REVIEW

Regulation of E3 ubiquitin ligases by homotypic and heterotypic assembly [version 1; peer review: 2 approved]

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Abstract

Protein ubiquitylation is essential for the maintenance of cellular homeostasis. E3 ubiquitin ligases are key components of the enzymatic machinery catalyzing the attachment of ubiquitin to substrate proteins. Consequently, enzymatic dysfunction has been associated with medical conditions including cancer, diabetes, and cardiovascular and neurodegenerative disorders. To safeguard substrate selection and ubiquitylation, the activity of E3 ligases is tightly regulated by post-translational modifications including phosphorylation, sumoylation, and ubiquitylation, as well as binding of alternative adaptor molecules and cofactors. Recent structural studies identified homotypic and heterotypic interactions between E3 ligases, adding another layer of control for rapid adaptation to changing environmental and physiological conditions. Here, we discuss the regulation of E3 ligase activity by combinatorial oligomerization and summarize examples of associated ubiquitylation pathways and mechanisms.

Keywords

C. elegans, ubiquitin, chaperone, proteostasis, E3 ligase, CHIP, RING, HECT

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Introduction

The covalent attachment of ubiquitin to substrate proteins is essential for the maintenance of organismal homeostasis by regulating diverse cellular signaling processes and protein quality control[1]. Substrate ubiquitylation is usually mediated by the sequential activity of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). The E3 ubiquitin ligases form the largest group with more than 600 members in humans, which provide a central role in catalyzing ubiquitin conjugation to internal lysine residues of specific substrates and thereby defining their fates[2]. Depending on the mechanism by which ubiquitin is transferred from the E2 enzyme to the substrate, E3 ligases are classified into Really Interesting New Gene (RING) finger domain-, Homologous to E6-associated protein C Terminus (HECT) domain-, or RING Between RING (RBR) domain-containing ubiquitin ligases[3]. While RING E3s facilitate the direct transfer of ubiquitin from E2~ubiquitin intermediates to the target protein, HECT and RBR E3s contain an active-site cysteine that forms a thioester with ubiquitin before transferring it to the substrate[3–5]. Despite a plethora of structurally unrelated proteins, their ubiquitylation is highly selective owing to the high number and the distinctive nature of E3 ligases. Usually, one E3 ligase can target and regulate several substrate proteins[6]. Therefore, the expression, activity, and turnover of E3 ligases is tightly regulated to prevent cellular dysfunctions[6,7]. E3 expression undergoes spatiotemporal control regulated by tissue-specific gene expression, gene imprinting, the cellular microenvironment, and levels of substrate protein[8–11]. Moreover, the activity and abundance of E3 ligases are defined by both post-translational modifications and binding of cofactors and/or adaptor molecules[12–14]. Besides these well-known control mechanisms, recent structural work identified an additional layer of regulation provided by homotypic and heterotypic combination of E3 ligases into oligomeric ubiquitylation complexes[4,5,15,16]. However, despite recent reports describing oligomer formation of E3 ligases, the underlying regulatory mechanisms and the physiological relevance largely remain unclear. Here we provide an overview on homotypic and heterotypic assembly of E3 ubiquitin ligases and potential implications in drug discovery and therapeutic interventions[17].

Oligomer formation: a shared principle of E3 ubiquitin ligases

Oligomer formation specifically modulates the catalytic activity of RING finger and HECT type E3 ubiquitin ligases (Figure 1 and Table 1)[5,13,15]. The HECT ligases SMURF1, NEDD4.1, and HUWE1 are negatively regulated by oligomerization, which limits the accessibility of the catalytic cysteine residues for ubiquitin binding[5,18,19]. Conversely, oligomerization can also promote the catalytic function, which was shown for the HECT ligase E6AP and the RING ligases BIRC7, cIAP, TRAF6, RNF4, and Mdm2–Mdmx[4,5,8,16,20,21]. E3 ubiquitin ligases form different types of oligomers including homotypic interactions where one monomer binds to one or more of its respective counterparts either symmetrically, as observed for SMURF1 and E6AP[18,22], or asymmetrically, as reported for the RING/U-box ligases Rad18 and CHIP[23,24]. In contrast, heterotypic oligomers are formed between different E3s, such as the RING ligases Brca1–Bard1 and Mdm2–Mdmx (Figure 1)[25,26]. Likewise, the multi-subunit Cullin–RING E3 ubiquitin ligases (CRLs) form complex oligomeric assemblies for nuanced regulation of their activity and effective substrate recruitment[27–30].

