JCB Journal of Cell Biology

REVIEW

Post-translational regulation of ubiquitin signaling

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Ubiquitination regulates many essential cellular processes in eukaryotes. This post-translational modification (PTM) is typically achieved by E1, E2, and E3 enzymes that sequentially catalyze activation, conjugation, and ligation reactions, respectively, leading to covalent attachment of ubiquitin, usually to lysine residues of substrate proteins. Ubiquitin can also be successively linked to one of the seven lysine residues on ubiquitin to form distinctive forms of polyubiquitin chains, which, depending upon the lysine used and the length of the chains, dictate the fate of substrate proteins. Recent discoveries revealed that this ubiquitin code is further expanded by PTMs such as phosphorylation, acetylation, deamidation, and ADP-ribosylation, on ubiquitin, components of the ubiquitination machinery, or both. These PTMs provide additional regulatory nodes to integrate development or insulting signals with cellular homeostasis. Understanding the precise roles of these PTMs in the regulation of ubiquitin signaling will provide new insights into the mechanisms and treatment of various human diseases linked to ubiquitination, including neurodegenerative diseases, cancer, infection, and immune disorders.

Introduction

Cells respond to endogenous development cues or insults from the environment by alternations in cellular processes via changes in protein abundancy or activity. Although many such responses eventually occur at the transcriptional level, altering the functional status of existing proteins allows for quick adjustments to cope with challenges, particularly in the initial phase of signal engagement. Changes in protein activity often are achieved by post-translational modifications (PTMs) that cleave precursor proteins, remove chemical moieties from side chains of amino acids, or covalently add modifying groups to one or more residues on the proteins. More than 200 types of PTMs have been identified (Mann and Jensen, 2003; Olsen and Mann, 2013). Among these, ubiquitination, a process that involves covalent attachment of the 76–amino acid protein ubiquitin onto protein substrates, is one of the best studied (Hershko and Ciechanover, 1998). This modification causes alternations in important properties of substrate proteins, including their activity, cellular localization, interactions with other proteins, and most extensively, their half-life in cells (Hershko and Ciechanover, 1998; Zheng and Shabek, 2017). Ubiquitination thus regulates a large cohort of important cellular processes and a dysfunction in ubiquitin signaling is implicated in the development many severe diseases, including cancer, neurodegeneration, immune disorders, and susceptibility to infections (Popovic et al., 2014; Heaton et al., 2016; Gilberto and Peter, 2017).

Biochemical reactions and enzymes that govern classical ubiquitination

Ubiquitination is a multistep process governed by the E1, E2, and E3 enzymes that successively activate, conjugate, and ligate ubiquitin to substrate proteins (Hershko and Ciechanover, 1998; Fig. 1). Among the three enzymes involved in ubiquitination, the number of E3s is the largest (>600 in humans); these structurally diverse enzymes are divided into three main families based on the presence of specific functional domains and on the mechanism of catalysis (Zheng and Shabek, 2017). The HECT (homologous to the E6-associated protein [E6AP] carboxyl terminus) domain E3s catalyze ubiquitin transfer to the substrate protein through a two-step reaction: ubiquitin is first transferred to a catalytic cysteine on the E3 and then from the E3 to the substrate. The name of this family is derived from its prototype, E6AP, which functions together with the E6 protein encoded by the oncogenic human papillomaviruses to target p53 for ubiquitin-dependent degradation (Rolfe et al., 1995). RING (Really Interesting New Gene) E3s mediate a direct transfer of ubiquitin to the substrate from ubiquitin-charged E2s. This family represents the most abundant type of ubiquitin ligases, which harbor either a zinc-binding domain termed RING or a U-box domain that mediates their interactions with the ubiquitin-charged E2. Some RING E3s such as the Cullin-RING ligases are composed by multiple subunits (Deshaies and Joazeiro, 2009). Finally, RING-between-RING (RBR) E3s can be considered a hybrid between HECT and RING. These enzymes

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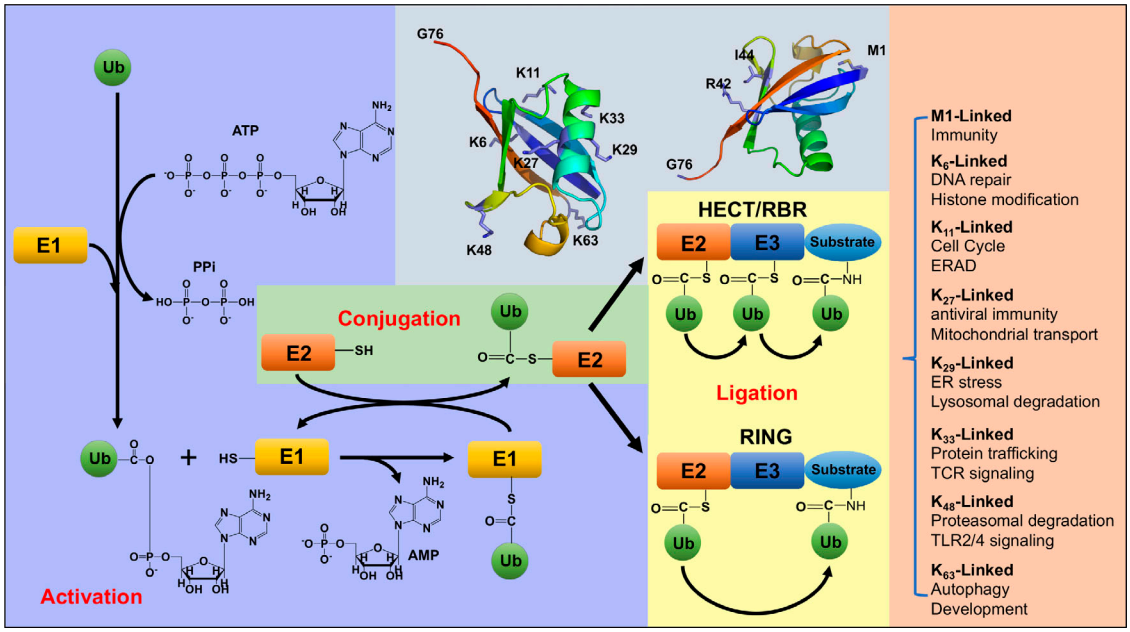

