Modes of allosteric regulation of the ubiquitination machinery

Martin L. Rennie, Viduth K. Chaugule, Helen Walden*

Institute of Molecular Cell and Systems Biology, College of Medical Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

*Corresponding author: Walden, Helen (Helen.Walden@glasgow.ac.uk)

Abstract

Ubiquitination is a post-translational modification critical for cellular signaling. A diverse range of enzymes constitute the machinery that mediate attachment of ubiquitin onto target proteins. This diversity allows the targeting of various proteins in a highly regulated fashion. Many of the enzymes have multiple domains or subunits that bind allosteric effectors and exhibit large conformational rearrangements to facilitate regulation. Here we consider recent examples of ubiquitin itself as an allosteric effector of RING and RBR E3 ligases, as well as advances in the understanding of allosteric regulatory elements within HECT E3 ligases.

Introduction

This review first covers the basics of ubiquitination and highlights the different modes of allosteric regulation of the ubiquitination machinery, then examines specific examples. Ubiquitination involves the covalent attachment of ubiquitin, an 8.6 kDa protein, to target proteins. This post-translational modification (PTM) is found in eukaryotes and is involved in proteasome degradation and many other signaling pathways [1,2]. An enzymatic cascade mediates the formation of an isopeptide bond between the C-terminus of ubiquitin and the amino group of a lysine sidechain on the target protein. Ubiquitination involves three steps: 1) activation of ubiquitin C-terminus by an E1 enzyme in a fast, ATP-dependent reaction, 2) conjugation onto an E2 via transthiolation with ubiquitin-charged E1, and 3) ligation onto the target by an E3 which interacts with both ubiquitin-charged E2 and the target (Figure 1a). When the target is ubiquitin itself, which contains seven lysines (Figure 1b), ubiquitin chains are formed [2]. Removal of ubiquitin from the target is achieved via de-ubiquitinases (DUBs) making ubiquitination a reversible PTM [3,4]. In addition to lysine, the N-terminal amino group, and some other side chains can be ubiquitinated [5].

While there are only a few E1 enzymes encoded in the human genome, there are tens of E2s and DUBs, and hundreds of E3s [8,9]. This diversity likely reflects the need to ubiquitinate a range of target proteins in a highly regulated fashion. Three types of E3 ligases – RING/U-box, HECT, and RBR (*vide infra*) – each have a conserved catalytic architecture and utilize different mechanisms to transfer ubiquitin from E2 onto the target [10,11]. In addition to the catalytic domain(s), E3 ligases contain other domains [3,4,12•,13•,14] or form multi-subunit complexes [15–18]. These ancillary components are generally involved in regulation

or target recognition. It is becoming increasingly apparent that the ancillary domains and subunits are flexibly linked with conformational rearrangement underlying regulation.

Allosteric regulation provides a molecular means to transfer information in biological systems involving a protein and an effector. Allosteric effectors of the ubiquitination machinery can interact with either the catalytic domain or the ancillary domains to regulate enzymatic activity. Effectors are commonly separate molecules [19] but can also be regions of the substrate such as a growing ubiquitin chain [20–22]. We refer to these modes of regulation as acting *in trans*, i.e. between separate molecules (Figure 2a). However, the multi-domain or multi-subunit architecture of many of these enzymes means effectors can also be regions of the enzyme itself [12•,13•,23]. We refer to this mode of regulation as acting *in cis*, i.e. within the same molecule or complex (Figure 2b). Negative *cis* allostery appears to be a common method for auto-inhibition of E3 enzymes. An additional complication of allostery in ubiquitination is that the protein substrates themselves may exhibit allosteric changes driven by distinct effectors or the ubiquitination machinery itself. Finally, regulation can occur without any conformational changes via augmenting or steric hinderance of the substrate binding interface, e.g. via an adapter that interacts with both substrate and enzyme to promote substrate binding (Figure 2c). Although this may not be strictly classed as allostery, the binding sites for this type of effector are often distal to the active site due to the tendency for extended substrate binding interfaces in the ubiquitination machinery. These modes may occur together, or in the context of oligomerization to regulate activity, as is the case for some RING and HECT E3s. Such regulation of oligomeric states via an effector was termed polystery in the 1970s [24]. These features make the study of allostery in ubiquitination a complicated but exciting area.

Biological effectors of the ubiquitination machinery range from short polypeptide motifs to large protein surfaces. Ubiquitin itself has drawn special attention recently for its ability to regulate ubiquitination, which has been suggested as a mechanism to aid processive ubiquitination [20–22]. The role of ubiquitin in many protein-protein interactions has been exploited to generate ubiquitin variants (UbVs) that target specific sites with high affinity using phage-display [22,25,26,27•]. Here we examine recent examples of allosteric regulation of E2, RING-E3, HECT-E3, and RBR-E3 enzymes.

Ubiquitin-conjugating E2 enzymes and RING/U-box E3 ligases

Ubiquitin-conjugating E2s (~35 members in humans) share a core ubiquitin conjugation (UBC) domain that bears a catalytic cysteine. They have a primary allosteric site where RING/U-box domains can bind to catalyse ubiquitin ligation onto the target lysine. Proteins comprising RING/U-box domains make up the largest family of ubiquitin E3 ligases (~600 members in humans) and can be sub-grouped into monomeric, homo-dimeric, hetero-dimeric, and cullin RING ligases (CRLs). In general, ancillary domains mediate target interaction while the catalytic domain promotes target ubiquitination by allosterically activating the E2~Ub thioester [28–30]. Briefly, RING/U-box binding of E2s stabilizes a closed/active E2~Ub conformation via direct interaction with ubiquitin and conformational dynamics of the E2 active site. In this ternary complex, an allosteric ‘linchpin’ arginine in RING/U-box contacts both the E2 and ubiquitin tail, restraining the E2~Ub thioester bond thus priming it for nucleophilic attack by the target lysine.

