Review

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Structural mechanisms of HECT-type ubiquitin ligases

https://doi.org/10.1515/hsz-2017-0184
Received June 20, 2017; accepted September 25, 2017; previously published online October 10, 2017

Abstract: Ubiquitin ligases (E3 enzymes) transfer ubiquitin from ubiquitin-conjugating (E2) enzymes to target proteins. By determining the selection of target proteins, modification sites on those target proteins, and the types of ubiquitin modifications that are formed, E3 enzymes are key specificity factors in ubiquitin signaling. Here, I summarize our knowledge of the structural mechanisms in the HECT E3 subfamily, many members of which play important roles in human disease. I discuss interactions of the conserved HECT domain with E2 enzymes, ubiquitin and target proteins, as well as macromolecular interactions with regulatory functions. While we understand individual steps in the catalytic cycle of HECT E3 enzymes on a structural level, this review also highlights key aspects that have yet to be elucidated. For instance, it remains unclear how diverse target proteins are presented to the catalytic center and how certain HECT E3 enzymes achieve specificity in ubiquitin linkage formation. The structural and functional properties of the N-terminal regions of HECT E3 enzymes that likely act as signaling hubs are also largely unknown. Structural insights into these aspects may open up routes for a therapeutic intervention with specific HECT E3 functions in distinct pathophysiological settings.

Keywords: E3 enzyme; enzyme mechanism; enzyme regulation; posttranslational modification; X-ray crystallography.

Introduction

Ubiquitin, a small posttranslational protein modifier, regulates an astounding range of cellular signaling pathways (Komander and Rape, 2012). It is attached to target proteins by a cascade of ubiquitin-activating (E1) enzymes, ubiquitin-conjugating (E2) enzymes, and ubiquitin ligases (E3 enzymes). With two E1 (Handley et al., 1991; Chiu et al., 2007; Jin et al., 2007; Pelzer et al., 2007), approximately 40 E2 (Michelle et al., 2009), and over 600 E3 enzymes (Li et al., 2008) in the human proteome, enzyme multiplicity increases dramatically throughout the catalytic cascade. Consistently, it is the battalion of E3 enzymes at the end of the cascade that has the major role in encoding the specificity of ubiquitin as a molecular signal. This specificity lies, at least in part, in the selection of target proteins and modification sites and in the types of ubiquitin modifications that are formed. Therefore, E3 enzymes are considered attractive targets for a therapeutic manipulation of specific ubiquitin-mediated signaling pathways in defined disease settings.

Based on their structural organization and mechanism, E3 enzymes are subdivided into three classes: RING (Really Interesting New Gene)/U-box-, HECT (Homologous to E6AP C-Terminus)- and RBR (RING-Between-RING)-type ligases. RING-type enzymes, the largest E3 subfamily, are characterized by a RING domain, a type of zinc finger domain, that interacts with ubiquitin-loaded E2 enzymes and facilitates the transfer of ubiquitin from the E2 to the target protein in a single step. This occurs typically through the RING domain-mediated stabilization of a particular 'closed' orientation of ubiquitin with respect to the E2 (Dou et al., 2012, 2013; Plechanovová et al., 2012; Pruneda et al., 2012; Branigan et al., 2015; Wright et al., 2016). In contrast, HECT- and RBR-type E3 enzymes contain a catalytic cysteine residue and form a thioester-linked complex with ubiquitin before passing ubiquitin on to a target protein (Huibregtse et al., 1995; Scheffner et al., 1995; Wenzel et al., 2011). From a structural perspective, however, these latter two classes of enzymes are distinct. The unique architecture, catalytic mechanism, and regulation of HECT E3 enzymes is the subject of this review. Their various physiological and pathophysiological functions have been surveyed extensively elsewhere (Bernassola et al., 2008; Rotin and Kumar, 2009; Scheffner and Kumar, 2014).

I will provide an overview of the domain architectures of all known human HECT E3 enzymes and highlight the

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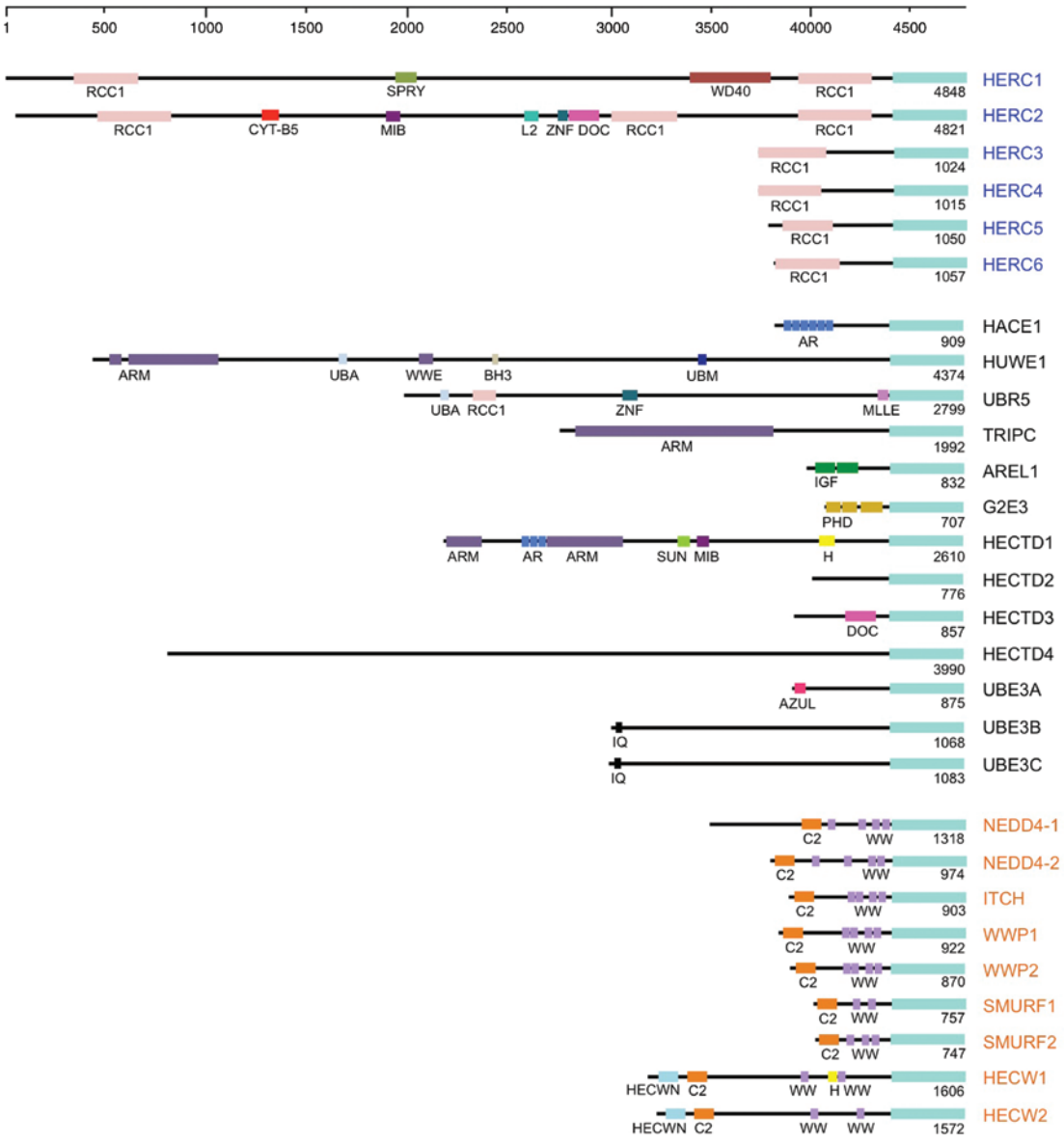

Figure 1: Domain organization of HECT E3 enzymes.

Overview of the domain organization of HECT-type E3 enzymes, as predicted by the InterPro server (Finn et al., 2017), including additional domains whose structures have been deposited in the PDB (see Table 1). Approximate domain sizes and positions are scaled along the primary sequence of the protein. The total length of each protein in number of amino acids is provided. The HERC subfamily (top section) consists of six members: HERC1 (p532, p619), HERC2, HERC3, HERC4, HERC5 (CEBP1) and HERC6. The middle section shows 13 heterogeneous members that are generally classified as 'other HECTs' and include HACE1, HUWE1 (ARF-BP1, HECTH9, MULE, LASU1, URE-B1), UBR5 (HYD, EDD), TRIPC (ULF, TRIP-12), AREL1, G2E3, HECTD1 (EULIR), HECTD2, HECTD3, HECTD4, UBE3A (E3A, E6AP, NY-REN-54), UBE3B (E3B) and UBE3C (E3C, HECTH2). The NEDD4 (also known as 'C2-WW-HECT') subfamily (bottom section) comprises nine members: NEDD4-1 (NEDD4), NEDD4-2 (NEDD4L), ITCH (AIP4, NAPP1), WWP1 (AIP5, TIUL1), WWP2 (AIP2), SMURF1, SMURF2, HECW1 (NEDL1) and HECW2 (NEDL2). Predicted domains are abbreviated as follows: RCC1, Regulator of Chromosome Condensation 1 repeat domain; SPRY, B30.2/SPRY (SPIA and RYanodine Receptor) domain (overlaps in HERC1 with predicted concanavalin A-like lectin/glucanase domain); WD40, WD40/YVTN repeat-like-containing domain; CYT-B5, Cytochrome B5-like heme/steroid binding domain; MIB, MIB-HERC2 domain (overlaps in HECTD1 with CPH domain); L2, ribosomal protein L2 domain (overlaps in HERC2 with predicted CPH domain); ZNF, zinc finger; DOC, APC10/DOC domain (overlaps in HERC2 and in HECTD3 with galactose-binding domain-like region; AR, ankyrin repeat-containing domain; ARM, armadillo-type fold domain (overlaps in TRIPC with a predicted WWE domain); UBA, ubiquitin-associated domain; WWE, WWE domain; BH3, BCL-2 homology region 3 domain; UBM, ubiquitin-binding motif; MLLE, Mademoiselle/PABC domain; IGF, immunoglobulin-like fold (overlaps in AREL1 with predicted filamin repeat-like fold); PHD, PHD-type zinc finger; SUN, SAD1/UNC domain (overlaps in HECTD1 with predicted galactose-binding-like domain); AZUL, AZUL domain/ N-terminal zinc-binding domain; IQ, IQ domain/ EF-hand binding site; C2, C2 domain; WW, WW domain; HECWN, HECW1/2 N-terminal domain; H, helical bundle (HECTD1) and helical box domain (HECW1), respectively. Predicted coiled-coil regions are not included.

common features of their reaction scheme, such as the catalytically imperative interactions of their canonical HECT domain with E2 enzymes, ubiquitin, and target proteins. Subsequently, I will discuss those regulatory interactions between HECT E3 enzymes and macromolecular factors that have been characterized structurally, with a particular focus on the role of HECT E3 oligomers. Finally, this review highlights the many open questions that currently limit our understanding of the structural mechanisms of HECT E3 enzymes.

Structural composition

The 28 human HECT E3 enzymes comprise between ~700 and ~4800 residues (Figure 1). They are characterized by an eponymous C-terminal HECT domain of ~40 kDa (Huibregtse et al., 1995), first defined structurally for E6AP (UBE3A) (Huang et al., 1999). The HECT domain consists of two lobes, a larger N-terminal lobe ('N-lobe') and a smaller C-terminal lobe ('C-lobe') that are connected by a short linker (Figure 2). Flexibility of this linker was found to be essential for catalysis (Verdecia et al., 2003) and allows for rearrangements of the two lobes with respect to each other, as illustrated by various different crystal structures of HECT domains (Figure 2) (Lorenz et al., 2012). The C-lobe contains a catalytic cysteine residue and interacts with ubiquitin during the transfer reaction. The very C-terminal region of the C-lobe ('C-tail') has also important, yet not entirely clarified roles in catalysis (Salvat et al., 2004; Kamadurai et al., 2013; Maspero et al., 2013). While the N-lobe associates with E2 enzymes (Huang et al., 1999), the recognition of target proteins is typically mediated by domains or motifs within the extended regions N-terminal to the catalytic HECT domain. The structural organization of these regions varies across the HECT E3 family (Figure 1).

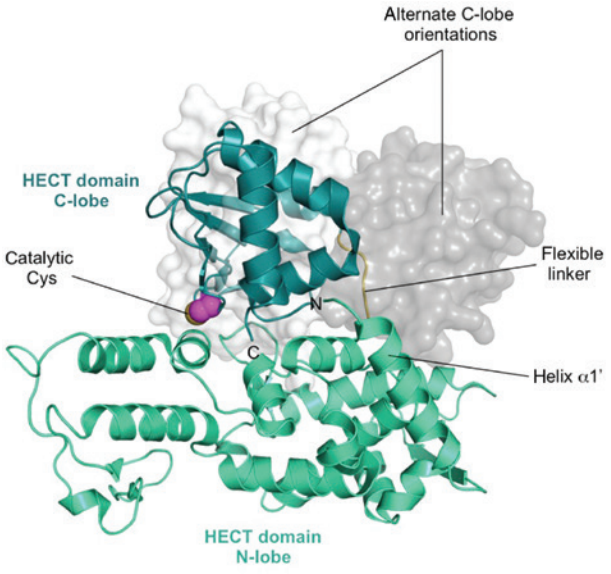

Figure 2: Architecture of the catalytic HECT domain.
The canonical catalytic HECT domain of WWP1 (PDB ID: 1ND7; Verdecia et al., 2003) is shown in ribbon representation. The side chain of the catalytic cysteine residue is displayed as balls; the flexible linker connecting the two lobes is highlighted in yellow. The N- and C-termini of the polypeptide chain are labeled. Two alternate C-lobe positions, as seen in the crystal structures of E6AP (PDB ID: 1C4Z; Huang et al., 1999), gray, and in a NEDD4-2 complex with an E2 and ubiquitin (PDB ID: 3JW0; Kamadurai et al., 2009), white, are displayed in surface rendition in the background; for clarity, the corresponding N-lobes (that were superposed with the N-lobe of WWP1) are not shown.