Figure 1. **The chemical reactions and enzymes used in the canonical ubiquitination cascade.** The structure of ubiquitin (Protein Data Bank accession number 1UBQ) with labeled landmark structural elements (including M1, the seven lysine residues, Arg_{42}, Ilu_{44}, and Gly_{76}) important for its functionality is shown (top). Note that the ribbon diagram has been oriented in two different angles to better view the relevant residues. The E1 enzyme uses ATP to activate ubiquitin by acyl-adenylation of its carboxyl terminus. Ubiquitin from the ubiquitin-AMP intermediate is transferred to the active site cysteine in E1 via the formation of a thioester bond between the carboxy-terminal carboxyl group of ubiquitin and the E1 cysteine sulfhydryl group; AMP is concomitantly released (light purple background). The E2 ubiquitin-conjugating enzyme catalyzes the transfer of ubiquitin from E1-thio-Ub to the active site cysteine of the E2 via a trans(thio)esterification reaction (light green background). Depending on the E3 ubiquitin ligase used, ubiquitin on the E2-thio-Ub conjugate can be transferred to the protein substrate by at least two mechanisms. For members of the HECT and RBR family, ubiquitin is delivered to the active site cysteine of the E3 ligase before being transferred to the substrate. For E3 ligases in the RING family, ubiquitin is directly transferred from E2 to the substrate in a process facilitated by the E3 (yellow background). The major roles of the several distinct polyubiquitin chains formed at the primary methionine or one of the seven lysine residues are indicated (orange background). PPi, inorganic pyrophosphate.

use an E2-binding RING domain and a second domain (called RING2) that contains an active Cys required for the formation of an E3~Ub intermediate, from which the ubiquitin is transferred to substrates (Walden and Rittinger, 2018).

Although the catalytic mechanism is conserved in all eukaryotic cells, the ubiquitin network is more complex in mammalian cells, partly due to the presence of larger number of E2 and E3 enzymes. Like many important PTMs, ubiquitination is reversible by reactions catalyzed by several distinct families of deubiquitinases (DUBs; Mevissen and Komander, 2017). Modification of a target protein may occur as a single ubiquitin on a single lysine (monoubiquitination), a single ubiquitin on multiple lysines (multiubiquitination), or as ubiquitinated chains in which lysines on the ubiquitin molecule initially linked to substrate proteins are further modified through sequential rounds of ubiquitination (polyubiquitination). Mono- and multiubiquitination often result in changes in the cellular localization, the activity of the protein, or the formation of multiprotein complexes (Pavri et al., 2006; Sasaki et al., 2011; Su et al., 2013). Polyubiquitination may occur on the amino-terminal methionine methionine (M1) or the amine group in one of the seven lysines of ubiquitin, resulting in eight biochemically different interubiquitin linkages (Fig. 1). The linkage created by the linear ubiquitin chain assembly complex is called linear or M1 linkage. Other types of poly-Ub chains are named by the lysine residue used to form the interubiquitin isopeptide bond. For example, those linked via Lys_{11} and Lys_{48} are called K_{11}- and K_{48}-type poly-Ub chains, respectively (Fig. 1). In many cases, E2 enzymes coordinate with E3 enzymes to control the formation of specific types of poly-Ub chains, which in turn function to regulate distinct cellular processes (Akutsu et al., 2016). For example, whereas modification by K_{48}-type poly-Ub chains often leads to proteasome degradation of the protein substrate (Komander and Rape, 2012), poly-Ub chains linked by K_{63} may activate kinases or facilitate intracellular trafficking of modified proteins (Chen and Sun, 2009; Erpapazoglou et al., 2014; Fig. 1). The architecture of ubiquitin chains governed by the lysine modified, the number of modified sites, and the length of the added ubiquitin molecules, as well as combinations of these parameters, have been referred to as the "ubiquitin code" (Komander and Rape, 2012). The ubiquitin code dictates the fate of the modified proteins by regulating their interactions with proteins harboring various ubiquitin-binding domains and determining their accessibility by DUBs, which specifically hydrolyze poly-Ub chains (Husnjak and Dikic, 2012).

Crosstalk between ubiquitination and other forms of PTMs

Ubiquitination extensively intertwines with other forms of PTMs. For example, mammalian cells express more than 500 protein kinases, which together with hundreds of phosphatases

regulate a wide variety of cellular processes (Mann and Jensen, 2003; Olsen and Mann, 2013). In parallel, there are ∼40 E2 enzymes and >600 E3 ligases that function in numerous combinations to ubiquitinate virtually every protein in the cell (Yau and Rape, 2016). As a result, the number of proteins that are both phosphorylated and ubiquitinated in the cell is large. It is thus clear that the crosstalk between these two forms of PTMs is extensive, and such crosstalk is important for numerous cellular functions (Deribe et al., 2010). Recent studies have revealed that components of the ubiquitination machinery, including E2 and E3 enzymes and the ubiquitin molecule itself, are subjected to being modified by various PTMs, thus providing further regulatory nodes for ubiquitin signaling. Such modifications have significantly expanded the ubiquitin code, thus allowing cells to more effectively respond to stimulations, particularly in the context of diseases or insults from the environment (Herhaus and Dikic, 2015; Swatek and Komander, 2016). Intriguingly, although prokaryotes do not have a ubiquitin system found in eukaryotes, many bacterial pathogens have evolved virulence factors that coopt the host ubiquitination machinery for their survival and replication, which has provided some extraordinary insights into our appreciation of the role of PTMs in ubiquitination. In this review, we will discuss recent progress in understanding how PTMs on ubiquitin itself and on components of the ubiquitination machinery impact cell signaling.

Regulation of E2 and E3 enzyme activity by PTMs

Substrate specificity in ubiquitination and the type of ubiquitin chains formed on proteins are controlled by E2, E3, or combinations of these two enzymes. For members of the HECT-type E3 family that directly transfer ubiquitin to the substrate, specificity is dictated by E3 enzymes (Rotin and Kumar, 2009). For RING- and RBR-type E3s, which constitute the majority of E3s, specificity is mostly conferred by E2s (Ye and Rape, 2009; Stewart et al., 2016). In many scenarios, the activity and substrate recognition of E2 and E3 enzymes are further regulated by PTMs. For the activation of Cullin-RING ubiquitin ligases by the conjugation of the Ub-like (Ubl) protein NEDD8 (neural precursor cell expressed developmentally down-regulated protein 8) to cullins (neddylation), please refer to two recent excellent reviews (Deshaies et al., 2010; Enchev et al., 2015).