In addition to the RING site, E2s also possess a 'backside' site to enhance affinity of the E3-E2 complex and allosterically regulate activity [31]. This backside supports interactions with a range of effectors including ubiquitin and ancillary regions of monomeric RING E3s [19,31,32]. The interplay of allostery between the primary and secondary sites on Ube2G2 by gp78/RNF45 (Figure 3a) has been dissected through structural and biophysical analysis [19] and recently with dynamics measurements using NMR and molecular dynamics (MD) [33••]. These studies suggest complex regulation whereby the backside effector reduces dynamics at both the Ube2G2 active site and RING binding site, which steadies E3-E2~Ub complex for ubiquitination. Subsequently, the RING partially restores conformational dynamics of Ube2G2 thereby reducing affinity for the backside effector. This releases Ube2G2 from the E3, thus permitting E2~Ub reloading for successive ubiquitination cycles. Recently, the bimodal (RING and backside effector) interaction of an E3 (FANCL and Rad18) was also shown to allosterically optimize the E2's active site (Ube2T and Ube2B, respectively) to stimulate specialized ubiquitination events [34•]. Interestingly in these enzyme pairs, E2~Ub activation occurs in absence of a 'linchpin' arginine in the cognate RING domains.

The histone H3 ubiquitinating enzyme, UHRF1, a multi-domain RING-E3, uses an internal ubiquitin-like (Ubl) domain to recruit the E2 Ube2D via backside interactions. In contrast to gp78/Ube2G2, this interaction appears to be solely a recruiting mechanism, which mediates target sensing [35,36••,37]. The various domains of UHRF1 to default to a condensed arrangement, however, chromatin binding reconfigures the inter-domain interactions to activate of the enzyme. Consequently, the Ubl and RING domains together recruit E2~Ub via backside and RING site interactions, respectively. In addition, the Ubl connects with a DNA

binding module of UHRF1 that senses hemi-methylated DNA and this interaction regulates H3 ubiquitination. Notably, certain cancer mutations in the Ubl lead to defects in H3 ubiquitination without altering E2 interaction or E3 auto-ubiquitination, indicating a loss in target sensing.

In dimeric RING/U-box E3s, one protomer supports the E2 while residues from both promoters stabilize a distinct ubiquitin surface of E2~Ub to enhance ubiquitination. Comparison of dimeric and monomeric RINGs reveals the presence of a tryptophan residue on the E2 binding helix in the latter family, which likely increases E3-E2 affinity and/or the allosteric activation of E2~Ub [38•]. Interestingly, engineering a tryptophan at the analogous location in dimeric RINGs can stimulate E3 activity in the absence of dimerization. Structural characterizations of TRIM proteins, a large RING E3 subfamily (~70 members), have revealed a correlation between dimerization and E3 activity [39,40]. Moreover, efforts to find effectors have uncovered dimeric UbVs that bind and stabilize the RING homodimers of XIAP [27•] thereby constitutively activating the E3 (Figure 3b). Structures of TRAF and Lnx family members, in complex with E2~Ub, also reveal dimeric RING conformations [41,42]. Notably, a zinc-finger neighbouring the RING facilitates additional ubiquitin interactions on E2~Ub that are critical for E3 activity. Conversely, in case of TRIM21 dimers, a B-Box 2 zinc-finger adjacent to the RING occupies the E2 binding pocket thus auto-inhibiting the E3 [43•]. Phosphorylation of the RING domain relieves this intra-molecular inhibition thus permitting E2-E3 interaction and ubiquitination. Overall, the dimerization requirement for activity allows for polysteric regulation of these enzymes by their targets. In this scenario, higher-order assemblies of the target, for example HIV-1 capsid, could shift the equilibrium of the

bound RING E3 enzyme (TRIM5α) to a multimeric state thus activating the enzyme for ubiquitination [44].

The basic architecture of CRLs is a RING subunit that binds E2, a cullin scaffold, and a substrate receptor. The substrate receptor of Cdc4, a WD40 domain, was recently shown to be allosterically regulated by multi-phosphorylated substrate [45]. Phospho-serine binding to the primary pocket appears to be reduced by binding of another phospho-serine on the substrate to a nearby allosteric site on the same domain. This may allow for fine-tuned regulation of the binding kinetics. Cullins have also been the target of UbVs [15,16,22]. In particular, a potential di-Ub binding site was identified for the APC/C complex, which unusually contains two RING domains. The di-Ub site has been proposed to facilitate switching activity between the two RINGs. As such it seems multiple, complex regulatory mechanism are at play.

HECT-type E3 ligases

For HECTs (~28 members) the catalytic domain architecture consists of an N- and C-lobe, which recruit E2~Ub and contain a catalytic cysteine, respectively. Rearrangement of these lobes is necessary to transfer ubiquitin from the E2~Ub onto the C-lobe catalytic cysteine via transthiolation and then onto the target. An allosteric binding site for ubiquitin on the N-lobe has been shown to regulate activity probably through promoting chain elongation [20,21]. UbVs targeting HECTs revealed that the role of this site may vary between different orthologs [26]. Ancillary domains upstream in the amino acid sequence regulate ubiquitination activity [46,47••]. Several studies have recently unraveled structural details of this regulation for the NEDD4 subfamily, suggesting a role of *cis* allostery. Crystal

structures of near-full length ITCH [13•], WWP1 [23], and WWP2 [12•] helped identify a conserved α-helix, flanked by repeat domains, that interacts with the HECT domain on the opposite face to the catalytic site (Figure 3c). This interaction seemingly restricts the conformational changes necessary for catalysis, maintaining the enzyme in an auto-inhibited state. Consistent with this, artificial deletion of the helical region increases auto-ubiquitination and target ubiquitination. Auto-inhibition is likely relieved *in vivo* through phosphorylation [12•,13•] or by binding of other proteins [13•,46] to the N-terminal domains. Interestingly, the interaction site on the HECT N-lobe overlaps with the ubiquitin allosteric site hinting at further complexity in the regulation of these enzymes.