Members of the NEDD4 subfamily (NEDD4-1, NEDD4-2, ITCH, WWP1, WWP2, SMURF1, SMURF2, HECW1, HECW2, as well as the yeast homologue RSP5) contain a Ca^{2+}-dependent, membrane-targeting C2 domain, followed by two to four WW domains that interact with particular proline-rich motifs (Ingham et al., 2004; Leon and Haguenauer-Tsapis, 2009). The structures of several individual C2 and WW domains have been determined in both *apo* and peptide-bound forms (Table 1).

Enzymes in the HERC subfamily (HERC1 to 6) are predicted to contain RCC1 (Regulator of Chromatin Condensation 1) repeat domains N-terminal to the HECT domain. In HERC3 to HERC6 (~1000 residues long) the predicted RCC1 repeats and the HECT domain are separated by a ~340- to 585-residue linker with no recognizable domain content. In the larger HERC1 and HERC2 proteins (~4800 residues each), the predicted RCC1 repeats are located a ~140-residue distance from the HECT domain, and a number of additional predicted domains are scattered across their N-terminal regions (Figure 1).

The remaining 13 HECT E3 enzymes are also predicted to contain diverse domains N-terminal to their HECT domains (Figure 1). As seen for the HERC subfamily, these structured domains only account for a small portion of the N-terminal regions. The remainder of these regions are not expected to adopt any known folds, but include stretches of low sequence complexity, indicative of intrinsic disorder. Interestingly, the amount of disorder in eukaryotic proteins was shown to be proportional to their interactivity (Haynes et al., 2006). It is, therefore, tempting to speculate that the N-terminal regions of HECT E3 enzymes have evolved as interaction hubs for the co-localization of various signaling factors.

No structures of full-length HECT E3 enzymes are available at present. In fact, the longest HECT E3 fragments for which structures have been determined are all relatively short and contain <100 residues adjacent

Table 1: Available structures of HECT E3 fragments and complexes thereof. NEDD4-type enzymes are highlighted in bold font.

E3	PDB ID	Description	Reference
HECT domain constructs			
E6AP	1D5F	Lacking the α1′-helix region	(Huang et al., 1999)
WWP1	**1ND7**		(Verdecia et al., 2003)
SMURF2	**1ZVD**		(Ogunjimi et al., 2005)
NEDD4-2	**2ONI**		
HUWE1	3G1N		
HUWE1	3H1D		(Pandya et al., 2010)
NEDD4-1	**2XBF**		(Maspero et al., 2011)
UBR5	3PT3	C-lobe only	(Matta-Camacho et al., 2012)
ITCH	**3TUG**		
NEDD4-1	**4BE8**		(Maspero et al., 2013)
WWP2	**4Y07**		(Gong et al., 2015)
NEDD4-1	**5C91**	**Covalent complex with small-molecule inhibitor**	(Kathman et al., 2015)
WWP2	**5TJQ**	**WW-2-linker-HECT domain construct; only HECT domain visible**	(Chen et al., 2017)
Extended HECT domain constructs			
RSP5	**3OLM**	**WW-3-HECT domain construct + ubiquitin**	(Kim et al., 2011)
RSP5	**4LCD**	**WW-3-HECT domain construct linked to ubiquitin and target peptide**	(Kamadurai et al., 2013)
HUWE1	5LP8	Dimerization region-HECT domain construct	(Sander et al., 2017)
WWP2	**5TJ7**	**WW-2-linker-HECT domain construct**	(Chen et al., 2017)
ITCH	**5XMC**	**Delta-C2 domain construct; PRR, WW-3, WW-4 domains disordered**	(Zhu et al., 2017)
HECT domain complexes with macromolecular partners			
E6AP	1C4Z	HECT domain (lacking the α1′-helix region) + UBCH7	(Huang et al., 1999)
NEDD4-2	**3JVZ**	**HECT domain + UBCH5B linked to ubiquitin**	(Kamadurai et al., 2009)
NEDD4-2	**3JWO**	**HECT domain + UBCH5B linked to ubiquitin**	(Kamadurai et al., 2009)
NEDD4-1	**2XBB**	**HECT domain + ubiquitin**	(Maspero et al., 2011)
RSP5	**3OLM**	**HECT domain + ubiquitin**	(Kim et al., 2011)
NEDD4-1	**4BBN**	**HECT domain linked to ubiquitin + ubiquitin**	(Maspero et al., 2013)
RSP5	**4LCD**	**HECT domain linked to ubiquitin and target peptide**	(Kamadurai et al., 2013)
NEDD4-1	**5C7J**	**HECT domain + ubiquitin variant**	(Zhang et al., 2016)
ITCH	**5C7M**	**HECT domain + ubiquitin variant**	(Zhang et al., 2016)
NEDD4-2	**5HPK**	**HECT domain + ubiquitin variant**	(Zhang et al., 2016)
RSP5	**5HPL**	**HECT domain + ubiquitin variant**	(Zhang et al., 2016)
WWP1	**5HPS**	**HECT domain + ubiquitin variant**	(Zhang et al., 2016)
WWP1	**5HPT**	**HECT domain + ubiquitin variant**	(Zhang et al., 2016)
Other domains			
E6AP	1EQX	E6 protein-interacting peptide	(Be et al., 2001)
UBR5	1I2T	MLLE domain	
NEDD4-2	**1WR3**	**Mus musculus WW-1 domain**	
NEDD4-2	**1WR4**	**Mus musculus WW-2 domain**	
NEDD4-2	**1WR7**	**Mus musculus WW-3 domain**	
ITCH	**1YIU**	**Mus musculus WW-3 domain**	
NEDD4-1	**2EZ5**	**Drosophila melanogaster WW-3 domain + peptide**	(Kanelis et al., 2006)
HECTD1	2DK3	MIB-HERC2 domain	
SMURF2	**2DJY**	**WW-3 domain + peptide**	(Chong et al., 2006)
ITCH	**2DMV**	**WW-2 domain**	
NEDD4-2	**2NSQ**	**C2 domain**	
ITCH	**2NQ3**	**C2 domain**	
WWP1	**2OP7**	**WW-4 domain**	
ITCH	**2JO9**	**Mus musculus WW-3 domain + peptide**	(Morales et al., 2007)
ITCH	**2JOC**	**Mus musculus WW-3 domain, phosphorylated**	(Morales et al., 2007)
ITCH	**2P4R**	**Proline-rich region in complex with an SH3 domain**	(Janz et al., 2007)
HUWE1	2EKK	UBA domain	
ITCH	**2YSF**	**WW-4 domain**	

Table 1 (continued)

E3	PDB ID	Description	Reference
SMURF2	**2JQZ**	**C2 domain**	(Wiesner et al., 2007)
UBR5	2QHO	UBA domain + ubiquitin	(Kozlov et al., 2007)
NEDD4-1	**3B7Y**	**C2 domain**	
HECTD1	3DKM	CPH domain	
HERC2	2KEO	Cytochrome-B5-like domain	
HERC2	3KCI	RCC1-3 repeat domain	
NEDD4-1	**2KQ0**	**WW-3 domain + peptide**	
NEDD4-1	**2KPZ**	**WW-3 domain + peptide**	
E6AP	2KR1	AZUL domain	(Lemak et al., 2011)
HECW1	**3L4H**	**Helical box and WW-2 domains**	
NEDD4-1	**3M7F**	**Mus musculus C2 domain complex**	(Huang and Szebenyi, 2010)
SMURF2	**2KXQ**	**Tandem WW-2-3 domains + peptide**	(Chong et al., 2010)
UBR5	3NTW	Rattus norvegicus MLLE domain + peptide	
SMURF1	**3PYC**	**C2 domain**	
NEDD4-2	**2LB2**	**WW-2 domain + phosphorylated peptide**	(Aragón et al., 2011)
SMURF1	**2LAZ**	**WW-1 domain + phosphorylated peptide**	(Aragón et al., 2011)
NEDD4-2	**2LAJ**	**WW-3 domain + phosphorylated peptide**	(Aragón et al., 2011)
SMURF1	**2LB0**	**WW-1 domain + phosphorylated peptide**	(Aragón et al., 2011)
SMURF1	**2LB1**	**WW-2 domain + peptide**	(Aragón et al., 2011)
HECTD1	2LC3	Helical bundle domain	
HECW2	**2LFE**	**N-terminal domain of unknown function**	
SMURF1	**2LTX**	**WW-2 domain + peptide**	(Aragón et al., 2011)
SMURF2	**2LTZ**	**WW-3 domain + peptide**	(Aragón et al., 2011)
NEDD4-2	**2LTY**	**WW-2 domain + peptide**	(Aragón et al., 2011)
E6AP	4GIZ	E6-interacting peptide + Alphapapillomavirus 9 E6 protein	(Zanier et al., 2013)
NEDD4-1	**2M3O**	**WW-1 domain + peptide**	(Bobby et al., 2013)
HERC2	4L1M	RCC1-1 domain	
NEDD4-1	**4N7F**	**WW-3 domain**	(Qi et al., 2014)
NEDD4-1	**4N7H**	**WW-3 domain + peptide**	(Qi et al., 2014)
HERC1	4O2W	RCC1-3 domain	
NEDD4-2	**2MPT**	**WW-3 domain + peptide**	(Escobedo et al., 2014)
HUWE1	2MUL	UBM domain	
HERC1	4QT6	SPRY domain	
ITCH	**4ROF**	**WW-3 domain + peptide**	
E6AP	4XR8	E6-interacting peptide + p53 + Alphapapillomavirus 9 E6 protein	(Martinez-Zapien et al., 2016)
NEDD4-1	**5AHT**	**WW-3 domain**	(Panwalkar et al., 2016)
ITCH	**5DWS**	**WW-3 domain + peptide**	
HUWE1	5C6H	BH3 domain	
ITCH	**5DZD**	**WW-4 domain + peptide**	
ITCH	**5CQ2**	**WW-3-4 domains + peptide**	(Liu et al., 2016)
NEDD4-1	**2N8S**	**Rattus norvegicus WW-1 domain**	(Spagnol et al., 2016)
NEDD4-1	**2N8T**	**Rattus norvegicus WW-2 domain + peptide**	(Spagnol et al., 2016)
ITCH	**5SXP**	**Proline-rich region in complex with an SH3 domain**	(Desrochers et al., 2017)

The structures, as deposited in the PDB, are grouped into 4 sections: (i) HECT domains, (ii) extended HECT domain constructs, including N-terminally flanking regions, (iii) HECT domains in complexes with other proteins, and (iv) motifs/domains found in the N-terminal regions of HECT E3 enzymes. In each section, the structures are arranged in chronological order of their release. Entries for enzymes of the NEDD4 subfamily are shown in bold font to highlight their over-representation compared to structures of other types of HECT E3 enzymes. Domains that occur in the corresponding proteins more than once are numbered in N- to C-terminal direction (e.g. WW-1 corresponding to the most N-terminal WW domain). Structures for which no reference is provided have been deposited in the PDB, but are currently not accompanied by a publication. Any potential omissions in this table are unintended.

to the catalytic domain (Table 1). Structural analyses of more complete HECT E3 constructs or even the full-length proteins and their macromolecular complexes are thus eagerly awaited and may help us understand if/how HECT E3 enzymes contribute to the assembly of signaling complexes or molecular scaffolds.

Catalytic mechanism

HECT E3 enzymes catalyze a two-step reaction (Figure 3A). The first step is a trans-thioesterification reaction in which the E3 enzyme takes over ubiquitin from a ubiquitin-loaded E2 enzyme and forms a thioester linkage between its catalytic cysteine residue and the C-terminus of ubiquitin. In the second step, the activated C-terminus of ubiquitin is nucleophilically attacked by a primary amino group of a target protein, giving rise to an isopeptide bond between ubiquitin and the target protein. During ubiquitin chain formation, ubiquitin itself functions as a target protein. Depending on which of the eight primary amino groups (seven lysine residues and the N-terminus) of ubiquitin acts as a nucleophile, different linkage types are generated. By convention, the ubiquitin molecule that is linked to the catalytic cysteine of the E3 (or E2) enzyme is referred to as the 'donor' ubiquitin, and the ubiquitin molecule that performs the nucleophilic attack on the donor during ubiquitin chain formation is called the 'acceptor' ubiquitin.

