaves in solution, molecular detail of how Parkin activation proceeds in its cellular context remains elusive. It remains to be determined whether, as some evidence suggests, in such a complex environment Parkin molecules dimerise around one E2 ~ UB to mediate UB-transfer to Rcat. Furthermore, little information exists on how activated Parkin can then modify such a wide variety of substrates. The next decade, therefore, is likely to reveal how these rearrangements and modes of activation support Parkin substrate targeting.

Credit author statement

MG, RT and GS wrote the manuscript with input from HW and GSS.

Declaration of Competing Interest

Authors declare no conflict of interest.

Acknowledgements

GSS was funded by Canadian Institutes of Health Research (CIHR) - PJT-166019. HW was funded by Wellcome Trust Investigator Award (209347/Z/17/Z)

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