The feature of upstream regulatory elements is also retained in HUWE1, which belongs to a different subfamily of HECTs. Recently it was shown that two distinct regions regulate dimerization of this enzyme in a complex fashion, with the oligomeric state linked to activity [47••]. A crystal structure of the dimer again suggested a locking of the HECT lobes in an inactive conformation, at least for one protomer. A pair of α-helices facilitate dimerization both in the crystal structure and in solution. Another set of predicted helices further upstream restricts dimerization, apparently by intra-molecular interaction with the dimerization helices. Enzymatic activity correlates well with the oligomeric state for truncation constructs, with an active monomer and inactive dimer. This system can be further regulated through binding another effector protein, which seems to interact with the predicted helices to facilitate inactivation through dimerization. Overall it seems that both *cis* and *trans* allostery are coupled to oligomerization in this enzyme.

RBR-type E3 ligases

The RBR (RING Between RING) family of E3 ligases (14 members) comprise a tripartite arrangement of RING1, in-between RING (IBR) and RING2 domains. RING1 adopts a zinc-coordinating cross-brace typically observed in RING domains, while, the IBR and RING2 share a linear bilobal zinc-binding fold. The RBRs follow an obligate two-step catalytic cycle where the RING1, along with the IBR, first facilitate ubiquitin transthiolation between the E2~Ub thioester and a catalytic cysteine in RING2 [49]. The loaded RING2~Ub intermediate subsequently catalyses substrate ubiquitination. Despite sharing the RBR module, structures of RBR fragments from different family members (HOIP [50••], Parkin [48•,51–53] and HHARI [54,55]) reveal distinct inter-domain arrangements which, along with ancillary domains and inter-domain linkers, regulate the catalytic cycle of the enzyme. The RING1 core of RBRs supports E2 docking, however, short insertions within this domain induces an extended/open conformation of the E2~Ub intermediate. Further, this extended Ub conformer is braced by a C-terminal helix of RING1 and the IBR. Notably, present on the opposing surface of RING1's C-terminal helix is an allosteric binding site for an effector ubiquitin molecule that stabilises E2~Ub-RING1 interactions thereby promoting the ubiquitin loading of RING2 (Figure 3d). In Parkin, a Ubl domain maintains an auto-inhibited state, with phosphorylation of this domain important in the release of auto-inhibition (Figure 3d) [8]. In most RBRs, transfer of ubiquitin between the E2 and RING2 is likely to occur in within the same complex, however a cooperative model involving multiple RBR molecules has also been proposed [48•,50••,52,53]. Finally, an N-terminal linker in proximity to the catalytic cysteine on RING2 is also reported to interact with ubiquitin and facilitate RING2~Ub loading and/or substrate ubiquitination [50••,56]. Overall, these structural studies of various RBRs have uncovered distinct ubiquitin-RBR interfaces that

regulate its catalytic cycle. These observations have prompted the search for allosteric UbVs that modulate these enzymes [57].

Conclusions

The ubiquitination machinery are complex enzymes often with multiple regulatory sites, effected in various ways (Table 1). Much progress has been made in identifying regulatory sites, in particular UbVs are a promising and general approach [25,26,27•]. However, untangling the mechanisms of regulation is a non-trivial task as multiple mechanisms are seemingly at work, even for individual regulatory sites [12•,23,33••,36••,45,47••]. The multiple steps of the ubiquitination pathway themselves can be regulated thus complicating the situation further. As such caution should be taken in concluding the mechanism(s) of allostery. Despite this, the combination of ubiquitination assays, affinity measurements, and structural characterization have, and will continue to, provide useful insight into allosteric regulation in ubiquitination. Additionally, computational methods, such as MD and comparison of residue contact networks [58], can reveal the details of conformational changes and dynamics and should not be overlooked. Particularly insightful will be structures with the regulatory domains or effectors present. Advances in cryoEM have already provided insight into regulation of the of the multi-subunit APC/C RING-E3 complex [15]. With smaller molecular weight samples becoming more tractable, cryoEM may prove very useful in characterizing inter-domain/subunit three-dimensional architectures of the ubiquitination machinery [59].

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the EMBO Young Investigator Programme to H.W.; the European Research Council (ERC-2015-CoG-681582 ICLUb) consolidator grant to H.W. We thank members of the Walden laboratory and Dr Ciara Kyne for useful discussions.