The chemical basis of isopeptide bond formation by HECT E3 enzymes is incompletely understood. For instance, it is unclear which mechanisms promote the deprotonation of the attacking primary amino group of the acceptor ubiquitin, thereby rendering this group nucleophilic at physiological pH. In the analogous case of E2 enzymes, the pk_a-value of the acceptor lysine was found to be suppressed through desolvation effects, as mediated by specific groups at the catalytic center (Yunus and Lima, 2006). One of these groups, an acidic side chain, can be provided either by the E2 itself (Yunus and Lima, 2006) or by ubiquitin in a mechanism of substrate-assisted catalysis (Wickliffe et al., 2011). In contrast, RBR ligases contain a histidine located two residues away from the catalytic cysteine that was proposed to act as a general base to promote deprotonation of the acceptor lysine (Stieglitz et al., 2013; Trempe et al., 2013). Interestingly, features reminiscent of both mechanisms can be found in the C-tail of HECT E3 enzymes: The acidic side chain of the C-terminal aspartate residue of NEDD4-1 was suggested to approach the active site during catalysis and is essential for isopeptide bond formation (Maspero et al., 2013). In case of WWP1 and RSP5, the C-terminus itself (rather than the acidic side chain) was suggested to contribute to the ligation reaction (Verdecia et al., 2003; Kamadurai et al., 2013). Like RBR ligases, HECT E3 enzymes also contain a conserved histidine two residues from the catalytic cysteine that impacts the chemical environment at the catalytic center (Kamadurai et al., 2009, 2013).

Another key residue in HECT E3-mediated isopeptide bond formation is a phenylalanine four residues from the C-terminus ('-4 Phe') that is conserved in the majority of HECT E3 enzymes (Salvat et al., 2004). While this residue was found to be disordered in most crystal structures of HECT domains, a structure of a chemically trapped proxy of RSP5, ubiquitin, and a target peptide during isopeptide bond formation shows that -4 Phe contacts the N-lobe (Kamadurai et al., 2013). It was therefore proposed that the function of -4 Phe is to anchor the lobes with respect to each other in a ligation-competent state (Kamadurai et al., 2013). Alternatively, -4 Phe has been hypothesized to contribute to the positioning of the donor or acceptor ubiquitin (Salvat et al., 2004; Kamadurai et al., 2013).

How ubiquitin chain formation occurs is also not fully mechanistically understood. A variety of different models have been discussed (Verdecia et al., 2003; Hochstrasser, 2006) and include, for instance, the pre-assembly of thioester-linked ubiquitin chains on the catalytic cysteine of the E3, followed by the transfer of the chain to an acceptor lysine *en bloc* ('indexation' mechanism); the step-wise transfer of individual ubiquitin moieties to the acceptor ('sequential addition' mechanism); and more complicated mechanisms that involve E2 or E3 dimers (e.g. a 'seesaw' mechanism). Based on mutational and kinetic analyses, E6AP was proposed to build chains *en bloc* (Wang and Pickart, 2005); however, direct evidence for ubiquitin chains attached to the E3 active site is lacking. In contrast, UBE3C was suggested to transfer ubiquitin sequentially (Wang and Pickart, 2005). The sequential addition mechanism has provided the basis for interpreting data in the majority of studies on NEDD4-type enzymes (French et al., 2009; Ogunjimi et al., 2010; Kim et al., 2011; Maspero et al., 2011; Herrador et al., 2013; Kathman et al., 2015) and has recently also been consolidated experimentally for a member of this subfamily, WWP1 (French et al., 2017).

E2 recognition by the HECT N-lobe

Two structures of HECT domains in complex with E2 enzymes are available at present, E6AP with UBCH7 (Huang et al., 1999) and NEDD4-2 with UBCH5B (Kamadurai et al., 2009) and reveal a common binding mode. The E2-E3-interface involves a critical Phe residue in a loop ('L4') region of the E2 that binds into a hydrophobic grove in the N-lobe of the E3 (Huang et al., 1999; Nuber and Scheffner, 1999; Kamadurai et al., 2009) (Figure 3B). Based on kinetic analyses, it has been suggested that the HECT domain of E6AP contains a second E2 binding site (Ronchi et al., 2013), but no structural model of this additional interaction is available at present.

The dissociation constant for the interaction between the E6AP HECT domain and UBCH7 falls in the low

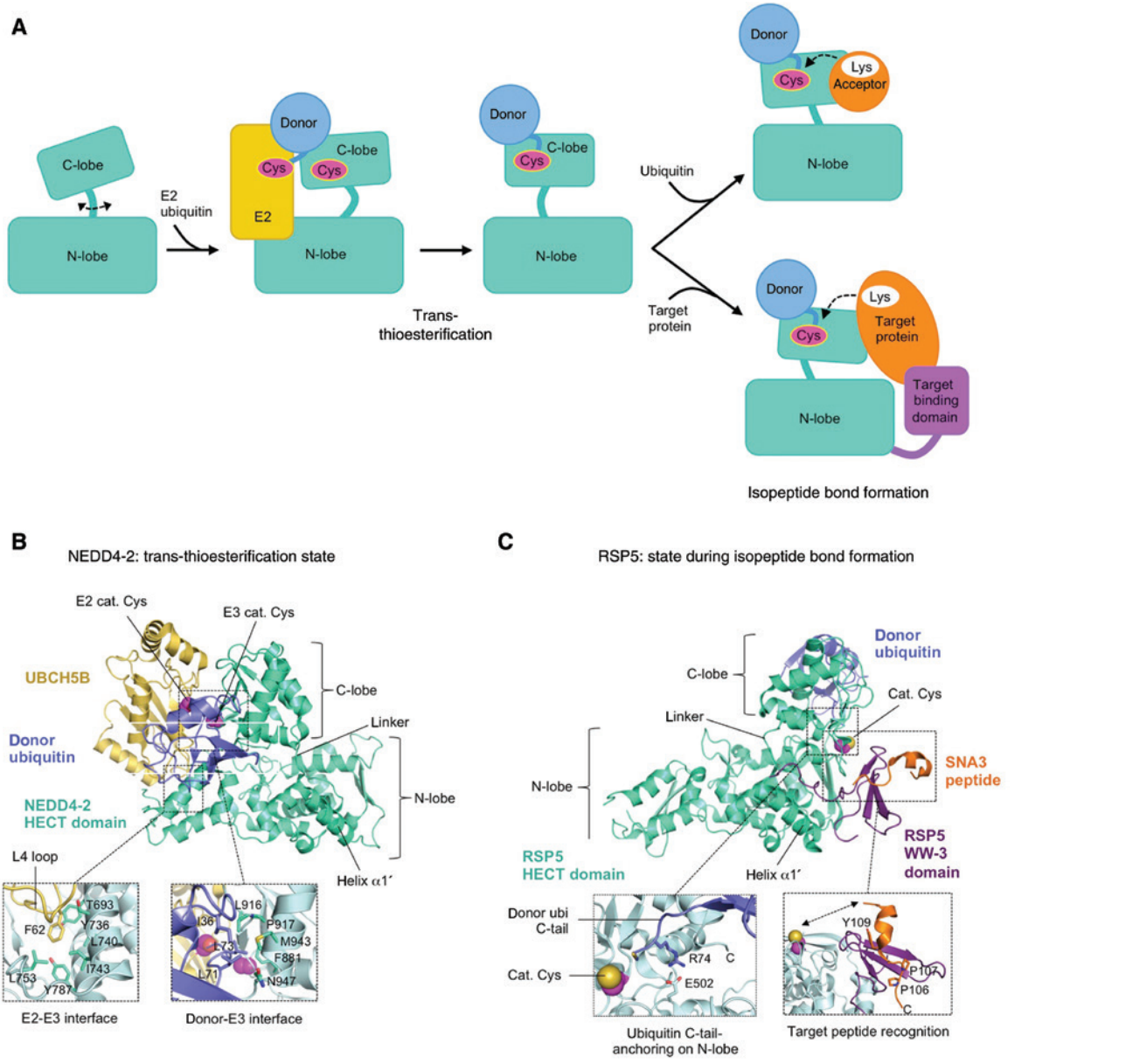

Figure 3: Structural mechanism of HECT E3 enzymes.
(A) Schematic of the reaction mechanism featuring the C-terminal HECT domain exclusively and including the *apo* state (in which the N- and C-lobe are flexible to each other); a non-covalent complex with a ubiquitin-loaded E2 enzyme (pre-trans-thioesterification state); a ubiquitin-loaded E3 state (post-trans-thioesterification state); and complexes with an acceptor ubiquitin or target protein that result in isopeptide bond formation between the donor ubiquitin and the acceptor or target protein. The recruitment of target proteins is typically mediated by target-binding domains that are located N-terminal to the HECT domain. (B) Crystal structure of a pre-trans-thioesterification state, including an oxyester-linked ubiquitin-E2 conjugate (UBCH5B, C85S variant) and a catalytically inactive variant of the HECT domain of NEDD4-2 (C922A) (PDB ID: 3JVZ; Kamadurai et al., 2009). The inserts show details of the E2-N-lobe interface, including the key phenylalanine residue of the E2 (F62 in the case of UBCH5B) and contacting residues on the E3 within a distance of 5 Å (left side); the donor ubiquitin-C-lobe interface, including Ile 36, Leu 71, and Leu 73 of ubiquitin and surrounding residues of the C-lobe within a distance of 5 Å (right side). (C) Crystal structure of a cross-linked, ternary complex of the HECT domain of RSP5, ubiquitin (C75C), and a SNA3-derived peptide (acceptor lysine residue replaced by an unnatural amino acid), for details see text (PDB ID: 4LCD; Kamadurai et al., 2013). The N-lobe of the HECT domain is shown in the same orientation as in (B). The cross-linker, connecting residues, and acceptor lysine-containing region of the peptide were not modeled, giving rise to a gap between the E3 active site and the N-terminus of the peptide. The insert (right side) shows details of the PY-motif of SNA3 binding to the WW domain; the missing peptide region (13 residues) between the PY-motif and the acceptor position is indicated by an arrow. The other insert (left side) shows that the C-terminal tail of ubiquitin is anchored by an ion pair on the N-lobe of the HECT domain. Protein backbones are shown in ribbon representation; the side chains of residues at the position of the catalytic cysteine are rendered as balls; and other labeled side-chains are shown as sticks.

micromolar range *in vitro*, independently of the loading of the E2 enzyme with ubiquitin (Eletr et al., 2005; Eletr and Kuhlman, 2007; Purbeck et al., 2010; Mortensen et al., 2015). In contrast, binding of UBCH5B to E6AP was found to be strengthened by the presence of E2-bound donor ubiquitin (Mortensen et al., 2015). Interestingly, the affinity between SMURF2 and UBCH7 is enhanced by the adapter protein SMAD7, which provides interaction sites for both enzymes (Ogunjimi et al., 2005). Additionally, co-localization effects can boost the occupancy of protein complexes in the cellular context, despite low affinities of the underlying pairwise protein-protein interactions. Context-dependent interactions have emerged as a recurring feature in the action of ubiquitination enzymes. They allow ubiquitination enzymes to mediate highly specific, yet transient, protein-protein interactions and enable an efficient handover of ubiquitin throughout the catalytic cascade (Eletr et al., 2005; Ye and Rape, 2009).

The functional compatibility of individual HECT E3 and E2 enzymes was delineated systematically *in vitro* (Sheng et al., 2012). However, it has proven difficult to pinpoint the structural determinants of their respective affinities and specificities, possibly due to a malleable nature of the relevant hydrophobic interactions (Eletr and Kuhlman, 2007; Kamadurai et al., 2009; Kar et al., 2012). Also, with a few exceptions (Kumar et al., 1997; Anan et al., 1998; Medintz et al., 1998; Dastur et al., 2006; Wong et al., 2006; Stoll et al., 2011; Sung et al., 2016), it is unclear which HECT E3 and E2 enzymes form physiological pairs. In consequence, it is also widely unknown whether HECT E3 enzymes transfer ubiquitin-like (Ubl) proteins besides ubiquitin. That this can indeed occur was shown for human HERC5 and murine HERC6, which transfer the Ubl ISG15 (Dastur et al., 2006; Wong et al., 2006; Oudshoorn et al., 2011; Ketscher et al., 2012) in collaboration with the E2 enzyme UBCH8 (Kim et al., 2004; Zhao et al., 2004) and the ISG15-specific E1 enzyme UBE1L (Yuan and Krug, 2001). It will be interesting to elucidate the structural underpinnings of the specificity of HERC5/6 for ISG15 and to interrogate whether other HECT E3 enzymes utilize Ubls in the cell.

Positioning of the donor ubiquitin on the HECT C-lobe

A structural characterization of the complexes formed between HECT domains, donor and acceptor ubiquitin is challenging, due to the low affinities of the underlying interactions and the hydrolytic susceptibility of the thioester linkage between the donor ubiquitin and the E3. Upon substitution of the native thioester bond by a stable analog, the structures of chemical mimics of donor-HECT domain complexes could be determined for three enzymes in the NEDD4 subfamily, NEDD4-1, NEDD4-2 and yeast RSP5, at different stages of the reaction cycle (Kamadurai et al., 2009, 2013; Maspero et al., 2013).

To crystallize a proxy of the E3-E2-ubiquitin complex in a state prior to trans-thioesterification, the thioester linkage between the E2 enzyme and donor ubiquitin was replaced by an oxyester (with a catalytic cysteine-to-serine variant) (Kamadurai et al., 2009). To mimic the product of the trans-thioesterification reaction, a donor ubiquitin variant with the C-terminal glycine substituted by cysteine was linked to the catalytic cysteine of the E3 by a disulfide bond (Maspero et al., 2013). Finally, to approximate the reaction complex during isopeptide bond formation, a three-way cross-linker was employed to bring together RSP5, ubiquitin, and a target protein-derived peptide (Kamadurai et al., 2013).