Most PTMs impact E2 activity by either lowering their cellular abundancy or directly affecting their ability in catalyzing ubiquitin conjugation. In yeast, the E2 enzyme Rad6 is activated by the cyclin-dependent kinase Bur1–Bur2 complex, thereby allowing it to function with the E3 ligase Bre1 to modify histones by monoubiquitination (Wood et al., 2005). For Ube2T in the Fanconi anemia DNA repair pathway, autoubiquitination occurs on at least three lysines, and modification of the one close to the active site reduces its activity (Machida et al., 2006). Differing from Ube2T, autoubiquitination at a lysine distal to the active site inhibits the activity of Ube2E1 (Schumacher et al., 2013). For Ube2K/E2-25K, which is involved in building poly-Ub chains, modification by the small Ubl modifier (SUMO) in a domain responsible for binding E3 enzymes blocks its ability to ubiquitinate substrates (Pichler et al., 2005). The yeast E2 Ubc7 is regulated by Cue1, a subunit of the ER-associated protein degradation complex. These two proteins directly interact and low cellular levels of Cue1 trigger proteasomal degradation of Ubc7 by attaching a poly-Ub chain to its catalytic cysteine (Ravid and Hochstrasser, 2007).

UBE2N/Ubc13 is an E2 important for signaling in immunity and DNA damage pathways. To fulfill its role in the production of K_{63}-type poly-Ub chains, this enzyme needs to form heterodimers with UBE2V1/UEV1a, a noncatalytic E2 variant (Hodge et al., 2016). The requirement of an additional protein for its E2 activity may be attributed to its complex regulation and diverse roles in cells. Although the enzymes involved are still unknown, UBE2N is ISGylated at Lys_{92} close to its catalytic cysteine (Cys87; Fig. 2 A). The modifier ISG15 is a 15-kD Ubl protein whose expression is induced by interferon (Blomstrom et al., 1986). ISGylation regulates a wide variety of cellular processes, particularly in cells infected by pathogens (Villarroya-Beltri et al., 2017). The inhibition of UBE2N activity by ISGylation may facilitate viral replication (Takeuchi and Yokosawa, 2005; Morales and Lenschow, 2013).

Consistent with its important roles in immunity, the activity of UBE2N is modulated by several bacterial pathogens by unique PTMs for their benefit. Successful infection by many pathogens relies on translocation of virulence factors called effectors into host cytoplasm by specialized secretion systems (Galán and Waksman, 2018). Whereas a large cohort of virulence proteins function as either E3 ligases or DUBs to modulate host processes (Maculins et al., 2016) by coopting with the host ubiquitin network, some pathogenic proteins interfere with the ubiquitin network by modulating the activity of E2 enzymes. Among these, OspI from *Shigella flexneri* attacks UBE2N by converting Gln_{100} of the E2 enzyme into a glutamate residue via a deamidation reaction (Fig. 2 B; Sanada et al., 2012). This modification impairs the ability of UBE2N to function with E3s such as TRAF6 to synthesize K_{63}-type poly-Ub chains, thus dampening NF-κB activation via the diacylglycerol–CARD11–BCL10–MALT1 signaling axis (Sanada et al., 2012). MavC from the bacterial pathogen *Legionella pneumophila* ubiquitinates UBE2N via a transglutamination reaction that links Gln_{40} of ubiquitin to Lys_{92} of the E2 by an isopeptide bond (Gan et al., 2019). This modification completely abolishes the activity of UBE2N most likely by steric hindrance due to the close proximity between Lys_{92} and Cys_{87}, the active site for this E2 enzyme (Fig. 2 C).

The activity of a number of E3 ligases is modulated by PTMs. One prominent example is the oncogenic Mdm2 (murine double minute 2; Hdm2 for human), an E3 ligase that regulates the tumor suppressor p53 by targeting its for proteasome degradation (Karni-Schmidt et al., 2016; Fig. 3). This enzyme is subjected to multiple forms of regulation by distinct PTMs. First, Mdm2 is phosphorylated at multiple sites by several kinases, which differently affects its activity. Phosphorylation of Ser_{395} by the ataxia telangiectasia mutated kinase destabilizes Mdm2 (Meek and Knippschild, 2003). Conversely, phosphorylation of Ser_{166} and Ser_{186} by PI3K/AktS stabilizes Mdm2 and destabilizes p53 (Mayo and Donner, 2001); phosphorylation of Ser_{17} by the DNA-dependent protein kinase reduces its binding affinity for p53 (Mayo et al., 1997; Fig. 3). Second, Mdm2 is acetylated by the CBP (cAMP response element–binding protein) and p300 at multiple

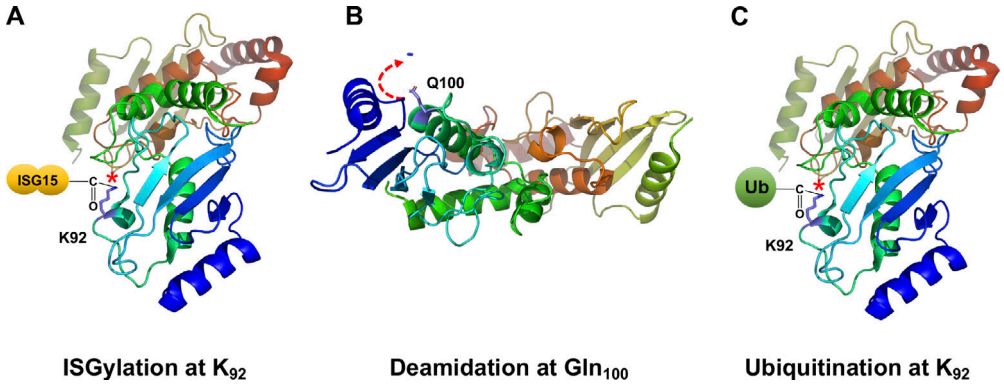

Figure 2. **Representative PTMs of the E2 enzyme UBE2N.** The structure of UBE2N (Protein Data Bank accession number 1JBB) is depicted by ribbon diagrams in which secondary structural elements such as α-helices, β-sheets, and links at different regions of the protein are shown in different colors. **(A)** Modification at Lys_{92} by ISG15. **(B)** Deamidation at Gln_{100} by OspI. **(C)** Ubiquitination at Lys_{92} by MavC. The addition of the bulky ISG15 or ubiquitin at Lys_{92}, which is in close proximity with the active cysteine located at the 87th position, will sterically preclude the incoming ubiquitin from being linked to Cys_{87}, thus blocking the activity of the E2 enzyme.