References and recommended reading

1. Hochstrasser M: **Origin and function of ubiquitin-like proteins**. *Nature* 2009, **458**:422–429.
2. Yau R, Rape M: **The increasing complexity of the ubiquitin code**. *Nat Cell Biol* 2016, **18**:579–586.
3. Komander D, Clague MJ, Urbé S: **Breaking the chains: Structure and function of the deubiquitinases**. *Nat Rev Mol Cell Biol* 2009, **10**:550–563.
4. Hanpude P, Bhattacharya S, Dey AK, Maiti TK: **Deubiquitinating enzymes in cellular signaling and disease regulation**. *IUBMB Life* 2015, **67**:544–555.
5. McDowell GS, Philpott A: **Non-canonical ubiquitylation: Mechanisms and consequences**. *Int J Biochem Cell Biol* 2013, **45**:1833–1842.
6. Olsen SK, Lima CD: **Structure of a Ubiquitin E1-E2 Complex: Insights to E1-E2 Thioester Transfer**. *Mol Cell* 2013, **49**:884–896.
7. Streich FC, Lima CD: **Capturing a substrate in an activated RING E3/E2-SUMO complex**. *Nature* 2016, **536**:304–308.
8. Chaugule VK, Walden H: **Specificity and disease in the ubiquitin system**. *Biochem Soc Trans* 2016, **44**:212–227.
9. Zhou J, Xu Y, Lin S, Guo Y, Deng W, Zhang Y, Guo A, Xue Y: **IUUCD 2.0: An update with rich annotations for ubiquitin and ubiquitin-like conjugations**. *Nucleic Acids Res* 2018, **46**:D447–D453.
10. Morreale FE, Walden H: **Types of Ubiquitin Ligases**. *Cell* 2016, **165**:248-248.
11. Zheng N, Shabek N: **Ubiquitin Ligases: Structure, Function, and Regulation**. *Annu Rev Biochem* 2017, **86**:129–157.

12. Chen Z, Jiang H, Xu W, Li X, Dempsey DR, Zhang X, Devreotes P, Wolberger C, Amzel LM, Gabelli SB, et al.: **A Tunable Brake for HECT Ubiquitin Ligases**. *Mol Cell* 2017, **66**:345–357.

• One of the first structures of a NEDD4 family member with the auto-inhibitory "WWL" region present.

13. Zhu K, Shan Z, Chen X, Cai Y, Cui L, Yao W, Wang Z, Shi P, Tian C, Lou J, et al.: **Allosteric auto-inhibition and activation of the Nedd4 family E3 ligase Itch**. *EMBO Rep* 2017, **18**:1618–1630.

• One of the first structures of a NEDD4 family member with the auto-inhibitory "WWL" region present.

14. Li YJ, Du L, Wang J, Vega R, Lee TD, Miao Y, Aldana-Masangkay G, Samuels ER, Li B, Ouyang SX, et al.: **Allosteric Inhibition of Ubiquitin-like Modifications by a Class of Inhibitor of SUMO-Activating Enzyme**. *Cell Chem Biol* 2019, **26**:278–288.
15. Brown NG, VanderLinden R, Watson ER, Weissmann F, Ordureau A, Wu KP, Zhang W, Yu S, Mercredi PY, Harrison JS, et al.: **Dual RING E3 architectures regulate multiubiquitination and ubiquitin chain elongation by APC/C**. *Cell* 2016, **165**:1440–1453.
16. Gorelik M, Manczyk N, Pavlenco A, Kurinov I, Sidhu SS, Sicheri F: **A Structure-Based Strategy for Engineering Selective Ubiquitin Variant Inhibitors of Skp1-Cul1-F-Box Ubiquitin Ligases**. *Structure* 2018, **26**:1226–1236.
17. Lampert F, Stafa D, Goga A, Soste MV, Gilberto S, Olieric N, Picotti P, Stoffel M, Peter M: **The multi-subunit GID/CTLH e3 ubiquitin ligase promotes cell proliferation and targets the transcription factor Hbp1 for degradation**. *Elife* 2018, **7**:1–23.
18. Maitland MER, Onea G, Chiasson CA, Wang X, Ma J, Moor SE, Barber KR, Lajoie GA, Shaw GS, Schild-Poulter C: **The mammalian CTLH complex is an E3 ubiquitin ligase that targets its subunit muskelin for degradation**. *Sci Rep* 2019, **9**:1–14.
19. Das R, Liang YH, Mariano J, Li J, Huang T, King A, Tarasov SG, Weissman AM, Ji X, Byrd RA: **Allosteric regulation of E2:E3 interactions promote a processive ubiquitination machine**. *EMBO J* 2013, **32**:2504–2516.
20. Maspero E, Mari S, Valentini E, Musacchio A, Fish A, Pasqualato S, Polo S: **Structure of**

the HECT:ubiquitin complex and its role in ubiquitin chain elongation. *EMBO Rep* 2011, **12**:342–349.

21. Kim HC, Steffen AM, Oldham ML, Chen J, Huibregtse JM: **Structure and function of a HECT domain ubiquitin-binding site**. *EMBO Rep* 2011, **12**:334–341.
22. Watson ER, Grace CRR, Zhang W, Miller DJ, Davidson IF, Prabu JR, Yu S, Bolhuis DL, Kulko ET, Vollrath R, et al.: **Protein engineering of a ubiquitin-variant inhibitor of APC/C identifies a cryptic K48 ubiquitin chain binding site**. *Proc Natl Acad Sci* 2019, **116**:17280–17289.
23. Wang Z, Liu Z, Chen X, Li J, Yao W, Huang S, Gu A, Lei Q-Y, Mao Y, Wen W: **A multi-lock inhibitory mechanism for fine-tuning enzyme activities of the HECT family E3 ligases**. *Nat Commun* 2019, **10**:3162.
24. Colosimo A, Brunori M, Wyman J: **Polysteric Linkage**. *J Mol Biol* 1976, **100**:47–57.
25. Ernst A, Avvakumov G, Tong J, Fan Y, Zhao Y, Alberts P, Persaud A, Walker JR, Neculai A, Neculai D, et al.: **A Strategy for Modulation of Enzymes in the Ubiquitin System**. *Science* 2013, **339**:590–595.
26. Zhang W, Wu KP, Sartori MA, Kamadurai HB, Ordureau A, Jiang C, Mercredi PY, Murchie R, Hu J, Persaud A, et al.: **System-Wide Modulation of HECT E3 Ligases with Selective Ubiquitin Variant Probes**. *Mol Cell* 2016, **62**:121–136.
27. Gabrielsen M, Buetow L, Nakasone MA, Ahmed SF, Sibbet GJ, Smith BO, Zhang W, Sidhu SS, Huang DT: **A General Strategy for Discovery of Inhibitors and Activators of RING and U-box E3 Ligases with Ubiquitin Variants**. *Mol Cell* 2017, **68**:456–470.