In all three crystal structures, the donor ubiquitin forms a conserved hydrophobic interface with the HECT C-lobe. This interface includes residues Ile 36, Leu 71, and Leu 73 of ubiquitin and a hydrophobic surface patch on the C-lobe that is removed from the active site (Figure 3B). This arrangement is enabled by an extended conformation of the C-terminal tail of ubiquitin and allows the catalytic cysteines of the E2 and the E3 to come into close proximity. The interface between ubiquitin and the C-lobe can thus be preserved throughout the trans-thioesterification reaction. Consistently, the integrity of this interface was found to be critical for ubiquitin transfer (Kamadurai et al., 2009; Maspero et al., 2013).

A similar hydrophobic tethering mechanism for the donor ubiquitin (or donor Ubl) promotes catalytic efficiency and processivity of E2 enzymes (Stewart et al., 2016) and was shown to be stabilized by RING-type E3 enzymes (Dou et al., 2012, 2013; Plechanovová et al., 2012; Pruneda et al., 2012; Scott et al., 2014; Branigan et al., 2015; Wright et al., 2016) as well as SUMO-ligases (Reverter and Lima, 2005; Cappadocia et al., 2015; Streich and Lima, 2016). Importantly, however, the interactions of HECT E3 or E2 enzymes with the donor ubiquitin do not determine the linkage specificity of ubiquitin chain formation. Instead, such specificity arises from the recognition of the acceptor ubiquitin, as discussed below.

Attack by the acceptor ubiquitin: the missing key to understanding linkage specificity

Which types of ubiquitin modifications individual HECT enzymes catalyze on cellular target proteins is

incompletely understood. Some HECT E3 enzymes have been reported to catalyze mono-ubiquitination preferentially, some exhibit linkage specificity or, at least, selectivity in ubiquitin chain formation, and others appear promiscuous (Rotin and Kumar, 2009; Komander and Rape, 2012; Swatek and Komander, 2016). Key to understanding how particular HECT E3 enzymes can encode linkage specificity is to reveal how those enzymes position the acceptor ubiquitin in such a manner that one particular primary amino group of the acceptor can nucleophilically attack the activated C-terminus of the donor that is bound at the E3 active site. Various studies have revealed that critical specificity determinants are encoded by the catalytic HECT domain (Wang and Pickart, 2005; Kim and Huibregtse, 2009; Sheng et al., 2012). Furthermore, using chimeric HECT domains composed of lobes from different E3 enzymes, it was demonstrated that linkage specificity is governed by the C-lobe exclusively (Kim and Huibregtse, 2009). This implies that the C-lobes of linkage-specific HECT E3 enzymes interact with acceptor ubiquitin specifically and independently of the N-lobe. In line with this notion, residues in ubiquitin adjacent to particular acceptor lysines were found to be essential for linkage-specific chain formation (Wang et al., 2006). Interestingly, the HECT C-tail was also found to impact linkage specificity in the case of NEDD4-1 (Maspero et al., 2013). Similar to E2 enzymes whose affinities for ubiquitin *in trans* fall into the millimolar K_D-range (Wickliffe et al., 2011), however, the interactions of HECT C-lobes with ubiquitin have thusfar proven too weak to be detected *in trans* (French et al., 2009; Kamadurai et al., 2009; Kim and Huibregtse, 2009). Moreover, no structures of HECT domains with ubiquitin in an acceptor position facing the active site have been solved. Hence, it remains unclear on a structural level how linkage specificity is achieved by certain HECT E3 enzymes.

Target recruitment

The recognition and modification of target proteins by E3 enzymes is generally difficult to study on a structural level due to the weak and transient nature of their interactions. Analyses of many HECT-type E3 enzymes are presented with the added complication that the target binding domains of the E3 have either not been identified or are removed from the catalytic HECT domain by sequence stretches that are likely disordered. Our knowledge of target recognition by HECT E3 enzymes is, therefore, limited to rather few cases.

WW domains in the NEDD4 subfamily

As shown in Figure 1, NEDD4-type enzymes contain a series of WW domains that can interact with specific proline-rich motifs, such as Leu/Pro-Pro-X-Tyr (L/PPXY, short 'PY') (Chen and Sudol, 1995), in target or adapter proteins (Ingham et al., 2004; Leon and Haguenauer-Tsapis, 2009). How a WW domain-bound target protein is presented to the catalytic center on the HECT domain was visualized for the first time in a crystal structure containing the WW-3-HECT domain region of RSP5, donor ubiquitin, and a PY-containing peptide derived from the target protein SNA3 (Kamadurai et al., 2013). In this study, a complex mimicking the catalytic intermediate during isopeptide bond formation was trapped by means of three-way cross-linking of the catalytic cysteine of RSP5 with an engineered C-terminal cysteine in ubiquitin (G75C variant), and an unnatural amino acid *in lieu* of the acceptor lysine of the SNA3-peptide (Figure 3C). As described above, this structure showed that the binding mode of donor ubiquitin to the C-lobe is the same during isopeptide bond formation as pre- and post-trans-thioesterification. However, the C-terminal tail of ubiquitin is also anchored on the N-lobe (through a tail residue, Arg 74), which, together with specific inter-lobe contacts, generates a composite catalytic center that was suggested to prime the thioester bond for the attack by a target lysine (Figure 3C). Initial evidence suggests that a similar catalytic architecture is used by other members of the NEDD4 subfamily (Kamadurai et al., 2013).

The study also shed light on the structural basis of acceptor lysine selection: While the peptide region harboring the critical acceptor lysine of SNA3 and the 3-way crosslinker could not be built into the electron density, a computational model of the unresolved peptide region was generated. Combined with activity assays, these studies suggest that the WW domain adopts a fixed orientation (as seen in the crystal structure) with respect to the HECT domain and, therefore, a minimal spacer of 10 residues between the acceptor lysine residue and the WW domain-binding PY-motif is required for productive target modification (Figure 3C) (Kamadurai et al., 2013).

Mademoiselle domain of UBR5

For HECT enzymes outside of the NEDD4 subfamily, little is known about the structural mechanisms of target protein modification. The HECT domain of UBR5 is flanked by a *mademoiselle* (MLLE) domain (also known as 'PABC' domain) (Deo et al., 2001) that interacts with a

particular motif (PABP-interacting motif, abbreviated as 'PAM') in the target protein PAIP2 (Lim et al., 2006). While a structure of the MLLE domain of UBR5 in complex with a PAM-peptide was determined and interactions between the MLLE and HECT domains were identified (Muñoz-Escobar et al., 2015), it has remained unclear how exactly the MLLE domain is positioned with respect to the HECT domain. As for many other HECT E3 enzymes, the missing link is a structure containing both the catalytic and the target-binding domain of UBR5 to unveil their relative orientations.

Viral hijacking of E6AP

E6AP provides an example of how viral pathogens can hijack the ubiquitin-proteasome system of their host. In the presence of the human papilloma virus (HPV) E6 oncoproteins E6AP was found to ubiquitinate the tumor suppressor p53 and other proteins, thus promoting HPV-induced cervical carcinogenesis (Scheffner et al., 1993; Beaudenon and Huibregtse, 2008). A peptide motif, 'LXXLL', in the N-terminal region of E6AP mediates the interaction with the E6 protein (Huibregtse et al., 1993; Ansari et al., 2012; Zanier et al., 2013). Recently, a structure containing the ternary complex of the LXXLL-peptide, E6, and the core domain of p53 was determined, and explains why the E6AP-E6 complex, but not the E6 protein alone, can recruit p53 (Martinez-Zapien et al., 2016). Yet, how p53 is presented to and modified by the HECT domain of E6AP is still unknown. Interestingly, while aberrant activation of E6AP promotes disease progression in cervical cancer, inactivation of this E6AP is linked to Angelman Syndrome, a neurodevelopmental disorder (Scheffner and Kumar, 2014). In the latter context the critical target proteins of E6AP have remained unclear, with the exception of the synaptic protein ARC (Greer et al., 2010).

Regulatory mechanisms

Our knowledge of the structural mechanisms that control the activities of HECT E3 enzymes is expanding at a rapid pace. Examples of virtually all imaginable regulatory principles have been identified in the HECT E3 family, including intramolecular domain interactions, changes in oligomeric states, interactions with macromolecular binding partners and small-molecule ligands, auto-ubiquitination, and other types of posttranslational modifications. The distinct regulatory mechanisms employed by individual HECT E3 enzymes are surveyed in the following paragraphs.

E6AP: to trimerize or not to trimerize

The activity of E6AP appears to be modulated by oligomerization; yet, the structural mechanisms of this phenomenon are incompletely understood. While the HECT domain of E6AP was the first one to be crystallized, both in its *apo* and E2-bound forms (Huang et al., 1999), no additional structures of the E6AP HECT domain have been reported since then (Table 1). In both available structures, the HECT domain forms a crystallographic trimer through N-lobe-N-lobe interactions (Figure 4A). The C-lobes of neighboring subunits make only minor, electrostatic contacts with each other in this context. The question whether this crystallographic trimer presents a functionally relevant state of E6AP has been the subject of several studies with varied outcomes.

On the one hand, it was suggested that the trimeric arrangement of E6AP in the crystal is due to adventitious packing (Huang et al., 1999). This interpretation was based on the observation that the crystallized E6AP construct is monomeric in solution, and that the substitution of a key interfacing residue in the crystallographic trimer, Phe 727, by alanine does not significantly impact the trans-thioesterification reaction (Huang et al., 1999). Size-exclusion chromatographic studies of mammalian cell lysates are in line with this interpretation (Huibregtse et al., 1991; Martinez-Noel et al., 2012): while full-length E6AP (~100 kDa) elutes as part of various macromolecular complexes, the protein was found to be enriched in elution fractions that likely correspond to a monomeric state (≤200 kDa, based on column calibration, i.e. assuming globular shape).

On the other hand, there has been a long-standing notion that E6AP self-associates, at least transiently, reaching back to the observation that, in the absence of the HPV E6 protein, the auto-ubiquitination of E6AP occurs predominantly *in trans* (Nuber et al., 1998). More recently, a non-conservative amino acid substitution at the trimer interface, F727D, was found to decrease the size of recombinant, full-length E6AP, consistent with a trimer-to-monomer transition (Ronchi et al., 2014). Moreover, various studies have referenced the crystallographic trimer to rationalize mutational results (Chan et al., 2013; Ronchi et al., 2014; Mortensen et al., 2015; Yi et al., 2015). For instance, several mutations at the crystallographic trimer interface, including F727D, R626A, D543A, Y533A (a mutation site in Angelman syndrome), and Y636D