sites (e.g., Lys_{182} and Lys_{185}), which not only increases its affinity for the DUB herpesvirus-associated ubiquitin-specific protease but also protects it from being autoubiquitinated (Wang et al., 2004; Nihira et al., 2017). Third, cellular abundancy of Mdm2 is regulated by ubiquitination either by itself (autoubiquitination) or by p300–CBP-associated factor, leading to its degradation (Wade et al., 2013). Furthermore, although the site of modification is unknown, another study showed that autoubiquitination activates the E3 ligase activity of Mdm2 in modifying p53, probably by increasing its affinity for the E2 UbcH5c (Ranaweera and Yang, 2013). Finally, Mdm2 is modified by SUMO by reactions catalyzed by at least three different SUMO E3 ligases (Ubc9, PIAS1, and RanBP2; Buschmann et al., 2001; Miyauchi et al., 2002; Fig. 3). The sequential SUMOylation of Mdm2 by RanBP2 and PIAS in the nucleus inhibits its E3 activity, leading to higher p53 stability (Miyauchi et al., 2002). The complexity of the regulation is further increased by enzymes that reverse these modifications. For example, phosphorylation and acetylation of Mdm2 are reversed by phosphatase 1D (PPM1D, also known as wild-type p53–induced phosphatase 1) and SIRT1 (silent mating type information regulation 2 homologue 1), respectively, leading to its stabilization thus lower cellular p53 levels (Lu et al., 2007; Nihira et al., 2017).

Several E3 ligases of the HECT subfamily are subjected to regulation by phosphorylation. For example, JNK1 serine/threonine kinase–induced phosphorylation within a proline-rich region relieves the E3 ITCH from an autoinhibitory status (Gao et al., 2004; Gallagher et al., 2010); similarly, phosphorylation by the c-Src kinase at Tyr_{43} and Tyr_{585} activates the E3 enzyme NEDD4.1 (Persaud et al., 2014). Phosphorylation by c-Abl at

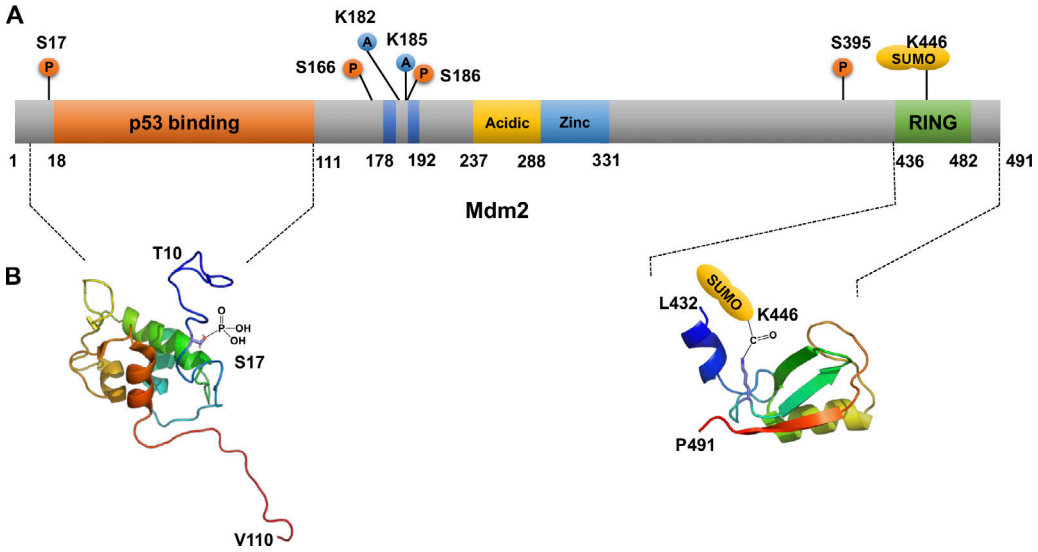

Figure 3. **A schematic diagram of the E3 ubiquitin ligase Mdm2 and the sites receiving diverse PTMs. (A)** Domain structure of Mdm2. S, serine; K, Lysine; P, phosphorylation; A, acetylation. Note the clustering of the modification sites at distinct domains. **(B)** The structure of the amino-terminal domain of Mdm2 (Protein Data Bank accession number 1Z1M; left) that harbors the phosphorylation site Ser_{17} and the carboxyl terminus RING domain (Protein Data Bank accession number 5MNJ; right) that contains the Lys_{446} SUMOylation site.

Tyr_{636} within the HECT domain of E6AP alters its substrate specificity (Chan et al., 2013). Instead of directly impacting activity, phosphorylation also modulates activity of E3s by altering their interactions with other proteins. One such example is serine phosphorylation of NEDD4.2 on Ser_{468} by the kinase SGK1 (serum/glucocorticoid-regulated kinase 1), which promotes its binding to the chaperone protein 14-3-3, leading to reduction in its affinity for the natural substrate ENaC (Debonneville et al., 2001; Snyder et al., 2002).

The activity of E3s is also regulated by ubiquitination or SUMOylation that either causes their degradation by the proteasome or changes their interactions with other proteins (de Bie and Ciechanover, 2011; Tomaic et al., 2011).

Induction of mitophagy by coordinated phosphorylation of an E3 ligase and ubiquitin

The impact of phosphorylation on ubiquitination is not limited to the regulation of E2 and E3 activity and the susceptibility of protein substrates; rather, it extends to the ubiquitin molecule itself. The ubiquitin protein harbors 11 amino acids able to accept a phosphate moiety from kinases, including three serines (Ser_{20}, Ser_{57}, and Ser_{65}), seven theronines (Thr_7, Thr_9, Thr_{12}, Thr_{14}, Thr_{22}, Thr_{55}, and Thr_{66}), and one tyrosine (Tyr_{58}). Although the kinases involved mostly remain elusive, phosphorylation has been detected in eight of these residues (Herhaus and Dikic, 2015).