• A dimeric UbV selectively binds and stabilizes the XIAP RING dimer thus activating its E3 ligase functions.

28. Pruneda JN, Littlefield PJ, Soss SE, Nordquist KA, Chazin WJ, Brzovic PS, Klevit RE: **Structure of an E3:E2~Ub Complex Reveals an Allosteric Mechanism Shared among RING/U-box Ligases**. *Mol Cell* 2012, **47**:933–942.
29. Plechanovov A, Jaffray EG, Tatham MH, Naismith JH, Hay RT: **Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis**. *Nature* 2012, **489**:115–120.
30. Dou H, Buetow L, Sibbet GJ, Cameron K, Huang DT: **BIRC7-E2 ubiquitin conjugate structure reveals the mechanism of ubiquitin transfer by a RING dimer**. *Nat Struct Mol Biol* 2012, **19**:876–883.

31. Stewart MD, Ritterhoff T, Klevit RE, Brzovic PS: **E2 enzymes: More than just middle men**. *Cell Res* 2016, **26**:423–440.

32. Li S, Liang YH, Mariano J, Metzger MB, Stringer DK, Hristova VA, Li J, Randazzo PA, Tsai YC, Ji X, et al.: **Insights into ubiquitination from the unique clamp-like binding of the RING E3 AO7 to the E2 UbcH5B**. *J Biol Chem* 2015, **290**:30225–30239.

33. Chakrabarti KS, Li J, Das R, Byrd RA: **Conformational Dynamics and Allostery in E2:E3 Interactions Drive Ubiquitination: gp78 and Ube2g2**. *Structure* 2017, **25**:794–805.

•• An elegant combination of relaxation dispersion experiments and molecular dynamics are used by Chakrabarti *et al* to probe the dynamics of UBE2G2 across a range of timescales and in the presence of the gp78 RING and backside effector regions. This provides an extremely detailed picture of the allostery present in UBE2G2.

34. Chaugule VK, Arkinson C, Rennie ML, Kämäräinen O, Toth R, Walden H. **Allosteric mechanism for site-specific ubiquitination of FANCD2.** *Nat Chem Biol*. 2019, https://doi.org/10.1038/s41589-019-0426-z.

• Residue network analysis combined with biochemical, crystallographic and molecular dynamics studies reveal an alternative E2 activation mechanism by RING E3s.

35. Harrison JS, Cornett EM, Goldfarb D, Darosa PA, Li ZM, Yan F, Dickson BM, Guo AH, Cantu D V., Kaustov L, et al.: **Hemi-methylated DNA regulates DNA methylation inheritance through allosteric activation of H3 ubiquitylation by UHRF1**. *Elife* 2016, **5**:e17101.

36. DaRosa PA, Harrison JS, Zelter A, Davis TN, Brzovic P, Kuhlman B, Klevit RE: **A Bifunctional Role for the UHRF1 UBL Domain in the Control of Hemi-methylated DNA-Dependent Histone Ubiquitylation**. *Mol Cell* 2018, **72**:753–765.

•• In this study the Ubl domain of the E3 UHRF1 is demonstrated to be an adaptor for the E2 Ube2D3. Other potential roles for the Ubl domain in the context of the full-lenth UHRF1 protein are also suggested.

37. Foster BM, Stolz P, Mulholland CB, Montoya A, Kramer H, Bultmann S, Bartke T: **Critical Role of the UBL Domain in Stimulating the E3 Ubiquitin Ligase Activity of UHRF1 toward Chromatin**. *Mol Cell* 2018, **72**:739–752.

38. Sarkar S, Behera AP, Borar P, Banka PA, Datta AB: **Designing active RNF4 monomers by introducing a tryptophan: Avidity towards E2∼Ub conjugates dictates the activity of ubiquitin RING E3 ligases**. *Biochem J* 2019, **476**:1465–1482.

• Analysis of RING domains reveals a specialized tryptophan in monomeric RINGs. Engineering the tryptophan in dimeric RING bypasses the need of dimerization for E3 activity.

39. Esposito D, Koliopoulos MG, Rittinger K: **Structural determinants of TRIM protein function**. *Biochem Soc Trans* 2017, **45**:183–191.
40. Stevens R V., Esposito D, Rittinger K: **Characterisation of class VI TRIM RING domains: Linking RING activity to C-terminal domain identity**. *Life Sci Alliance* 2019, **2**:e201900295.
41. Middleton AJ, Budhidarmo R, Das A, Zhu J, Foglizzo M, Mace PD, Day CL: **The activity of TRAF RING homo-and heterodimers is regulated by zinc finger 1**. *Nat Commun* 2017, **8**:1788.
42. Nayak D, Sivaraman J: **Structure of LNX1:Ubc13 ~ Ubiquitin Complex Reveals the Role of Additional Motifs for the E3 Ligase Activity of LNX1**. *J Mol Biol* 2018, **430**:1173–1188.
43. Dickson C, Fletcher AJ, Vaysburd M, Yang JC, Mallery DL, Zeng J, Johnson CM, McLaughlin SH, Skehel M, Maslen S, et al.: **Intracellular antibody signalling is regulated by phosphorylation of the Fc receptor TRIM21**. *Elife* 2018, **7**:e32660.