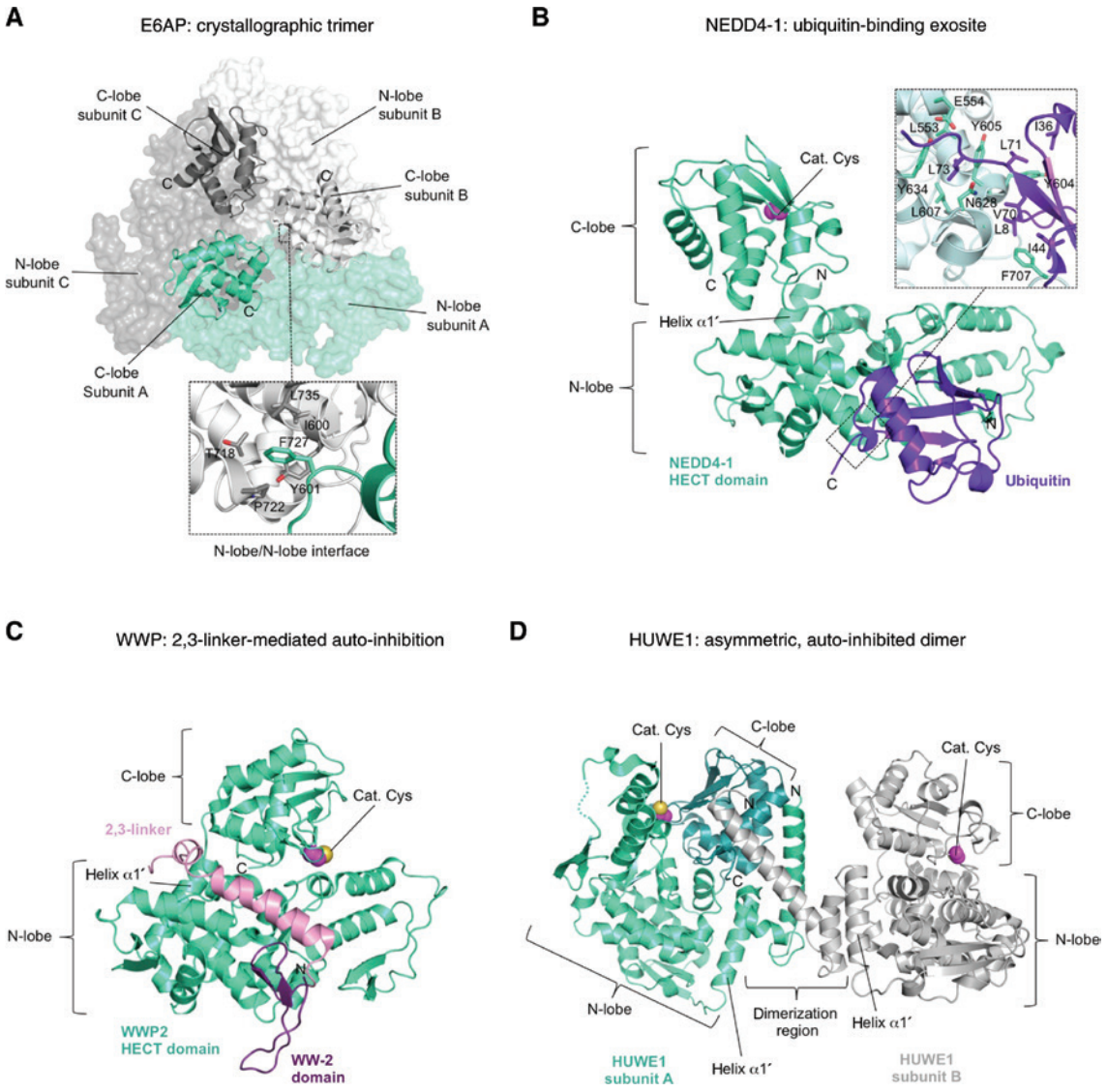

Figure 4: Regulatory mechanisms of HECT E3 enzymes.
(A) Structure of the crystallographic trimer formed by a truncated HECT domain construct of E6AP (viewed along the 3-fold axis). For clarity, the N-lobes of the three subunits are shown as surfaces, the C-lobes as ribbons. The insert provides details of the N-lobe-N-lobe interaction between two subunits, focusing on Phe 727 (the subject of mutational analyses) and its inter-subunit contacts within a distance of 5 Å. (B) The ubiquitin-binding 'exosite' of NEDD4 subfamily enzymes, illustrated by a structure of the non-covalent complex of the NEDD4-1 HECT domain and ubiquitin (PDB ID: 2XBB; Maspero et al., 2011). (C) Auto-inhibitory interaction of the 2,3-linker with the HECT domain of WWP2, as seen in a crystal structure of a WW-2-(2,3-linker)-HECT fusion construct (PDB ID: 5TJ7; Chen et al., 2017). The HECT domain is shown in the same orientation as in (B) to illustrate that the position of the 2,3-linker occludes the exosite. (D) Crystal structure of the asymmetric dimer formed by an extended HECT domain construct of HUWE1 (PDB ID: 5LP8; Sander et al., 2017). Dimerization is mediated by an α-helical region that flanks the HECT domain N-terminally. The green subunit, in which the C-lobe is locked conformationally at the dimer interface, adopts an auto-inhibited state.

(a variant mimicking phosphorylation of Y636 by the tyrosine kinase c-ABL) were found to reduce the activity of E6AP, nourishing the idea that the E6AP trimer represents an activated state (Chan et al., 2013; Ronchi et al., 2014).

An interesting cue for interpreting this complex body of data can be found in the crystal structure of E6AP: The crystallized construct is devoid of an N-terminal α-helical element [residues 472-496 of isoform 1, denoted here as 'α1′-helix' (Verdecia et al., 2003)] that was found to be important for the stability of the HECT domain in the case of HUWE1 (Pandya et al., 2010). The α1′-helix is present in all other crystallized HECT domain constructs (Figure 1A) and adopts a conserved position with respect to the remainder of the HECT domain in all of these structures,

including those that contain extensions N-terminal to the HECT domain (Table 1). When modeled into the structure of E6AP, this conserved position of the α1′-helix clashes with the crystallographic trimer interface, suggesting that the presence of the α1′-helix may promote a monomeric state. Yet, the sequence of the α1′-helix region of E6AP diverges from other HECT domains whose structures have been solved, so it remains unknown, how the α1′-helix region of E6AP is arranged structurally and if its presence would preclude trimer formation.

Interestingly, the addition of an α1′-helix-derived peptide to recombinant full-length E6AP, which was reported to trimerize *in vitro*, decreases the size of the protein, similar to the effect of the F272D substitution at the trimer interface (Ronchi et al., 2014). In the context of the trimer model, this observation implies that the α1′-helix of E6AP is structurally shielded in the full-length protein and, hence, does not interfere *in cis* with trimer formation. When added *in trans*, however, the α1′-helix peptide has the ability to disrupt the trimer.

Cell-based analyses have revealed yet another interesting facet in the oligomerization behavior of E6AP (Yi et al., 2015). Full-length E6AP was found to self-associate upon mutation of a physiological phosphorylation site for PKA in the α1′-helix region (T485A). In this study, however, self-association of the WT protein or a phospho-mimetic variant, T485D, respectively, was not detected, which points to a role of phosphorylation in inhibiting oligomerization of E6AP in the cell. The phosphorylation-deficient mutation (T485A) was found to enhance the interaction of E6AP with target proteins compared to the WT and the T485D variant and to stimulate both activity towards target proteins and auto-ubiquitination, as reflected in an increased proteasomal turnover of E6AP. However, the introduction of the trimer interface mutation, F727D, in the T485A background does not affect E6AP levels in this context. Therefore, it remains unclear if the effects observed in this study are related to the crystallographic E6AP trimer or a yet uncharacterized, alternate mode of oligomerization.

Taken together, it emerges that full-length E6AP has the ability to self-associate in the cell and that this process is modulated by phosphorylation. To understand the structural details of how oligomerization occurs and how it impacts the catalytic activity of this ligase, we may need to assess the conformational space of the α1′-helix. As will be discussed later, a study on RSP5 has posited that the position of the α1′-helix may be altered by regulatory events (Attali et al., 2017). If applicable to E6AP, such a model could, perhaps, provide a way to integrate available experimental data. It will also be interesting to decipher at the structural level how the oligomeric state and activity of E6AP is regulated by posttranslational modifications other than phosphorylation as well as intra- and intermolecular interactions, indicated in several studies (Kühnle et al., 2011; Chan et al., 2013; Mortensen et al., 2015).

The ubiquitin-binding 'exosite' of NEDD4-type enzymes

The activities of NEDD4-type enzymes can be modulated by the binding of ubiquitin to an area on the N-lobe, known as the 'exosite' (French et al., 2009; Ogunjimi et al., 2010; Kim et al., 2011; Maspero et al., 2011, 2013; Zhang et al., 2016; French et al., 2017) (Figure 4B). This interaction is mediated by of series of conserved residues on the E3 that contact a region of ubiquitin that includes the canonical 'hydrophobic patch', centered on Ile 44, and extends towards the C-terminus by including Ile 36, Leu 71, and Leu 73 (Figure 4B, insert). The affinity of ubiquitin for the exosite falls into the micromolar K_D-range *in vitro* (Kim et al., 2011; Maspero et al., 2011; Zhang et al., 2016; French et al., 2017).

The functional consequences of the association of ubiquitin with the exosite have been analyzed extensively. NEDD4-1, SMURF2, WWP1, and RSP5, were reported to depend on this interaction for the processive formation of target protein-bound, E3-bound or unattached ubiquitin chains (Ogunjimi et al., 2010; Kim et al., 2011; Maspero et al., 2011; French et al., 2017). The consequences of the interaction for the mono-ubiquitination of target proteins varied between the studies (Ogunjimi et al., 2010; Kim et al., 2011). It was suggested collectively, however, that the exosite site functions in tethering of the distal ubiquitin in a growing chain to the E3, thus stabilizing ubiquitinated target proteins on the E3 (Ogunjimi et al., 2010; Kim et al., 2011; Maspero et al., 2011). In-line with this concept, a small-molecule inhibitor of NEDD4-1 that blocks the exosite was shown to switch the enzyme from a processive to a distributive mode of target modification (Kathman et al., 2015).

A similar concept has been utilized to explain how the E2-like UEV domain of the ESCRT-I subunit VPS23 stimulates mono-ubiquitination of target proteins by RSP5: The ubiquitin-interacting UEV domain is thought to compete with the E3 exosite for the binding of target protein-bound ubiquitin, thus suppressing chain elongation and favoring mono-ubiquitination (Herrador et al., 2013).

Perplexingly, ubiquitin binding to the exosite of NEDD4-1 and RSP5 has also been reported to restrict ubiquitin chain elongation (French et al., 2009). Recently,

ubiquitin variants with enhanced affinities for the exosite were employed to dissect the role of the exosite in several HECT E3 enzymes (Zhang et al., 2016). These comparative studies demonstrated that the occupation of the exosite with ubiquitin variants has rather complex effects with regard to processivity, chain elongation, target lysine choice, and domain-domain interactions and can impact individual parts of the reaction in different ways, depending on the identities of the E3 and target proteins and precise constructs used (Zhang et al., 2016).

A role for the C2 domain in controlling NEDD4-type enzymes

The activities of NEDD4-type enzymes are also modulated by intramolecular interactions of the HECT domain with the N-terminal C2 and WW domains and intervening regions. For instance, it was shown by NMR spectroscopy that the C2 domain of SMURF2 associates with the HECT domain (Wiesner et al., 2007). The C2 domain-binding site of SMURF2 overlaps largely with the ubiquitin-binding exosite on the N-lobe, but it also involves a region of the C-lobe in proximity to the catalytic cysteine (Wiesner et al., 2007; Mari et al., 2014). Binding of the C2 domain to the HECT domain is therefore expected to restrict the flexibility of the two lobes. Consistent with the observation that inter-lobe flexibility is important for HECT activity (Verdecia et al., 2003), the intramolecular interaction was found to inhibit the activity of SMURF2 and to interfere with both the binding of ubiquitin to the exosite, required for processive ubiquitin chain formation, and the transfer of ubiquitin to the E3 active site (Wiesner et al., 2007; Mari et al., 2014). The auto-inhibitory interaction can be released by the adapter protein SMAD7, which associates with the HECT domain of SMURF2 and displaces the C2 domain (Wiesner et al., 2007).

Interestingly, the HECT domain binding site on the C2 domain of SMURF2 overlaps with a binding site for specific phospholipids, which may provide an additional layer of regulation: C2 domain-phospholipid interactions are thought to anchor SMURF2 at the cytoplasmic membrane, thus co-localizing it with its targets (TGF-β receptor complexes), while at the same time releasing the HECT domain from its intramolecular engagement to keep the ligase in an active state (Wiesner et al., 2007). A similar mechanism was proposed for NEDD4-2, in which the auto-inhibitory intramolecular association of the C2 and HECT domains competes with Ca^{2+}-binding to the C2 domain and Ca^{2+}-dependent interactions of the HECT domain with phospholipids (Wang et al., 2010; Escobedo et al., 2014).

Auto-inhibitory functions have also been assigned to the C2 domains of NEDD4-1 and WWP2 (Kamynina et al., 2001; Snyder et al., 2001; Wiesner et al., 2007). Interestingly, the activation state of NEDD4-1 is sensitive to phosphorylation. FGFR1-mediated activation of the tyrosine kinase SRC promotes the phosphorylation of two tyrosine residues in the HECT and C2 domains of this ligase, which interferes with the ability of these domains to associate with each other and hence activates ligase activity (Persaud et al., 2014).

WW domains and linker regions: additional regulatory elements in the NEDD4 subfamily

A recent study on WWP2 identified the helical linker region between its second and third WW domain (the '2,3-linker'), rather than the C2 domain, as a key element mediating auto-inhibition (Chen et al., 2017). A crystal structure of a WW-2 construct, in which the WW-2 domain–2,3-linker region was fused N-terminally to the HECT domain reveals extensive interactions between the 2,3-linker and the N-lobe, including the ubiquitin-binding exosite (Figure 4C). Consequently, the association of a ubiquitin variant with this WWP2 construct is significantly weakened. The 2,3-linker also engages with the inter-lobe linker of the HECT domain, which is expected to contribute to auto-inhibition by restricting the flexibility of the two lobes. Notably, the 2,3-linker of WWP2 contains two tyrosines, which stimulate both ubiquitin binding and catalytic activity when phosphorylated, thus providing another level of regulation (Chen et al., 2017). Another recent structural study delineated a 2,3-linker-mediated auto-inhibitory mechanism for ITCH that closely resembles the one described for WWP2 (Zhu et al., 2017). Interestingly, this study also showed that ITCH can be activated upon mutational disruption of a contact at the interface between the N-lobe and the C-lobe in the auto-inhibited state, attesting to the idea that inter-lobe flexibility is a critical component of ligase activation.

In contrast to WWP1, WWP2 and ITCH, deletion of the 2,3-linker does not stimulate the activity of NEDD4-1 (Chen et al., 2017). Instead, a region C-terminal to the first WW domain of NEDD4-1 was found to have an auto-inhibitory role (Chen et al., 2017).

The WW domains themselves have been reported to serve regulatory functions (Gallagher et al., 2006; Bruce et al., 2008; Mund and Pelham, 2009; Mund et al., 2015; Riling et al., 2015; Chen et al., 2017; Zhu et al., 2017). On the one hand, inhibitory interactions between WW and HECT domains were suggested to be mediated by PY-motifs in

the C-lobe of NEDD4-2 (Bruce et al., 2008; Escobedo et al., 2014) and in the N- and C-lobes of ITCH (Riling et al., 2015), respectively. However, available crystal structures show that these motifs are not accessible in the context of the folded HECT domain, so their contributions to the auto-inhibition of the native proteins have remained unclear (Bruce et al., 2008; Riling et al., 2015).