The study of the interplay among phospho-Ub at Ser_{65}, the E3 ligase Parkin, and the phosphatase and tensin homolog induced putative kinase 1 (PINK1) responsible for phosphorylation of both ubiquitin and Parkin, has generated important insights into the roles and mechanisms of elimination of damaged mitochondria in neurodegenerative diseases (Greene et al., 2003; Durcan and Fon, 2015). Mutations in the gene coding for PINK1 are linked to Parkinson's disease and PINK1 genetically interacts with the E3 ubiquitin ligase Parkin (Valente et al., 2004; Clark et al., 2006). At the cellular level, dysfunctions in Parkin lead to accumulation of missfolded, aggregated proteins and degenerated mitochondria. Under normal conditions, Parkin assumes an inactive conformation and localizes in the cytoplasm. Newly synthesized PINK1 is imported into the mitochondria in a process accompanied by a series of proteolytic cleavage steps, which convert the 64-kD full-length form into 60- and 52-kD fragments (Jin et al., 2010). The 52-kD fragment is degraded by the proteasome through the N-end rule pathway once it is exported back into the cytosol (Yamano and Youle, 2013). Thus, very low PINK1 levels are maintained in healthy cells. Mitochondrial damage triggered by events such as the loss of membrane potential causes PINK1 to accumulate on the surface of damaged mitochondria (Narendra et al., 2008), where it phosphorylates ubiquitin at Ser_{65} (Koyano et al., 2014; Sauvé et al., 2015; Wauer et al., 2015a; Figs. 4 and 5 A). The local increase of phospho-Ub abundancy recruits Parkin to the mitochondria, making it accessible to PINK1, which phosphorylates Ser_{65} within its Ubl domain (Fig. 4). This modification causes the movement of the Ubl domain and the release of the catalytic RING2, one of the four zinc-binding domains in the E3 ligase (Condos et al., 2018; Sauvé et al., 2018). PINK1 also phosphorylates Ser_{65} in poly-Ub chains associated with mitochondria, which further promotes the tethering of Parkin to the organelle (Shiba-Fukushima et al., 2014; Wauer et al., 2015b). Activated Parkin ubiquitinates numerous mitochondrial and cytosolic proteins, including mitofusins and Miro, and eventually induces mitophagy to eliminate damaged mitochondria (Harper et al., 2018; Fig. 4).

A number of DUBs regulate the function of Parkin by removing ubiquitin chains from its substrates (Harper et al., 2018). However, whether modified ubiquitin can be converted to its original form by specific phosphatases remains unclear. Because phosphorylation of ubiquitin affects its transfer from E2 to build poly-Ub chains by several E2 and E3 enzyme pairs, including UBE2N–UBE2V1 and TRAF6, that are pivotal for several important signaling cascades (Wauer et al., 2015b; Hodge et al., 2016), it seems necessary to restore modified ubiquitin back to its unmodified form after damaged mitochondria have been cleared. Whether phospho-Ub is assembled into any poly-Ub chain under any physiological condition remains unknown, let alone the abundancy and impact of such chains on cellular processes. Finally, ubiquitin phosphorylation also occurs at several other residues (Herhaus and Dikic, 2015), but virtually nothing is known about the impact of the modification of these sites on ubiquitin in terms of its usefulness for ubiquitination, poly-Ub chain synthesis, or activity of the chains formed by the modified ubiquitin species.

The impact of acetylation, glutamine deamidation, or SUMOylation on ubiquitin

In addition to phosphorylation, ubiquitin is modified by several other PTMs, particularly acetylation and SUMOylation. These modifications may affect the functionality of ubiquitin by steric hindrance or alternations in surface properties such as charge. Acetylation at Lys_6 and Lys_{48} can be detected in cells (Fig. 5 B). Acetylation at Lys_6 or Lys_{48} does not affect ubiquitin activation by E1 and subsequent transfer to the substrate because monoubiquitination reactions using these two forms of ubiquitin are indistinguishable from those containing native ubiquitin (Ohtake et al., 2015). Acetylation at Lys_6 and Lys_{48} will clearly block the synthesis of poly-Ub chains linked through these two positions. Unexpectedly, this modification also interferes with the synthesis of K_{11}- and K_{63}-type poly-Ub chains by a number of E2 enzymes (Ohtake et al., 2015). Interestingly, acetylation of ubiquitin at Lys_6 and Lys_{48} appears to be reversed by histone deacetylases (Seto and Yoshida, 2014). Given the importance of these types of poly-Ub chains in signaling (Akutsu et al., 2016), AcK6Ub and AcK48Ub likely have a profound yet unrecognized impact on at least some cellular processes.

Modifications by small chemical moieties such as phosphoryl and acetyl groups impact ubiquitin signaling by altering the charge or structure property of ubiquitin. Such changes can also be achieved by deamidation, a reaction that converts an amide into an acid on asparagine or glutamine residues. For example, the cycle inhibiting factor from enteropathogenic *Escherichia coli* (Cif) homolog in *Burkholderia pseudomallei* is a deamidase that converts Gln_{40} of both ubiquitin (Fig. 5 C) and the Ubl modifier NEDD8 into a glutamate residue (Cui et al., 2010). Deamidated ubiquitin is less suitable for the synthesis of poly-Ub chains by