• Struture of TRIM21 RING-B-Box 2 fragment reveals how the B-Box2 domain auto-inhibits E3 activity by occluding the E2 binding surface of the RING.

44. Fletcher AJ, Vaysburd M, Maslen S, Zeng J, Skehel JM, Towers GJ, James LC: **Trivalent RING Assembly on Retroviral Capsids Activates TRIM5 Ubiquitination and Innate Immune Signaling**. *Cell Host Microbe* 2018, **24**:761–775.
45. Csizmok V, Orlicky S, Cheng J, Song J, Bah A, Delgoshaie N, Lin H, Mittag T, Sicheri F, Chan HS, et al.: **An allosteric conduit facilitates dynamic multisite substrate recognition by the SCF Cdc4 ubiquitin ligase**. *Nat Commun* 2017, **8**:13943.
46. Riling C, Kamadurai H, Kumar S, O'Leary CE, Wu KP, Manion EE, Ying M, Schulman BA, Oliver PM: **Itch WW domains inhibit its E3 ubiquitin ligase activity by blocking E2-E3**

ligase trans-thiolation. *J Biol Chem* 2015, **290**:23875–23887.

47. Sander B, Xu W, Eilers M, Popov N, Lorenz S: **A conformational switch regulates the ubiquitin ligase HUWE1**. *Elife* 2017, **6**:e21036.

•• In this study a series of truncations are biophysically characterized identifying key regulatory elements of the dimerization of the HECT-E3 HUWE1.

48. Kumar A, Chaugule VK, Condos TEC, Barber KR, Johnson C, Toth R, Sundaramoorthy R, Knebel A, Shaw GS, Walden H: **Parkin-phosphoubiquitin complex reveals cryptic ubiquitin-binding site required for RBR ligase activity**. *Nat Struct Mol Biol* 2017, **24**:475–483.

• Structure of Parkin in complex with an allosteric Ub that is phosphorylated on Ser65. Conformation changes in Parkin/pUb complex reveals a binding pocket that accommodates the donor Ub of the E2~Ub intermediate. A model for cooperative action between multiple RBR molecules is also presented.

49. Wenzel DM, Lissounov A, Brzovic PS, Klevit RE: **UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids**. *Nature* 2011, **474**:105–108.

50. Lechtenberg BC, Rajput A, Sanishvili R, Dobaczewska MK, Ware CF, Mace PD, Riedl SJ: **Structure of a HOIP/E2~ubiquitin complex reveals RBR E3 ligase mechanism and regulation**. *Nature* 2016, **529**:546–550.

•• First structure of an RBR E3 in complex with the E2~Ub intermediate and an allosteric Ub. The allosteric Ub stimulates RBR-E2~Ub interaction and the E2~Ub is poised for Ub transfer to the RBR catalytic site.

51. Condos TE, Dunkerley KM, Freeman EA, Barber KR, Aguirre JD, Chaugule VK, Xiao Y, Konermann L, Walden H, Shaw GS: **Synergistic recruitment of UbcH7~Ub and phosphorylated Ubl domain triggers parkin activation**. *EMBO J* 2018, **37**:e100014.

52. Sauvé V, Sung G, Soya N, Kozlov G, Blaimschein N, Miotto LS, Trempe JF, Lukacs GL, Gehring K: **Mechanism of parkin activation by phosphorylation**. *Nat Struct Mol Biol* 2018, **25**:623–630.

53. Gladkova C, Maslen SL, Skehel JM, Komander D: **Mechanism of parkin activation by PINK1**. *Nature* 2018, **559**:410–414.

54. Yuan L, Lv Z, Atkison JH, Olsen SK: **Structural insights into the mechanism and E2 specificity of the RBR E3 ubiquitin ligase HHARI**. *Nat Commun* 2017, **8**:211.

55. Dove KK, Olszewski JL, Martino L, Duda DM, Wu XS, Miller DJ, Reiter KH, Rittinger K, Schulman BA, Klevit RE: **Structural Studies of HHARI/UbcH7∼Ub Reveal Unique E2∼Ub Conformational Restriction by RBR RING1**. *Structure* 2017, **25**:890–900.

56. Dove KK, Stieglitz B, Duncan ED, Rittinger K, Klevit RE: **Molecular insights into RBR E3 ligase ubiquitin transfer mechanisms** . *EMBO Rep* 2016, **17**:1221–1235.

57. Ordureau A, Paulo JA, Zhang W, Ahfeldt T, Zhang J, Cohn EF, Hou Z, Heo JM, Rubin LL, Sidhu SS, et al.: **Dynamics of PARKIN-Dependent Mitochondrial Ubiquitylation in Induced Neurons and Model Systems Revealed by Digital Snapshot Proteomics**. *Mol Cell* 2018, **70**:211–227.

58. Kayikci M, Venkatakrishnan AJ, Scott-Brown J, Ravarani CNJ, Flock T, Babu MM: **Visualization and analysis of non-covalent contacts using the Protein Contacts Atlas**. *Nat Struct Mol Biol* 2018, **25**:185–194.

59. Lyumkis D: **Challenges and opportunities in cryo-EM single-particle analysis**. *J Biol Chem* 2019, **294**:5181–5197.