On the other hand, interactions of WW-domains with PY-containing protein ligands, such as NDFIP1 (NEDD4-family interacting protein 1) were shown to activate some HECT E3 enzymes, such as ITCH and WWP2 (Mund and Pelham, 2009; Mund et al., 2015; Riling et al., 2015; Zhu et al., 2017), likely by displacing the WW-2 domain and 2,3-linker from the HECT domain (Zhu et al., 2017). Moreover, activation of ITCH can be brought about by serine/threonine kinase JNK1-mediated phosphorylation within a proline-rich region preceding the WW domains (Gallagher et al., 2006; Zhu et al., 2017).

RSP5: auto-ubiquitination, oligomerization, and the exosite

The activity of yeast RSP5, a NEDD4-type E3, has recently been proposed to be regulated through a mechanism of auto-ubiquitination-dependent oligomerization, in which the α1′-helix is thought to act as a structural switch (Attali et al., 2017). This model is based on the following main observations: RSP5 self-associates in cells (Dunn and Hicke, 2001; Attali et al., 2017), and a truncated HECT domain construct lacking the α1′-helix oligomerizes *in vitro*, at least partially in a trimeric form. This oligomerization behavior can be recapitulated by genetically fusing ubiquitin to the N-terminus of the α1′-helix. This construct is thought to mimic an auto-ubiquitinated state of RSP5, in which a lysine residue near the N-terminus of this helix is modified with ubiquitin. In this context, mutation of the hydrophobic patch of ubiquitin (I44A) and the exosite of RSP5 (I537D) were found to interfere with oligomerization and to stimulate activity. Therefore, it was hypothesized that the ubiquitin moiety that was fused to the α1′-helix may be able to dock via its hydrophobic patch onto the exosite of the HECT domain *in cis*, thereby removing the α1′-helix from its canonical location. This, in turn, may promote the formation of a RSP5 trimer, analogous to the crystallographic trimer formed by the truncated E6AP construct that is lacking the α1′-helix. It should be noted, however, that the trimerization of E6AP was suggested to be associated with hyper-activation (Chan et al., 2013; Ronchi et al., 2014), whereas the proposed RSP5 trimer model confers auto-inhibition. This model of RSP5 regulation has profound structural implications, in particular for the role of the α1′-helix, but also for the adjacent N-terminal regions and intramolecular domain interactions that await to be explored.

HUWE1: a unique dimerization switch

The regulation of HUWE1 has been proposed to involve a conformational switch between an active monomeric and an auto-inhibited dimeric state (Sander et al., 2017). The dimerization is mediated by two amphipathic helices that flank the HECT domain and form an asymmetric, hydrophobic interface in a crystal structure of the C-terminal region of this enzyme (Figure 4D). The dimeric arrangement conformationally locks the C-lobe of one subunit and buries its catalytically important C-tail. If HUWE1 were to bind donor ubiquitin in the same way as NEDD4-type enzymes, this interaction would also be obstructed in the context of the dimer. The dimerization of C-terminal constructs of HUWE1 in solution was shown to be modulated by a region, the 'activation segment', located ~50 residues N-terminally to the dimerization region. This segment is thought to interact with the dimerization region in *cis*, hence counteracting dimer formation and stimulating ligase activity.

Remarkably, the activation segment also provides a binding site for the tumor suppressor, p14ARF, (Sander et al., 2017) a physiological inhibitor of HUWE1 (Chen et al., 2005). It was therefore proposed that p14ARF inhibits HUWE1 by sequestering the activation segment and shifting the conformational equilibrium towards the inhibited dimer (Sander et al., 2017).

Future studies ought to consolidate these ideas and provide structural views of extended HUWE1 constructs and complexes with p14ARF. It will also be important to investigate how the activity of HUWE1 is tuned by other physiological factors in the cellular context.

Outlook

Since the original structural description of the catalytic HECT domain (Huang et al., 1999), many significant insights into the structural mechanisms and the conformational plasticity of this domain have been obtained. This is particularly true for enzymes in the NEDD4 subfamily, which have been disproportionally well characterized (Table 1). However, it has not been systematically analyzed to what extent the mechanistic features of

NEDD4-type enzymes are conserved in the entire HECT E3 family. For instance, it is unknown if the modes of donor ubiquitin recognition and exosite binding seen in NEDD4-type enzymes are also used by enzymes outside of this subfamily.

Furthermore, central questions remain open at all stages of the catalytic cycle across the HECT E3 family. For one, the orientation of acceptor ubiquitin at the E3 active site has not yet been structurally visualized. Consequently, it is unknown how certain HECT E3 enzymes can encode linkage specificity in ubiquitin chain formation – a key determinant of ubiquitin function. It will also be important to gain more insights into the interactions of HECT E3 enzymes with their target proteins in order to understand how distinct target proteins are presented to the catalytic center, how individual lysine residues are selected for modification, how ubiquitin chains are formed on target proteins, and how chain length is controlled. One key to answering these questions on a structural level will be to overcome the low affinities that typically underlie E3-substrate interactions and to stabilize relevant protein complexes in *vitro*. To this end, semi-synthetic and click chemistry strategies (Spasser and Brik, 2012) will continue to provide valuable tools.

To understand how HECT E3 enzymes – as opposed to HECT domains – work, we need to uncover the structural and functional properties of the extended N-terminal regions of these enzymes. The rather large size of these regions, their scarcity in conserved domains, and abundance of low sequence complexity stretches suggests that these regions may act as platforms for the assembly of supramolecular complexes. Proteomic analyses have started to shed light on such roles (Lu et al., 2008; Martinez-Noel et al., 2012; Galligan et al., 2015). For instance, over 300 interaction partners have been identified for the giant HECT E3 enzyme HERC2 that implicate this enzyme in diverse cellular pathways, including DNA damage repair, translation, energy metabolism, and protein trafficking (Galligan et al., 2015). How HECT E3-mediated protein complexes are organized in space and time, what their structural underpinnings are, and if/how their physiological functions relate to the catalytic activity of the E3 are exciting questions for future studies. Answering these questions may open up avenues toward rational, therapeutic manipulations of specific HECT E3 functions. Targeting individual HECT E3 enzymes is an appealing strategy, particularly since the existing structural data has revealed diverse and possibly even enzyme-specific mechanisms of regulation. While this may be due to the limited number of HECT E3 family proteins characterized to date, it also holds the intriguing potential of exploiting enzyme-specific regulatory features for therapeutic benefit.

Acknowledgments: I would like to thank Jon Huibregtse and members of my laboratory for insightful discussions; Aaron Cantor for critical reading of the manuscript. My laboratory is funded by the Emmy Noether-Program of the German Research Foundation (DFG; LO 2003/1-1), the Research Training Group 2243 (DFG; 'Understanding ubiquitylation: from molecular mechanisms to disease'), and the Wilhelm Sander-Stiftung (2015.147.1).

References

Anan, T., Nagata, Y., Koga, H., Honda, Y., Yabuki, N., Miyamoto, C., Kuwano, A., Matsuda, I., Endo, F., Saya, H., et al. (1998). Human ubiquitin-protein ligase Nedd4: expression, subcellular localization and selective interaction with ubiquitin-conjugating enzymes. Genes Cells *3*, 751–763.

Ansari, T., Brimer, N., and Vande Pol, S.B. (2012). Peptide interactions stabilize and restructure human papillomavirus type 16 E6 to interact with p53. J. Virol. *86*, 11386–11391.

Aragón, E., Goerner, N., Zaromytidou, A.-I., Xi, Q., Escobedo, A., Massague, J., and Macias, M.J. (2011). A Smad action turnover switch operated by WW domain readers of a phosphoserine code. Genes Dev. *25*, 1275–1288.

Attali, I., Tobelaim, W.S., Persaud, A., Motamedchaboki, K., Simpson-Lavy, K.J., Mashahreh, B., Levin-Kravets, O., Keren-Kaplan, T., Pilzer, I., Kupiec, M., et al. (2017). Ubiquitylation-dependent oligomerization regulates activity of Nedd4 ligases. EMBO J. *36*, 425–440.

Be, X., Hong, Y., Wei, J., Androphy, E.J., Chen, J.J., and Baleja, J.D. (2001). Solution structure determination and mutational analysis of the papillomavirus E6 interacting peptide of E6AP. Biochemistry *40*, 1293–1299.

Beaudenon, S. and Huibregtse, J.M. (2008). HPV E6, E6AP and cervical cancer. BMC Biochem. *9* (Suppl 1), S4.

Bernassola, F., Karin, M., Ciechanover, A., and Melino, G. (2008). The HECT family of E3 ubiquitin ligases: multiple players in cancer development. Cancer Cell *14*, 10–21.

Bobby, R., Medini, K., Neudecker, P., Lee, T.V., Brimble, M.A., McDonald, F.J., Lott, J.S., and Dingley, A.J. (2013). Structure and dynamics of human Nedd4-1 WW3 in complex with the alpha ENaC PY motif. Biochim. Biophys. Acta Proteins Proteomics *1834*, 1632–1641.

Branigan, E., Plechanovová, A., Jaffray, E.G., Naismith, J.H., and Hay, R.T. (2015). Structural basis for the RING-catalyzed synthesis of K63-linked ubiquitin chains. Nat. Struct. Mol. Biol. *22*, 597–602.

Bruce, M.C., Kanelis, V., Fouladkou, F., Debonneville, A., Staub, O., and Rotin, D. (2008). Regulation of Nedd4-2 self-ubiquitination and stability by a PY motif located within its HECT-domain. Biochem. J. *415*, 155–163.

Cappadocia, L., Pichler, A., and Lima, C.D. (2015). Structural basis for catalytic activation by the human ZNF451 SUMO E3 ligase. Nat. Struct. Mol. Biol. *22*, 968–975.

Chan, A.-L., Grossman, T., Zuckerman, V., Campigli Di Giammartino, D., Moshel, O., Scheffner, M., Monahan, B., Pilling, P., Jiang, Y.-H., Haupt, S., et al. (2013). c-Abl phosphorylates E6AP and regulates its E3 ubiquitin ligase activity. Biochemistry *52*, 3119–3129.

Chen, H.I. and Sudol, M. (1995). The WW domain of Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules. Proc. Natl. Acad. Sci. USA *92*, 7819–7823.

Chen, D., Kon, N., Li, M., Zhang, W., Qin, J., and Gu, W. (2005). ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. Cell *121*, 1071–1083.

Chen, Z., Jiang, H., Xu, W., Li, X., Dempsey, D.R., Zhang, X., Devreotes, P., Wolberger, C., Amzel, L.M., Gabelli, S.B., et al. (2017). A tunable brake for HECT ubiquitin ligases. Mol. Cell *66*, 345–357.e6.

Chiu, Y.-H., Sun, Q., and Chen, Z.J. (2007). E1-L2 activates both ubiquitin and FAT10. Mol. Cell. *27*, 1014–1023.

Chong, P.A., Lin, H., Wrana, J.L., and Forman-Kay, J.D. (2006). An expanded WW domain recognition motif revealed by the interaction between Smad7 and the E3 ubiquitin ligase Smurf2. J. Biol. Chem. *281*, 17069–17075.

Chong, P.A., Lin, H., Wrana, J.L., and Forman-Kay, J.D. (2010). Coupling of tandem Smad ubiquitination regulatory factor (Smurf). WW domains modulates target specificity. Proc. Natl. Acad. Sci. USA *107*, 18404–18409.

Dastur, A., Beaudenon, S., Kelley, M., Krug, R.M., and Huibregtse, J.M. (2006). Herc5, an interferon-induced HECT E3 enzyme, is required for conjugation of ISG15 in human cells. J. Biol. Chem. *281*, 4334–4338.

Deo, R.C., Sonenberg, N., and Burley, S.K. (2001). X-ray structure of the human hyperplastic discs protein: an ortholog of the C-terminal domain of poly(A)-binding protein. Proc. Natl. Acad. Sci. USA *98*, 4414–4419.

Desrochers, G., Cappadocia, L., Lussier-Price, M., Ton, A.-T., Ayoubi, R., Serohijos, A., Omichinski, J.G., and Angers, A. (2017). Molecular basis of interactions between SH3 domain-containing proteins and the proline-rich region of the ubiquitin ligase Itch. J. Biol. Chem. *292*, 6325–6338.

Dou, H., Buetow, L., Sibbet, G.J., Cameron, K., and Huang, D.T. (2012). BIRC7-E2 ubiquitin conjugate structure reveals the mechanism of ubiquitin transfer by a RING dimer. Nat. Struct. Mol. Biol. *19*, 876–883.

Dou, H., Buetow, L., Sibbet, G.J., Cameron, K., and Huang, D.T. (2013). Essentiality of a non-RING element in priming donor ubiquitin for catalysis by a monomeric E3. Nat. Struct. Mol. Biol. *20*, 982–986.

Dunn, R. and Hicke, L. (2001). Domains of the Rsp5 ubiquitin-protein ligase required for receptor-mediated and fluid-phase endocytosis. Mol. Biol. Cell *12*, 421–435.

Eletr, Z.M. and Kuhlman, B. (2007). Sequence determinants of E2-E6AP binding affinity and specificity. J. Mol. Biol. *369*, 419–428.