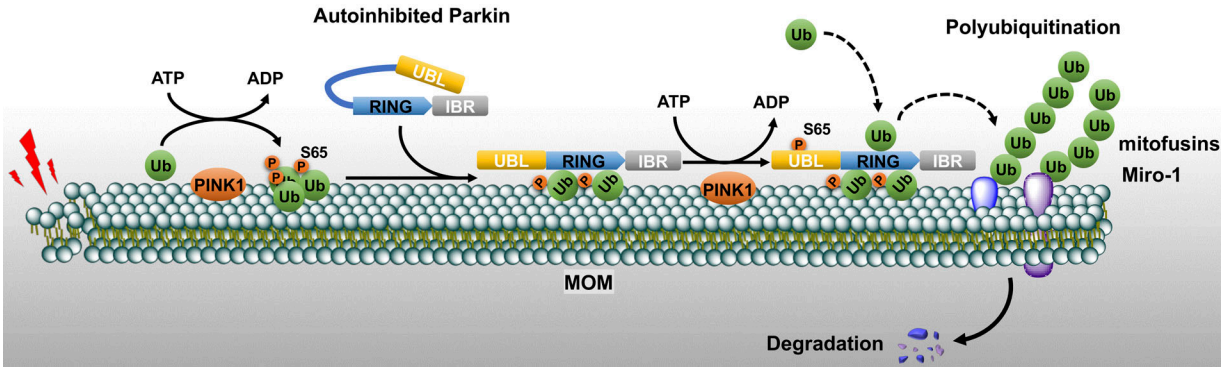

Figure 4. **Activation of the E3 ligase Parkin by PINK1-induced phosphorylation and phosphorylated ubiquitin.** Under unstressed conditions, mature PINK1 is inserted into mitochondrial membranes via its amino-terminal domain and Parkin assumes an autoinhibitory conformation in the cytoplasm. Mitochondrial damage caused by events such as the loss of membrane potential causes PINK1 to accumulate on mitochondrial surface where it phosphorylates ubiquitin at Ser_{65}, the accumulation of phospho-Ub on the damaged mitochondria recruits Parkin. PINK1 also phosphorylates Parkin, leading to its complete activation. Fully activated Parkin ubiquitinates a number of mitochondrial proteins such as Miro-1 and GTPases mitofusins involved in mitochondria fusion, which eventually leads to elimination of damaged mitochondria by mitophagy. UBL, ubiquitin-like; IBR, R1-in-between-ring; MOM, mitochondrial outer membrane.

several E2 and E3 enzyme pairs important for immunity (Cui et al., 2010). In contrast, Cif specifically deamidates NEDD8 at Gln_{40}. Although deamidated NEDD8 can still be used to modify Cullin, it potently inhibits the ubiquitin ligase activity of the neddylated Cullin-RING complex (Cui et al., 2010). Deamidation is an irreversible modification, suggesting proper signaling mediated by the affected enzymes cannot be restored in infected cells, which is consistent with the notion that cells attacked by CHBP or Cif eventually undergo apoptosis (Samba-Louaka et al., 2009; Yao et al., 2012).

Ubiquitin has also been shown to be modified by SUMOylation, again at lysine residues (Wilson and Heaton, 2008), which suggests the existence of more complex ubiquitin architecture made by multiple modifiers on substrate proteins. Heat shock and inhibition of the proteasome appear to promote ubiquitin SUMOylation, suggesting a role of SUMOylated ubiquitin in stress response (Tatham et al., 2011), yet the impact of this modification on the activity of ubiquitin, as well as the impact of SUMOylated ubiquitin on the function of modified proteins, is not known. Overexpression of the Ubl NEDD8 produced neddylated ubiquitin, but the physiological significance of this modification is unknown (Enchev et al., 2015). Clearly, the addition of the modifiers with molecular weights similar to ubiquitin will further increase the complexity in their synthesis, ligation, and potential removal from protein substrates (Swatek and Komander, 2016). Future challenges are the identification of

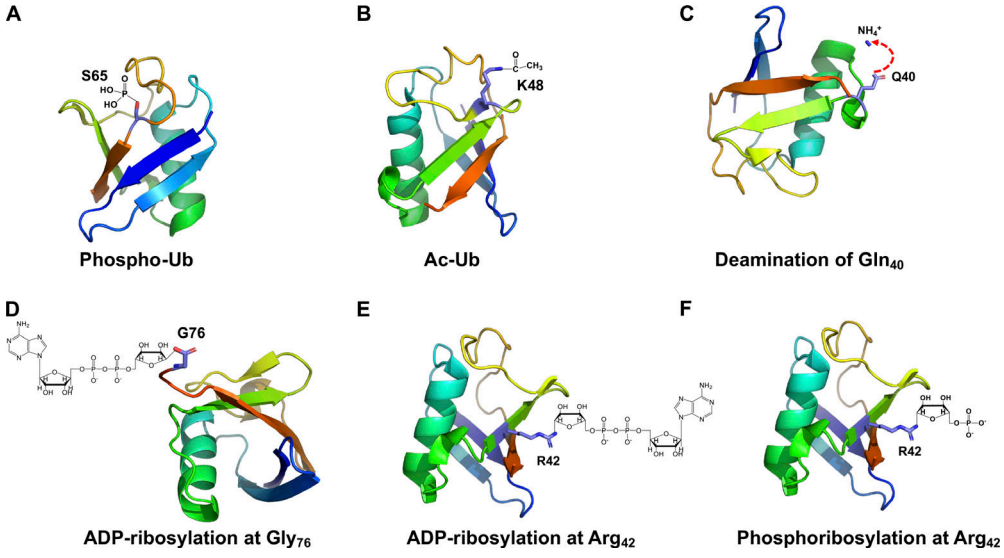

Figure 5. **Representative modifications of ubiquitin.** The structure of ubiquitin (Protein Data Bank accession number 1UBQ) was shown in ribbon diagrams. Secondary structural features such as α-helices, β-sheets, and links at different regions of the protein are shown in different colors. **(A)** Phosphorylation can occur on multiple sites of ubiquitin, including Thr_7, Thr12, $Thr1_4$, Ser_{20}, Ser_{57}, Tyr_{59}, Ser_{65}, and Thr_{66}. Shown is the location of S_{65}, the best-studied phosphorylation on ubiquitin. **(B)** Acetylation of ubiquitin. Both Lys_8 and Lys_{48} can be modified by acetylation, and shown is the modification on Lys_{48}. **(C)** Deamidation at Gln_{40}. **(D)** ADP-ribosylation at Gly_{76}. **(E)** ADP-ribosylation at Arg_{42}. **(F)** Phosphoribosylation at Arg_{42}.

the enzymes involved in the production of the modifier chimeras and the cellular processes affected by these molecules.

New territories in ubiquitination revealed by the old ADP-ribosylation modification

ADP-ribosylation is a reaction that transfers the ADP-ribose (ADPR) moiety from NAD^+ to substrate proteins accompanied by the release of nicotinamide catalyzed by a family of enzymes that contain the ADP-ribosyltransferase (ART) domain. ADP-ribosylation was originally discovered by studying the diphtheria toxin from the bacterial pathogen *Corynebacterium diphtheriae*; proteins containing ART motifs were later identified in eukaryotic cells (Honjo et al., 1968; Corda and Di Girolamo, 2003). Mammalian ART proteins catalyze covalent link of either a single ADPR or ADPR polymers (poly-ADP ribose) to substrate proteins, but most of its 17 family members induce the attachment of a single ADPR to substrate protein (mono-ADP-ribosylation) to regulate various cellular processes, including DNA repair and tumorigenesis (Kraus, 2015). In contrast, virtually all bacterial ART proteins induce the transfer of a single ADPR to substrate proteins by a conserved mono-ART (mART) motif (Simon et al., 2014).