Oligomer formation of E3 ligases is mechanistically regulated by post-translational modifications including phosphorylation, sumoylation, and even ubiquitylation (Figure 1)[8,14,31,32]. For example, the HECT ligase E6AP is active in its trimeric form whereas monomerization inhibits its catalytic function, which is triggered by c-Abl kinase-dependent phosphorylation[22,32]. This phenomenon is intriguingly different from other HECT ligases, which are inactive as oligomers. Ronchi *et al*. reported that most HECT ligases contain a conserved α-helix, which inhibits oligomerization but is absent in E6AP immediately N-terminal to Asn[497]. Adding evidence to this structural condition, increasing concentrations of the α-helix-related peptide abrogate the oligomerization and catalytic activity of E6AP[22]. However, the HECT domain of E6AP is also observed to be a monomer in solution[33]. Therefore, further studies are required to shed light on the role of monomers and oligomers as well as the stimuli for their molecular switch. Alternatively, the yeast and human HECT ligases Rsp5 and NEDD4 adopt auto-inhibitory homotrimer conformations upon ubiquitylation[31]. Trimerization is achieved by exposure of a hidden oligomeric interface due to the attraction of the conjugated ubiquitin to a ubiquitin-binding patch at the other side of the HECT domain. This allosteric mechanism restricts an essential motion between the N-terminal and the C-terminal lobes of the HECT domain[34]. Similarly, dimer-dependent activation of the RING ligase Cbl-b is mediated by ubiquitin binding[35]. The RING domain-containing SUMO-targeted ubiquitin ligase (STUbL) RNF4 is predominantly monomeric and inactive under normal conditions. Upon proteotoxic stress, poly-SUMO chains accumulate and recruit RNF4, which facilitates its dimerization and activity[8].

Besides post-translational modifications, homotypic and heterotypic interaction between E3 ligases is supported by adaptor proteins and specialized cofactors (Figure 1). For instance, homodimerization of SMURF1 mediates auto-inhibition, which is disrupted upon allosteric interaction with CDH1 and CKIP[36]. Another E3 ligase, HUWE1, has a distinct oligomerization mechanism where its active and inactive states are promoted by intramolecular and intermolecular interactions[19]. One monomer of HUWE1 is auto-inhibited upon dimerization, which might trigger overall inhibition of its catalytic function[19]. Interestingly, HUWE1 usually counteracts its auto-inhibitory state by an intramolecular interaction with a segment located 50 residues upstream of the dimer-binding region to remain active. HUWE1 inhibitors like p14ARF have been reported to bind to this segment and promote the auto-inhibitory dimeric conformation[5,19]. In contrast, the dimerization interface of cIAP1 stays in a closed inactive conformation until it is bound and stabilized by IAP antagonists such as SMAC mimetics, which open up the interface and facilitate dimerization-dependent cIAP1 activation[4,37–39]. Adaptor proteins can also fine-tune the balance