Eletr, Z.M., Huang, D.T., Duda, D.M., Schulman, B.A., and Kuhlman, B. (2005). E2 conjugating enzymes must disengage from their E1 enzymes before E3-dependent ubiquitin and ubiquitin-like transfer. Nat. Struct. Mol. Biol. *12*, 933–934.

Escobedo, A., Gomes, T., Aragón, E., Martín-Malpartida, P., Ruiz, L., and Macias, M.J. (2014). Structural basis of the activation and degradation mechanisms of the E3 ubiquitin ligase Nedd4L. Structure *22*, 1446–1457.

Finn, R.D., Attwood, T.K., Babbitt, P.C., Bateman, A., Bork, P., Bridge, A.J., Chang, H.-Y., Dosztányi, Z., El-Gebali, S., Fraser, M., et al. (2017). InterPro in 2017-beyond protein family and domain annotations. Nucleic Acids Res. *45*, D190–D199.

French, M.E., Kretzmann, B.R., and Hicke, L. (2009). Regulation of the RSP5 ubiquitin ligase by an intrinsic ubiquitin-binding site. J. Biol. Chem. *284*, 12071–12079.

French, M.E., Klosowiak, J.L., Aslanian, A., Reed, S.I., Yates, J.R., and Hunter, T. (2017). Mechanism of ubiquitin chain synthesis employed by a HECT ubiquitin ligase. J. Biol. Chem. *292*, 10398–10413.

Gallagher, E., Gao, M., Liu, Y.-C., and Karin, M. (2006). Activation of the E3 ubiquitin ligase Itch through a phosphorylation-induced conformational change. Proc. Natl. Acad. Sci. USA *103*, 1717–1722.

Galligan, J.T., Martinez-Noel, G., Arndt, V., Hayes, S., Chittenden, T.W., Harper, J.W., and Howley, P.M. (2015). Proteomic analysis and identification of cellular interactors of the giant ubiquitin ligase HERC2. J. Proteome Res. *14*, 953–966.

Gong, W., Zhang, X., Zhang, W., Li, J., and Li, Z. (2015). Structure of the HECT domain of human WWP2. Acta Crystallogr. F Struct. Biol. Commun. *71*, 1251–1257.

Greer, P.L., Hanayama, R., Bloodgood, B.L., Mardinly, A.R., Lipton, D.M., Flavell, S.W., Kim T-K, Griffith, E.C., Waldon, Z., Maehr, R., et al. (2010). The Angelman Syndrome protein Ube3A regulates synapse development by ubiquitinating arc. Cell *140*, 704–716.

Handley, P.M., Mueckler, M., Siegel, N.R., Ciechanover, A., and Schwartz, A.L. (1991). Molecular cloning, sequence, and tissue distribution of the human ubiquitin-activating enzyme E1. Proc. Natl. Acad. Sci. USA *88*, 258–262.

Haynes, C., Oldfield, C.J., Ji, F., Klitgord, N., Cusick, M.E., Radivojac, P., Uversky, V.N., Vidal, M., and Iakoucheva, L.M. (2006). Intrinsic disorder is a common feature of hub proteins from four eukaryotic interactomes. PLoS Comput. Biol. *2*, e100.

Herrador, A., Leon, S., Haguenauer-Tsapis, R., and Vincent, O. (2013). A mechanism for protein monoubiquitination dependent on a *trans*-acting ubiquitin-binding domain. J. Biol. Chem. *288*, 16206–16211.

Hochstrasser, M. (2006). Lingering mysteries of ubiquitin-chain assembly. Cell *124*, 27–34.

Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P.M., Huibregtse, J.M., and Pavletich, N.P. (1999). Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. Science *286*, 1321–1326.

Huang, Q. and Szebenyi, D.M.E. (2010). Structural basis for the interaction between the growth factor-binding protein GRB 10 and the E3 ubiquitin ligase NEDD4. J. Biol. Chem. *285*, 42130–42139.

Huibregtse, J.M., Scheffner, M., and Howley, P.M. (1991). A cellular protein mediates association of P53 with the E6 oncoprotein of human papillomavirus type-16 or type-18. EMBO J. *10*, 4129–4135.

Huibregtse, J.M., Scheffner, M., and Howley, P.M. (1993). Localization of the E6-Ap regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins. Mol. Cell Biol. *13*, 4918–4927.

Huibregtse, J.M., Scheffner, M., Beaudenon, S., and Howley, P.M. (1995). A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc. Natl. Acad. Sci. USA *92*, 2563–2567.

Ingham, R.J., Gish, G., and Pawson, T. (2004). The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. Oncogene *23*, 1972–1984.

Janz, J.M., Sakmar, T.P., and Min, K.C. (2007). A novel interaction between atrophin-interacting protein 4 and β-p21-activated kinase-interactive exchange factor is mediated by an SH3 domain. J. Biol. Chem. *282*, 28893–28903.

Jin, J., Li, X., Gygi, S.P., and Harper, J.W. (2007). Dual E1 activation systems for ubiquitin differentially regulate E2 enzyme charging. Nature *447*, 1135–1138.

Kamadurai, H.B., Qiu, Y., Deng, A., Harrison, J.S., MacDonald, C., Actis, M., Rodrigues, P., Miller, D.J., Souphron, J., Lewis, S.M., et al. (2013). Mechanism of ubiquitin ligation and lysine prioritization by a HECT E3. eLife *2*, e00828.

Kamadurai, H.B., Souphron, J., Scott, D.C., Duda, D.M., Miller, D.J., Stringer, D., Piper, R.C., and Schulman, B.A. (2009). Insights into ubiquitin transfer cascades from a structure of a UbcH5B approximately ubiquitin-HECT(NEDD4L) complex. Mol. Cell *36*, 1095–1102.

Kamynina, E., Tauxe, C., and Staub, O. (2001). Distinct characteristics of two human Nedd4 proteins with respect to epithelial Na^+ channel regulation. Am. J. Physiol. Renal Physiol. *281*, F469–F477.

Kanelis, V., Bruce, M.C., Skrynnikov, N.R., Rotin, D., and Forman-Kay, J.D. (2006). Structural determinants for high-affinity binding in a Nedd4 WW3* domain-Comm PY motif complex. Struct. Fold Des. *14*, 543–553.

Kar, G., Keskin, O., Nussinov, R., and Gursoy, A. (2012). Human proteome-scale structural modeling of E2-E3 interactions exploiting interface motifs. J. Proteome Res. *11*, 1196–1207.

Kathman, S.G., Span, I., Smith, A.T., Xu, Z., Zhan, J., Rosenzweig, A.C., and Statsyuk, A.V. (2015). A small molecule that switches a ubiquitin ligase from a processive to a distributive enzymatic mechanism. J. Am. Chem. Soc. *137*, 12442–12445.

Ketscher, L., Basters, A., Prinz, M., and Knobeloch, K.-P. (2012). mHERC6 is the essential ISG15 E3 ligase in the murine system. Biochem. Biophys. Res. Commun. *417*, 135–140.

Kim, H.C. and Huibregtse, J.M. (2009). Polyubiquitination by HECT E3s and the determinants of chain type specificity. Mol. Cell. Biol. *29*, 3307–3318.

Kim, K.I., Giannakopoulos, N.V., Virgin, H.W., and Zhang, D.-E. (2004). Interferon-inducible ubiquitin E2, Ubc8, is a conjugating enzyme for protein ISGylation. Mol. Cell Biol. *24*, 9592–9600.

Kim, H.C., Steffen, A.M., Oldham, M.L., Chen, J., and Huibregtse, J.M. (2011). Structure and function of a HECT domain ubiquitin-binding site. EMBO Rep. *12*, 334–341.

Komander, D. and Rape, M. (2012). The ubiquitin code. Annu. Rev. Biochem. *81*, 203–229.

Kozlov, G., Nguyen, L., Lin, T., De Crescenzo, G., Park, M., and Gehring, K. (2007). Structural basis of ubiquitin recognition by the ubiquitin-associated (UBA) domain of the ubiquitin ligase EDD. J. Biol. Chem. *282*, 35787–35795.

Kumar, S., Kao, W.H., and Howley, P.M. (1997). Physical interaction between specific E2 and Hect E3 enzymes determines functional cooperativity. J. Biol. Chem. *272*, 13548–13554.

Kühnle, S., Kogel, U., Glockzin, S., Marquardt, A., Ciechanover, A., Matentzoglu, K., and Scheffner, M. (2011). Physical and functional interaction of the HECT ubiquitin-protein ligases E6AP and HERC2. J. Biol. Chem. *286*, 19410–19416.

Lemak, A., Yee, A., Bezsonova, I., Dhe-Paganon, S., and Arrowsmith, C.H. (2011). Zn-binding AZUL domain of human ubiquitin protein ligase Ube3A. J. Biomol. NMR *51*, 185–190.

Leon, S., and Haguenauer-Tsapis, R. (2009). Ubiquitin ligase adaptors: regulators of ubiquitylation and endocytosis of plasma membrane proteins. Exp. Cell Res. *315*, 1574–1583.

Li, W., Bengtson, M.H., Ulbrich, A., Matsuda, A., Reddy, V.A., Orth, A., Chanda, S.K., Batalov, S., and Joazeiro, C.A.P. (2008). Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. PLoS One *3*, e1487.

Lim, N.S., Kozlov, G., Chang, T.-C., Groover, O., Siddiqui, N., Volpon, L., De Crescenzo, G., Shyu, A.-B., and Gehring, K. (2006). Comparative peptide binding studies of the PABC domains from the ubiquitin-protein isopeptide ligase HYD and poly(A)-binding protein. Implications for HYD function. J. Biol. Chem. *281*, 14376–14382.

Liu, Y., Lau, J., Li, W., Tempel, W., Li, L., Dong, A., Narula, A., Qin, S., and Min, J. (2016). Structural basis for the regulatory role of the PPxY motifs in the thioredoxin-interacting protein TXNIP. Biochem. J. *473*, 179–187.

Lorenz, S., Cantor, A.J., Rape, M., and Kuriyan, J. (2012). Macromolecular juggling by ubiquitylation enzymes. BMC Biol. *11*, 65.

Lu, J.-Y., Lin, Y.-Y., Qian, J., Tao, S.-C., Zhu, J., Pickart, C., and Zhu, H. (2008). Functional dissection of a HECT ubiquitin E3 ligase. Mol. Cell Proteomics *7*, 35–45.

Mari, S., Ruetalo, N., Maspero, E., Stoffregen, M.C., Pasqualato, S., Polo, S., and Wiesner, S. (2014). Structural and functional framework for the autoinhibition of Nedd4-family ubiquitin ligases. Structure *22*, 1639–1649.

Martinez-Noel, G., Galligan, J.T., Sowa, M.E., Arndt, V., Overton, T.M., Harper, J.W., and Howley, P.M. (2012). Identification and proteomic analysis of distinct UBE3A/E6AP protein complexes. Mol. Cell Biol. *32*, 3095–3106.

Martinez-Zapien, D., Ruiz, F.X., Poirson, J., Mitschler, A., Ramirez, J., Forster, A., Cousido-Siah, A., Masson, M., Vande Pol, S., Podjarny, A., et al. (2016). Structure of the E6/E6AP/p53 complex required for HPV-mediated degradation of p53. Nature *529*, 541–545.

Maspero, E., Mari, S., Valentini, E., Musacchio, A., Fish, A., Pasqualato, S., and Polo, S. (2011). Structure of the HECT:ubiquitin complex and its role in ubiquitin chain elongation. EMBO Rep *12*, 342–349.

Maspero, E., Valentini, E., Mari, S., Cecatiello, V., Soffientini, P., Pasqualato, S., and Polo, S. (2013). Structure of a ubiquitin-loaded HECT ligase reveals the molecular basis for catalytic priming. Nat. Struct. Mol. Biol. *20*, 696–701.

Matta-Camacho, E., Kozlov, G., Menade, M., and Gehring, K. (2012). Structure of the HECT C-lobe of the UBR5 E3 ubiquitin ligase. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. *68*, 1158–1163.

Medintz, I., Jiang, H., and Michels, C.A. (1998). The role of ubiquitin conjugation in glucose-induced proteolysis of *Saccharomyces* maltose permease. J. Biol. Chem. *273*, 34454–34462.

Michelle, C., Vourc'h, P., Mignon, L., and Andres, C.R. (2009). What was the set of ubiquitin and ubiquitin-like conjugating enzymes in the eukaryote common ancestor? J. Mol. Evol. *68*, 616–628.

Morales, B., Ramirez-Espain, X., Shaw, A.Z., Martín-Malpartida, P., Yraola, F., Sánchez-Tilló, E., Farrera, C., Celada, A., Royo, M., and Macias, M.J. (2007). NMR structural studies of the ItchWW3 domain reveal that phosphorylation at T30 inhibits the interaction with PPxY-containing ligands. Struct./Fold. Des. *15*, 473–483.

Mortensen, F., Schneider, D., Barbic, T., Sladewska-Marquardt, A., Kühnle, S., Marx, A., and Scheffner, M. (2015). Role of ubiquitin and the HPV E6 oncoprotein in E6AP-mediated ubiquitination. Proc. Natl. Acad. Sci. USA *112*, 9872–9877.

Mund, T. and Pelham, H.R.B. (2009). Control of the activity of WW-HECT domain E3 ubiquitin ligases by NDFIP proteins. EMBO Rep. *10*, 501–507.