A recent study showed that the ART protein Parp9 interacts with the histone E3 ligase Dtx3L to form heterodimers that modify ubiquitin at its carboxyl terminus by NAD^+-dependent mono-ADP-ribosylation (Yang et al., 2017; Fig. 5 D). Ubiquitination by the canonical mechanism is completed by the formation of an isopeptide bond between the carboxyl terminus of ubiquitin molecule and lysine residues in substrate proteins; mono-ADP-ribosylation at Gly_{76} will clearly inhibit substrate modification or the synthesis of poly-Ub chains. This modification enables Parp9 to regulate the E3 activity of Dtx3L in response to fluctuations in NAD^+ concentrations (Yang et al., 2017). Interestingly, mono-ADP-ribosylation of ubiquitin by the Parp9–Dtx3L heterodimer requires the E1 and E2 enzymes as well as ATP and is reversible, pointing to potentially complex regulation (Yang et al., 2017). Parp9 may catalyze the addition of ADPR to E2-ubiquitin thioester before its attachment to the substrate by Dtx3L, or it may modify inactivated free ubiquitin by an unknown mechanism that requires E1, E2, and ATP. No matter the mechanism, further investigation is needed to address how Parp9-mediated ADP-ribosylation regulates the E3 activity of Dtx3L in vivo and whether dysregulation in this process is involved in any disease process.

Crosstalk between mono-ADP-ribosylation and ubiquitin signaling also is the foundation of a new form of ubiquitination catalyzed by chemical reactions that completely differ from those used by the canonical mechanism participated by the E1, E2, and E3 enzymes. This discovery was made by studying the SidE effector family from *L. pneumophila,* which utilizes hundreds of effectors with diverse biochemical activities to create an intracellular niche permissive for its replication (Qiu and Luo, 2017). Members of the SidE family such as SdeA contain a conserved mART motif (Fig. 6 A) that catalyzes the transfer of the mono-ADP-ribosyl moiety from NAD^+ to Arg_{42} of ubiquitin (Qiu et al., 2016). The ADP-ribosylated ubiquitin (ADPR-Ub; Fig. 5 E) is equivalent to the Ub-AMP conjugate produced by E1 and ATP in classical ubiquitination. A phosphodiesterase (PDE)-like domain also embedded in members of the SidE family utilizes ADPR-Ub as the substrate; it cleaves the phosphoanhydride bond in ADPR-Ub and transfers phosphoribosylated ubiquitin (PR-Ub; Fig. 5 F) to serine residues of substrate proteins, concomitant with the release of AMP (Bhogaraju et al., 2016; Kotewicz et al., 2017; Fig. 6 B). In the absence of protein substrates, ubiquitin and NAD^+ in reactions will be completely converted into PR-Ub, nicotinamide, and AMP by SidEs (Bhogaraju et al., 2016). Furthermore, these two reactions are separable. Large quantities of ADPR-Ub can be produced by reactions containing ubiquitin, NAD^+, and a SdeA mutant defective in PDE activity or a fragment of SdeA harboring an active mART domain (Bhogaraju et al., 2016). SdeA mutants defective in the mART domain or a domain containing an active PDE domain can use ADPR-Ub to ubiquitinate protein substrates (Bhogaraju et al., 2016; Kotewicz et al., 2017). Similar to the reversibility seen in canonical ubiquitination, PR-Ub can be reversed by a specific enzyme that does not share homology to any protein involved in deubiquitination (Qiu et al., 2017).

Mechanistically, ubiquitin activation occurs in a chamber formed by an α-helical lobe and the mART core that binds NAD^+ and carries out ADP-ribosylation of ubiquitin. This catalytic mechanism is similar to other bacterial toxins with ADP-ribosylation activity (Akturk et al., 2018; Dong et al., 2018; Kalayil et al., 2018). The transfer of PR-Ub produced from ADPR-Ub by PDE activity is a two-step process: PR-Ub is first delivered to the catalytic His_{277} before being transferred to a serine residue in substrate proteins (Kalayil et al., 2018). Interestingly, structural analysis revealed that the mART motif is ∼50 Å from the PDE domain (Akturk et al., 2018; Dong et al., 2018; Kalayil et al., 2018). How ADPR-Ub made at the mART motif is delivered to the PDE domain without being released into the cytoplasm remains unknown. Under native conditions, SidEs may form oligomers in which these two domains are configured into close proximity to channel the reactions.

SidEs ubiquitinate a number of Rab small GTPases associated with the ER and the ER protein Rtn4 (Qiu et al., 2016; Kotewicz et al., 2017). Whereas ubiquitination by SidEs slightly inhibits the GTPase activity of Rab33b, modification of Rtn4 causes it to aggregate and enrich on the vacuole containing *L. pneumophila* (Kotewicz et al., 2017). A substrate-binding cleft in the PDE domain dictates substrate specificity by recognizing hydrophobic residues surrounding the serine residues (Akturk et al., 2018; Dong et al., 2018; Kalayil et al., 2018), which may explain the recognition of structurally diverse proteins by SidEs. This feature also predicts that the number of host proteins targeted by SidEs may be large. One future challenge is to identify these substrates and determine their roles in *L. pneumophila* infection.