Figure captions and tables

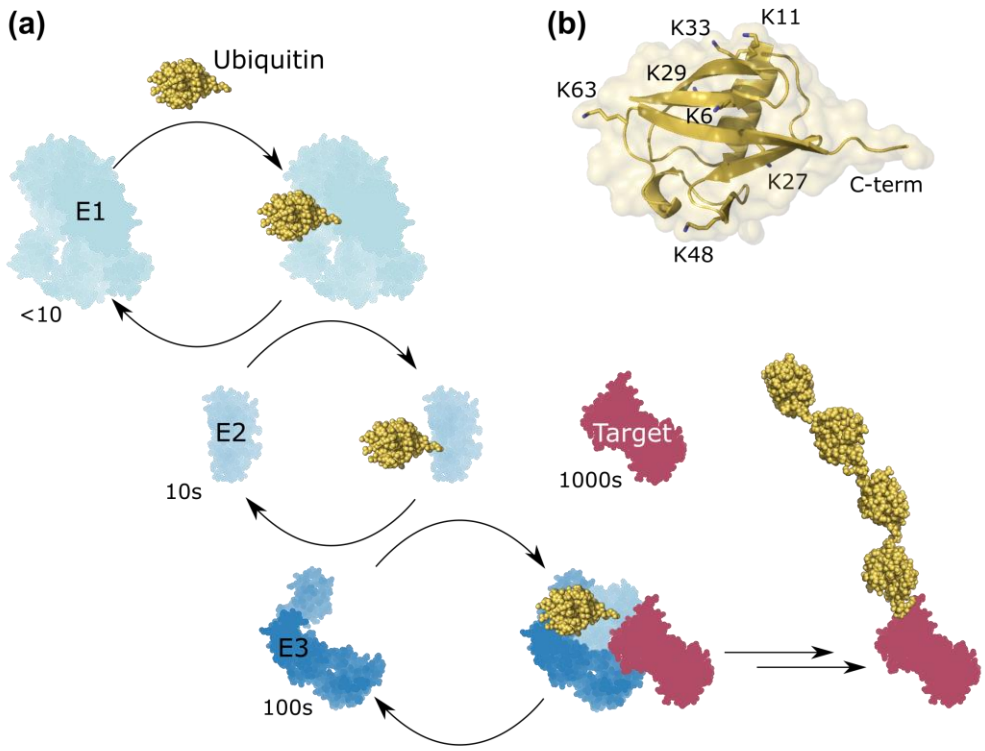

Figure 1. The ubiquitination machinery. **(a)** The ubiquitination pathway with E1 represented by Uba1 (PDB: 4II2 chain A [6]), E2 represented by Ubc9 (PDB: 5JNE chain B [7]), E3 represented by Siz1 (PDB: 5JNE chain A), and target represented by PCNA (PDB: 5JNE chain D); Ub and tetra-Ub represented by 1UBQ and 3HM3, respectively). A RING-E3 mechanism is shown and the approximate number of each type of protein are shown. **(b)** Ubiquitin with lysine sidechains represented as sticks (1UBQ).

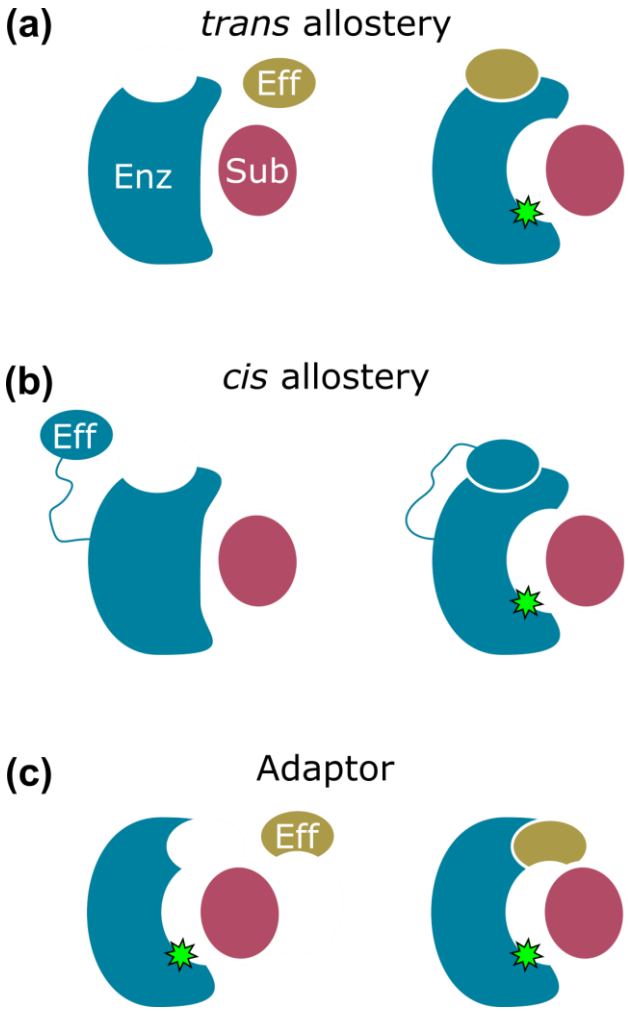

Figure 2. Regulation of the ubiquitination machinery arises from multiple origins. **(a)** Classical, *trans* allostery is triggered by binding of an effector (gold) distinct from the enzyme (blue) as shown, however may also arise from an additional domain on the substrate (red). **(b)** Allostery can also be triggered in *cis* by additional domains/subunits within the enzyme. **(c)** Substrate binding can also be mediated by an effector that extends the binding interface, as shown, or may act to sterically obscure the binding interface. All schematics illustrate positive regulation, although negative regulation is also possible.