Mund, T., Graeb, M., Mieszczanek, J., Gammons, M., Pelham, H.R.B., and Bienz, M. (2015). Disinhibition of the HECT E3 ubiquitin ligase WWP2 by polymerized Dishevelled. Open Biol. *5*, 150185.

Muñoz-Escobar, J., Matta-Camacho, E., Kozlov, G., and Gehring, K. (2015). The MLLE domain of the ubiquitin ligase UBR5 binds to its catalytic domain to regulate substrate binding. J. Biol. Chem. *290*, 22841–22850.

Nuber, U. and Scheffner, M. (1999). Identification of determinants in E2 ubiquitin-conjugating enzymes required for hect E3 ubiquitin-protein ligase interaction. J. Biol. Chem. *274*, 7576–7582.

Nuber, U., Schwarz, S.E., and Scheffner, M. (1998). The ubiquitin-protein ligase E6-associated protein (E6-AP) serves as its own substrate. Eur. J. Biochem. 254, 643–649.

Ogunjimi, A.A., Briant, D.J., Pece-Barbara, N., Le Roy, C., Di Guglielmo, G.M., Kavsak, P., Rasmussen, R.K., Seet, B.T., Sicheri, F., and Wrana, J.L. (2005). Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. Mol. Cell *19*, 297–308.

Ogunjimi, A.A., Wiesner, S., Briant, D.J., Varelas, X., Sicheri, F., Forman-Kay, J., and Wrana, J.L. (2010). The ubiquitin binding region of the Smurf HECT domain facilitates polyubiquitylation and binding of ubiquitylated substrates. J. Biol. Chem. *285*, 6308–6315.

Oudshoorn, D., van Boheemen, S., Sánchez-Aparicio, M.T., Rajsbaum, R., García-Sastre, A., and Versteeg, G.A. (2011). HERC6 is the main E3 ligase for global ISG15 conjugation in mouse cells. PLoS One *7*, e29870.

Pandya, R.K., Partridge, J.R., Love, K.R., Schwartz, T.U., and Ploegh, H.L. (2010). A structural element within the HUWE1 HECT domain modulates self-ubiquitination and substrate ubiquitination activities. J. Biol. Chem. *285*, 5664–5673.

Panwalkar, V., Neudecker, P., Schmitz, M., Lecher, J., Schulte, M., Medini, K., Stoldt, M., Brimble, M.A., Willbold, D., and Dingley, A.J. (2016). The Nedd4-1 WW domain recognizes the PY motif peptide through coupled folding and binding equilibria. Biochemistry *55*, 659–674.

Pelzer, C., Kassner, I., Matentzoglu, K., Singh, R.K., Wollscheid, H.-P., Scheffner, M., Schmidtke, G., and Groettrup, M. (2007). UBE1L2, a novel E1 enzyme specific for ubiquitin. J. Biol. Chem. *282*, 23010–23014.

Persaud, A., Alberts, P., Mari, S., Tong, J., Murchie, R., Maspero, E., Safi, F., Moran, M.F., Polo, S., and Rotin, D. (2014). Tyrosine phosphorylation of NEDD4 activates its ubiquitin ligase activity. Sci. Signal *7*, ra95.

Plechanovová, A., Jaffray, E.G., Tatham, M.H., Naismith, J.H., and Hay, R.T. (2012). Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis. Nature *489*, 115–120.

Pruneda, J.N., Littlefield, P.J., Soss, S.E., Nordquist, K.A., Chazin, W.J., Brzovic, P.S., and Klevit, R.E. (2012). Structure of an E3:E2~Ub complex reveals an allosteric mechanism shared among RING/U-box ligases. Mol. Cell *47*, 933–942.

Purbeck, C., Eletr, Z.M., and Kuhlman, B. (2010). Kinetics of the transfer of ubiquitin from UbcH7 to E6AP. Biochemistry *49*, 1361–1363.

Qi, S., O'Hayre, M., Gutkind, J.S., and Hurley, J.H. (2014). Structural and biochemical basis for ubiquitin ligase recruitment by arrestin-related domain-containing protein-3 (ARRDC3). J. Biol. Chem. *289*, 4743–4752.

Reverter, D., and Lima, C.D. (2005). Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup358 complex. Nature *435*, 687–692.

Riling, C., Kamadurai, H., Kumar, S., O'Leary, C.E., Wu, K.-P., Manion, E.E., Ying, M., Schulman, B.A., and Oliver, P.M. (2015). Itch WW domains inhibit its E3 ubiquitin ligase activity by blocking E2-E3 transthiolation. J. Biol. Chem. *290*, 23875–23887.

Ronchi, V.P., Klein, J.M., and Haas, A.L. (2013). E6AP/UBE3A ubiquitin ligase harbors two E2~ubiquitin binding sites. J. Biol. Chem. *288*, 10349–10360.

Ronchi, V.P., Klein, J.M., Edwards, D.J., and Haas, A.L. (2014). The active form of E6-associated protein (E6AP)/UBE3A ubiquitin ligase is an oligomer. J. Biol. Chem. *289*, 1033–1048.

Rotin, D. and Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. Nat. Rev. Mol. Cell Biol. *10*, 398–409.

Salvat, C., Wang, G., Dastur, A., Lyon, N., and Huibregtse, J.M. (2004). The -4 phenylalanine is required for substrate ubiquitination catalyzed by HECT ubiquitin ligases. J. Biol. Chem. *279*, 18935–18943.

Sander, B., Xu, W., Eilers, M., Popov, N., and Lorenz, S. (2017). A conformational switch regulates the ubiquitin ligase HUWE1. eLife *6*, e21036.

Scheffner, M. and Kumar, S. (2014). Mammalian HECT ubiquitin-protein ligases: biological and pathophysiological aspects. Biochim. Biophys. Acta *1843*, 61–74.

Scheffner, M., Nuber, U., and Huibregtse, J.M. (1995). Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature *373*, 81–83.

Scheffner, M., Huibregtse, J.M., Vierstra, R.D., and Howley, P.M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell *75*, 495–505.

Scott, D.C., Sviderskiy, V.O., Monda, J.K., Lydeard, J.R., Cho, S.E., Harper, J.W., and Schulman, B.A. (2014). Structure of a RING E3 trapped in action reveals ligation mechanism for the ubiquitin-like protein NEDD8. Cell *157*, 1671–1684.

Sheng, Y., Hong, J.H., Doherty, R., Srikumar, T., Shloush, J., Avvakumov, G.V., Walker, J.R., Xue, S., Neculai, D., Wan, J.W., et al. (2012). A human ubiquitin conjugating enzyme (E2)-HECT E3 ligase structure-function screen. Mol. Cell. Proteomics *11*, 329–341.

Snyder, P.M., Olson, D.R., McDonald, F.J., and Bucher, D.B. (2001). Multiple WW domains, but not the C2 domain, are required for inhibition of the epithelial Na^+ channel by human Nedd4. J. Biol. Chem. *276*, 28321–28326.

Spagnol, G., Kieken, F., Kopanic, J.L., Li, H., Zach, S., Stauch, K.L., Grosely, R., and Sorgen, P.L. (2016). Structural studies of the Nedd4 WW domains and their selectivity for the connexin43 (Cx43) carboxyl terminus. J. Biol. Chem. *291*, 7637–7650.

Spasser, L. and Brik, A. (2012). Chemistry and biology of the ubiquitin signal. Angew. Chem. Int. Ed. *51*, 6840–6862.
Stewart, M.D., Ritterhoff, T., Klevit, R.E., and Brzovic, P.S. (2016). E2 enzymes: more than just middle men. Cell Res. 26, 423–440.
Stieglitz, B., Rana, R.R., Koliopoulos, M.G., Morris-Davies, A.C., Schaeffer, V., Christodoulou, E., Howell, S., Brown, N.R., Dikic, I., and Rittinger, K. (2013). Structural basis for ligase-specific conjugation of linear ubiquitin chains by HOIP. Nature *503*, 422–426.
Stoll, K.E., Brzovic, P.S., Davis, T.N., and Klevit, R.E. (2011). The essential Ubc4/Ubc5 function in yeast is HECT E3-dependent, and RING E3-dependent pathways require only monoubiquitin transfer by Ubc4. J. Biol. Chem. *286*, 15165–15170.
Streich, F.C. and Lima, C.D. (2016). Capturing a substrate in an activated RING E3/E2-SUMO complex. Nature *536*, 304–308.
Sung, M.-K., Porras-Yakushi, T.R., Reitsma, J.M., Huber, F.M., Sweredoski, M.J., Hoelz, A., Hess, S., and Deshaies, R.J. (2016). A conserved quality-control pathway that mediates degradation of unassembled ribosomal proteins. eLife *5*, e19105.
Swatek, K.N. and Komander, D. (2016). Ubiquitin modifications. Cell Res. *26*, 399–422.
Trempe, J.-F., Sauvé, V., Grenier, K., Seirafi, M., Tang, M.Y., Menade, M., Al-Abdul-Wahid, S., Krett, J., Wong, K., Kozlov, G., et al. (2013). Structure of parkin reveals mechanisms for ubiquitin ligase activation. Science *340*, 1451–1455.
Verdecia, M.A., Joazeiro, C.A.P., Wells, N.J., Ferrer, J.-L., Bowman, M.E., Hunter, T., and Noel, J.P. (2003). Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. Mol Cell *11*, 249–259.
Wang, M. and Pickart, C.M. (2005). Different HECT domain ubiquitin ligases employ distinct mechanisms of polyubiquitin chain synthesis. EMBO J. *24*, 4324–4333.
Wang, M., Cheng, D., Peng, J., and Pickart, C.M. (2006). Molecular determinants of polyubiquitin linkage selection by an HECT ubiquitin ligase. EMBO J. *25*, 1710–1719.
Wang, J., Peng, Q., Lin, Q., Childress, C., Carey, D., and Yang, W. (2010). Calcium activates Nedd4 E3 ubiquitin ligases by releasing the C2 domain-mediated auto-inhibition. J. Biol. Chem. *285*, 12279–12288.
Wenzel, D.M., Lissounov, A., Brzovic, P.S., and Klevit, R.E. (2011). UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. Nature *474*, 105–108.
Wickliffe, K.E., Lorenz, S., Wemmer, D.E., Kuriyan, J., and Rape, M. (2011). The mechanism of linkage-specific ubiquitin chain elongation by a single-subunit E2. Cell *144*, 769–781.
Wiesner, S., Ogunjimi, A.A., Wang, H.-R., Rotin, D., Sicheri, F., Wrana, J.L., and Forman-Kay, J.D. (2007). Autoinhibition of the HECT-type ubiquitin ligase Smurf2 through its C2 domain. Cell *130*, 651–662.
Wong, J.J.Y., Pung, Y.F., Sze, N.S.-K., and Chin, K.-C. (2006). HERC5 is an IFN-induced HECT-type E3 protein ligase that mediates type I IFN-induced ISGylation of protein targets. Proc. Natl. Acad. Sci. USA *103*, 10735–10740.
Wright, J.D., Mace, P.D., and Day, C.L. (2016). Secondary ubiquitin-RING docking enhances Arkadia and Ark2C E3 ligase activity. Nat. Struct. Mol. Biol. *23*, 45–52.
Ye, Y. and Rape, M. (2009). Building ubiquitin chains: E2 enzymes at work. Nat. Rev. Mol. Cell Biol. *10*, 755–764.
Yi, J.J., Berrios, J., Newbern, J.M., Snider, W.D., Philpot, B.D., Hahn, K.M., and Zylka, M.J. (2015). An autism-linked mutation disables phosphorylation control of UBE3A. Cell *162*, 795–807.
Yuan, W. and Krug, R.M. (2001). Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. EMBO J. *20*, 362–371.
Yunus, A.A. and Lima, C.D. (2006). Lysine activation and functional analysis of E2-mediated conjugation in the SUMO pathway. Nat. Struct. Mol. Biol. *13*, 491–499.
Zanier, K., Charbonnier, S., Sidi, A.O.M.O., McEwen, A.G., Ferrario, M.G., Poussin-Courmontagne, P., Cura, V., Brimer, N., Babah, K.O., Ansari, T., et al. (2013). Structural basis for hijacking of cellular LxxLL motifs by papillomavirus E6 oncoproteins. Science *339*, 694–698.
Zhang, W., Wu, K.-P., Sartori, M.A., Kamadurai, H.B., Ordureau, A., Jiang, C., Mercredi, P.Y., Murchie, R., Hu, J., Persaud, A., et al. (2016). System-wide modulation of HECT E3 ligases with selective ubiquitin variant probes. Mol. Cell *62*, 121–136.
Zhao, C., Beaudenon, S.L., Kelley, M.L., Waddell, M.B., Yuan, W., Schulman, B.A., Huibregtse, J.M., and Krug, R.M. (2004). The UbcH8 ubiquitin E2 enzyme is also the E2 enzyme for ISG15, an IFN-α/β-induced ubiquitin-like protein. Proc. Natl. Acad. Sci. USA *101*, 7578–7582.
Zhu, K., Shan, Z., Chen, X., Cai, Y., Cui, L., Yao, W., Wang, Z., Shi, P., Tian, C., Lou, J., et al. (2017). Allosteric auto-inhibition and activation of the Nedd4 family E3 ligase Itch. EMBO Rep., *18*, 1618–1630.

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