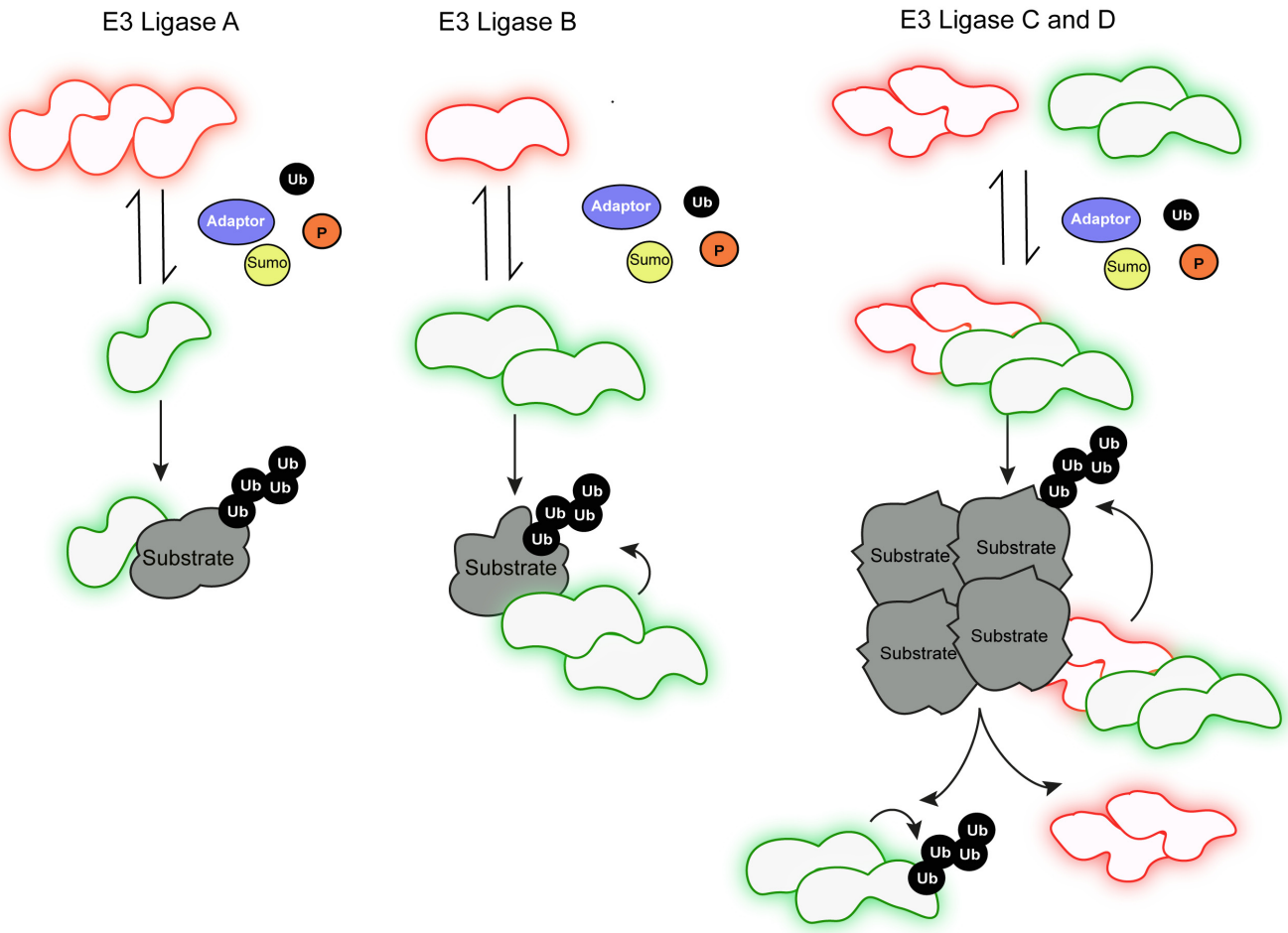

Figure 1. Different types of E3 ligase regulation and assembly. E3 ligase A is inactive (red) as an oligomer and converted into an active monomer (green) upon post-translational modification or binding to adaptor molecules, indicated with orange, yellow, black, and violet circles, representing phosphate (P), sumo, ubiquitin (Ub), and adaptor molecules, respectively. Conversely, E3 ligase B is inactive as a monomer and activated upon dimerization. Heterotypic interaction of inactive E3 ligase C and active E3 ligase D results in the formation of a multimeric E3 ligase complex, which is able to target oligomeric substrates for ubiquitylation. Upon substrate degradation, the remaining, active ligase D undergoes auto-ubiquitylation and turnover. The different substrates are indicated in other shapes.

between dimer and oligomer assemblies of E3 ligases, as seen in CRL3. Here, the adaptor protein SPOP, which is a positive regulator of oligomerization, teams up with the negative regulator SPOPL in controlling the catalytic activity of the E3 ligase[27].