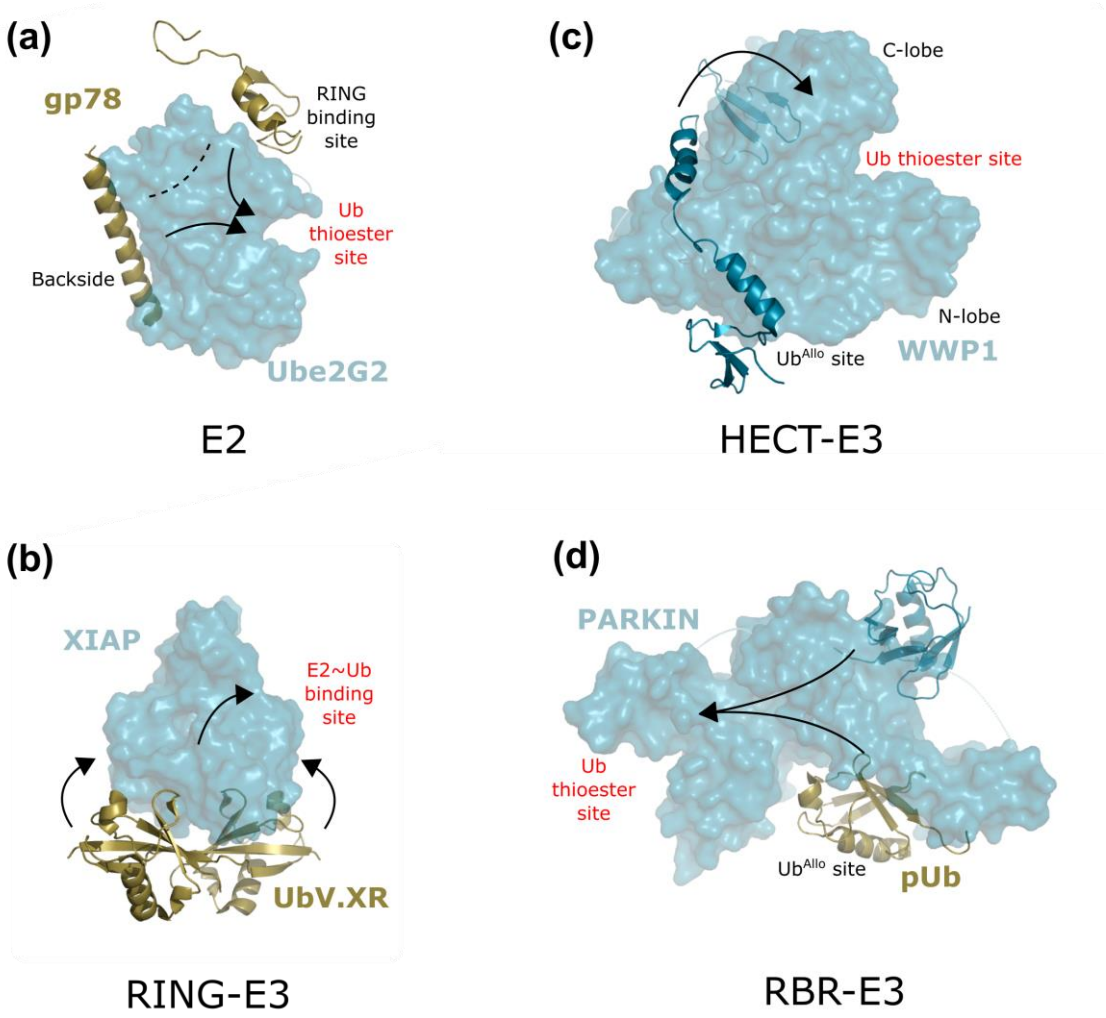

Figure 3. Examples of structures determined with effectors. Effectors are represented as ribbons, the active site highlighted by red text, and interactions of between the allosteric and active sites suggested with arrows. Dashed lines indicate interactions between allosteric sites. **(a)** Crystal structure of Ube2G2 in complex with gp78$^{RING\text{-}G2BR}$ (PDB: 4LAD [19]). The RING binding site is considered an effector site from the perspective of the E2. **(b)** Crystal structure of WWP1^{2L34HECT} (PDB: 6J1X [23]). **(c)** Crystal structure of XIAP^{RING} dimer in complex with UbV.XR dimer (PDB: 5O6T [27•]). UbV.XR is presumed to stabilize the RING dimer. The E2~Ub binding site is considered the active site with respect to the RING domain. **(d)** Crystal structure of Parkin^{UblR0RBR} in complex with pUb (PDB: 5N2W [47•]).

Table 1

Selected examples of regulation of the ubiquitination enzymes

Enzyme	Effector	Type	References
Ube2G2	gp78 GB2R domain	*trans* and adaptor	[19,33••]
	gp78 RING domain	*trans* and adaptor	[19,33••]
Ube2T	FANCL	*trans* and adaptor	[34•]
Ube2D	UHRF1 Ubl domain	adaptor, possible *trans*	[35,36••,37]
UHRF1	chromatin	*trans*	[35]
XIAP	UbV.XR	*trans*	[27•]
Itch	Itch WW2-linker region	*cis*	[13•]
WWP2	WWP2 WW2-linker region	*cis*	[12•]
HUWE1	HUWE1 dimerization region	*trans* (between two HUWE1 molecules)	[47••]
	HUWE1 activation region	*cis*	[47••]
Parkin	Parkin Ubl domain	*cis*	[8]
	pUb	*trans*	[48•,51–53]