ADPR-Ub and PR-Ub produced by SidEs potently interfere with ubiquitination catalyzed by the canonical mechanism (Bhogaraju et al., 2016). Furthermore, diubiquitins modified by ADPR are resistant to cleavage by various DUBs that use different mechanisms for isopeptide bond hydrolysis (Puvar et al., 2017). Although one can produce ADPR-Ub and PR-Ub in large quantities by biochemical reactions with mutants lacking PDE

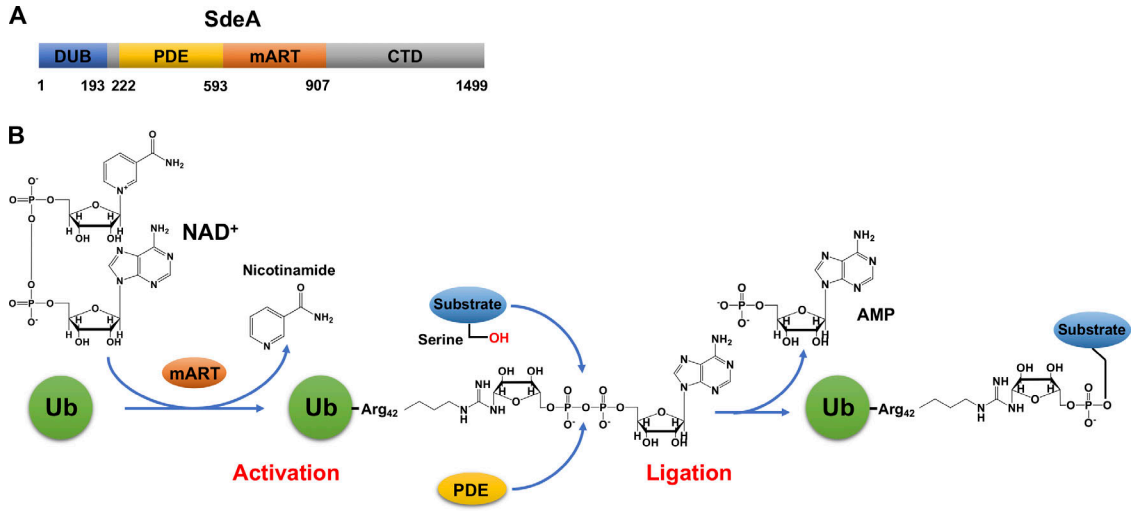

Figure 6. **Ubiquitination catalyzed by ADP-ribosylation. (A)** Domain structure of SdeA, a member of the SidE effector family. This protein contains a canonical DUB domain in its first 200 residues (Sheedlo et al., 2015), a PDE-like domain between residues 222 and 593, and a mono-ADP-ribosylation domain between residues 594 and 907; the function of the domain that comprises the last 500 residues is unknown. CTD, carboxy-terminal domain. **(B)** Biochemical reactions that lead to ubiquitination. The mART domain activates ubiquitin by transferring the ADP-ribosyl moiety from NAD^+ to Arg_{42} of ubiquitin. A PDE activity cleaves the phosphoanhydride bond in the reaction intermediate ADPR-Ub and transfers PR-Ub to serine residues of substrate proteins accompanied by the release of AMP.

activity or with wild-type SdeA (Bhogaraju et al., 2016; Kotewicz et al., 2017), these forms of modified ubiquitin likely are scarce, even in cells infected with *L. pneumophila*. Consistent with this notion, canonical ubiquitination is required for successful bacterial infection, and it appears to operate normally in infected cells (Dorer et al., 2006). The ADPR moiety on modified proteins can be removed by ADP-ribosylhydrolase enzymes (Lüscher et al., 2018); it is possible that ubiquitin in both ADPR-Ub and PR-Ub can be restored to the unmodified form by cleaving the N-glycosidic bond between arginine and ribose by specific enzymes. However, the enzymes responsible for such events have yet to be identified.

Summary and future perspectives

Advancement in technologies such as mass spectrometry has allowed the detection of not only novel PTMs but also known PTMs on previously unrecognized modified proteins. As a result, ubiquitin and the enzymes involved in its conjugation have been found to be increasingly intertwined with PTMs that often critically impact ubiquitin signaling. While such modifications will inevitably increase the complexity of the ubiquitin code, they likely will provide more intricate regulation under certain conditions. Among the 11 residues known to be phosphorylated on ubiquitin, only the role of phosphorylation at Ser_{65} has been studied in detail. The fact that PINK1 is linked to Parkinson's disease, a condition in which ubiquitin phosphorylated at Ser_{65} becomes evident, suggests that the importance of phosphorylation at other sites may only be relevant under specific disease or stressed conditions. In resting cells, phosphorylation at these sites may be fine-tuned to an equilibrium by specific kinases and phosphatases. Detection of phosphorylated ubiquitin under different stress conditions may reveal the role of modification at these sites.

Infection by viral or bacterial pathogens constitutes an important form of stress that requires reorchestration of the ubiquitin network. The study of pathogenic elements directly involved in coopting ubiquitin signaling has revealed several unique PTMs that tilt the ubiquitin system toward their benefit. It will be interesting to determine whether an abundance of phospho-Ub species undergoes any change in infected cells, particularly those infected by pathogens known to modulate the function of specific organelles. The same principle may be applied to the analysis of other forms of modifications on ubiquitin such as acetylation.

In light of the discovery of ubiquitination driven by NAD^+ and ADP-ribosylation, one important question is whether eukaryotic cells employ this mechanism to ubiquitinate proteins. One strategy is to identify ubiquitin moieties attached to proteins by the phosphoribosyl linkage in eukaryotic cells, which can be facilitated by developing antibodies that specifically recognize this linkage. Because protein ubiquitination by this mechanism is performed by a two-step process with enzymes of distinct activity, eukaryotic cells may use proteins that are either loosely associated or functioning independently to execute the modification. Once proteins modified by PR-Ub catalyzed by endogenous enzymes have been identified, one can identify the proteins for the second step reaction using ADPR-Ub produced by the mART domain of SidEs.

Modifications on ubiquitin or enzymes involved in ubiquitination often occur under specific conditions. Once the cues become diminished or the insults have been properly dealt with, modified ubiquitin or ubiquitin conjugation enzymes can either be recycled after degradation or converted into their unmodified forms by specific enzymes. The identification of such enzymes will aid our understanding of the role of these modifications. A better understanding of the mechanism of proteins involved in

the regulation will lay the foundation for the development of therapeutics for treating relevant diseases.

Acknowledgments

We apologize to scientists whose important contributions were not referenced in this review due to space limitations. We would like to thank members of our laboratory for helpful discussions.

This work was supported by the National Institutes of Health (grant R01AI127465) and the First Hospital of Jilin University research fund.

The authors declare no competing financial interests.

Author contributions: Z.-Q. Luo conceived the content and wrote and revised the text. L. Song drew the figures under the guidance of Z.-Q. Luo.

Submitted: 13 February 2019
Revised: 29 March 2019
Accepted: 3 April 2019

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