Regulatory mechanisms and physiological relevance of E3 ligase assembly

The dimerization of the E3 ligase TRAF6 occurs via its RING domain, which primes for oligomerization via the coiled-coil (CC) region. This elegant assembly supports binding of the RING domains to numerous E2~ubiquitin molecules and formation of extended poly-ubiquitin chains. In addition, the CC domain of TRAF6 fosters recruitment and on-site recharging of E2 with ubiquitin without complete dissociation from the E3 ligase. This effective mechanism further increases the rate of polyubiquitin chain formation[16,40]. Binding of E2~ubiquitin by RING domains is also required for the backside binding of E2s like the UBCH5 family, which provide a specialized role in polyubiquitylation of substrate proteins[15,41].

Regarding the homodimeric RING E3s BIRC7, RNF4, cIAP, and IDOL, both the monomer subunits are intrinsically capable of interacting with E2 enzymes[4,15,42]. Whereas for the heterodimeric RING ligases BRCA1–BARD1 and RING1B–Bmi1, only one of the monomer subunits is able to interact with the E2 enzyme; the other one is mostly inactive while serving to stabilize the complex, target substrates, and support the enzymatic activity[4]. Remarkably, Mdm2 and Mdmx assemble both Mdm2–Mdm2 and Mdmx–Mdmx homodimers *in vitro,* but when mixed together they prefer to form Mdm2–Mdmx heterodimers[4,43,44]. The Mdm2–Mdmx heterodimer has the potential to form tetramers, especially to target the putative substrate p53, which is primarily a tetramer[4,45].

Table 1. List of E3 ubiquitin ligases forming oligomers.

S No	E3 ligase	Class	Oligomeric state	References
1	SMURF1	HECT	Inactive	Wan *et al.*[36]
2	NEDD4.1	HECT	Inactive	Attali *et al.*[31]
3	HUWE1	HECT	Inactive	Sander *et al.*[19]
4	E6AP	HECT	Active	Ronchi *et al.*[22]
5	BIRC7	RING	Active	Dou *et al.*[21]
6	cIAP1	RING	Active	Mace *et al.*[39]
7	TRAF6	RING	Active	Yin *et al.*[40]
8	RNF4	RING	Active	Plechanovová *et al.*[46]; Rojas-Fernandez *et al.*[8]
9	MDM2	RING	Active	Poyurovsky *et al.*[44]; Cheng *et al.*[12]
10	RAD18	RING	Active	Huang *et al.*[23]
11	Brca1	RING	Active	Brzovic *et al.*[25]
12	Cbl-b	RING	Active	Peschard *et al.*[35]
13	IDOL	RING	Active	Zhang *et al*[42]
14	SIAH1	RING	Active	Hu G and Fearon ER[47]
15	CHIP	U-box	Active	Zhang *et al.*[24]

For the E3 ligases MDM2 and SIAH1, homo-oligomerization might also provide a role in auto-degradation[14]. Remarkably, upon degradation of their substrates, the increased cellular level of these ligases triggers homo-dimerization and subsequently pushes the equilibrium towards auto-ubiquitylation *in trans* and subsequent proteasomal degradation (Figure 1)[14,47,48]. This mechanism removes the excessive E3 ligase molecules and thereby regulates the level of the enzyme. Especially in the case of Mdm2, the stringent control of E3 ligase level seems to be important to prevent tumorigenesis[49–51].

As described before, the RING domain has a direct role in binding E2~ubiquitin conjugates. Interestingly, dimers of the RING ligases RNF4, cIAP, and BIRC7 have higher affinity to E2~ubiquitin than their monomeric counterparts[15]. RING dimers preferentially bind charged E2~ubiquitin rather than E2 alone[21]. Most monomeric RING E3 ligases possess a conserved tryptophan residue, which is critical for binding to E2~ubiquitin conjugates and optimal ligase activity, while the dimeric RING E3s present different residues at this position. Strikingly, RING dimers, when endowed with this tryptophan residue, are hyperactive[52]. During the course of evolution, this particular tryptophan residue seemed to be modified in dimeric E3 ligases to prevent aberrant functioning and to enable regulation of the catalytic activity only by oligomer formation[52]. It has been demonstrated that RNF4 is present in a basal inactive monomer form and only proteotoxic and genotoxic stress conditions increase polySUMO chain levels to potentially induce dimer formation and enzymatic activation[8,52]. As a common feature, dimeric ligases are critical for several signaling pathways and their misregulation results in cellular defects and cancer progression[6,50,53,54].

Interestingly, the conserved U-box domain protein Ufd2p/UFD-2 functions as both an E3 and an E4 ligase[55]. Unlike the U-box containing E3 ligase CHIP, which forms an asymmetric homodimer, UFD-2 exists as a monomer[24,56]. The structure of UFD-2 shows that it can readily bind to E2~ubiquitin conjugates as a monomer in a similar fashion to dimeric CHIP[55,56]. The question of why some proteins exist as monomers while some are dimers is addressing an interesting aspect considering that UFD-2 teams up with CHIP to enhance polyubiquitylation of the myosin assembly chaperone UNC-45 in *Caenorhabditis elegans*. Therefore, it is interesting to speculate that UFD-2 and CHIP form a heterodimeric complex providing altered substrate specificity and processing[56].

Conclusion

E3 ubiquitin ligases regulate a myriad of proteins and therefore their expression and activity need to be tightly controlled to prevent dysfunction and toxicity[6]. Besides multiple regulatory principles, oligomerization appears to be a key mechanism in the adaptation of E3 ligase activity to cellular requirements[37,46,47,55]. Ubiquitylation results in either proteolytic or non-proteolytic fates of conjugated substrates[14]. Therefore, it is intriguing to speculate that oligomeric E3 ligases promote polyubiquitylation and proteasomal degradation, whereas oligomer disassembly supports monoubiquitylation and non-proteolytic substrate regulation[27]. Depending on the concentration, Mdm2 is able

to polyubiquitylate or monoubiquitylate p53, which results in proteasomal degradation or nuclear export[57]. Conclusively, oligomer formation provides an elegant mechanism, which defines E3 ligase function.

Studying the underlying regulation involves various challenges. For many E3 ligases, the regulation and physiological relevance of oligomer formation is not completely understood because of the limitation of methods to follow the dynamic (dis)assembly of E3 ligase complexes *in vivo*. Indeed, many studies were performed under non-physiological conditions by analyzing the structure of recombinantly expressed protein domains *in vitro* or transgenic overexpression *in cellulo*, where the dynamic regulation is different compared to endogenous conditions. For example, studies on the yeast Rad18 RING domain or *Zebrafish* CHIP U-box domain suggested a symmetric homo-dimer assembly and altered E2 binding in contrast to results obtained for both full-length proteins[15,23,58]. Another limitation is that some E3 ligases exist in a monomer–dimer transition state in solution[15], suggesting that binding of E2, adaptor molecules, or chaperones is able to modulate the equilibrium[18,59]. In addition, the expression of E3 ligases is often tissue-specifically regulated and can trigger concentration-dependent changes in oligomer formation[5].

The regulatory role of homotypic and heterotypic combination of E3 ligases appears to be an attractive mechanism to target for drug discovery. Indeed, IAP antagonists are known to promote cIAP dimerization and activity for treating cancer[15,38]. Similarly, homo- or hetero-Proteolysis-Targeting Chimeras (PROTACs) are synthetic small molecules that promote dimerization of specific E3 ligases. For example, the homobifunctional compounds CML1 and 15a induce effective dimerization of the CRL2 subunits VHL and CRBN, respectively, which results in the self-degradation of VHL and CRBN[17,60]. Alternatively, the heterobifunctional compounds 14a and CRBN-6-4-5-5-VHL, synthesized to target both VHL and CRBN, preferentially degrade CRBN over VHL[61,62]. In the case of the E3 ligase CHIP, specific peptides were shown to inhibit its dimerization and E3 ligase activity[63]. More studies and technological advances will provide better insights and understanding of the oligomerization mechanism, which will help to design compounds to manipulate E3 ligase assembly for therapeutic applications.

Abbreviations

CC, coiled-coil; CRL, Cullin–RING E3 ubiquitin ligase; HECT, Homologous to E6-associated protein C Terminus; RBR, RING Between RING; RING, Really Interesting New Gene.

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