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Principles of Ubiquitin-Dependent Signaling

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Abstract

Ubiquitylation is an essential posttranslational modification that controls cell division, differentiation, and survival in all eukaryotes. By combining multiple E3 ligases (writers), ubiquitin-binding effectors (readers), and deubiquitylases (erasers) with functionally distinct ubiquitylation tags, the ubiquitin system constitutes a powerful signaling network that is employed in similar ways from yeast to humans. Here, we discuss conserved principles of ubiquitin-dependent signaling that illustrate how this posttranslational modification shapes intracellular signaling networks to establish robust development and homeostasis throughout the eukaryotic kingdom.

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INTRODUCTION

"The language of science is universal and is a powerful force in bringing the peoples of the world closer together." This statement of Arthur H. Compton, spoken on the occasion of celebrating the 1927 Nobel Prize in Physics for his discovery of the particle nature of electromagnetic radiation, is more important than ever. Akin to languages, cells often transfer information and control proliferation, differentiation, or survival through communication systems that employ recurrent building blocks arranged in variable sequences. An intriguing example is posttranslational modification with the highly conserved protein ubiquitin, a process referred to as ubiquitylation. Ubiquitylation is essential in every eukaryotic cell, and ubiquitin modifications of different topologies have the same meaning in organisms spanning the evolutionary distance from yeast to humans. How the universal language of ubiquitin directs cell behavior is at the center of this review.

Ubiquitylation describes the process during which the 76-amino-acid protein ubiquitin is covalently attached to cellular proteins (**Figure 1*a***). In most cases, the carboxy terminus of ubiquitin forms an isopeptide bond with the ϵ amino group of Lys residues in substrates, yet ubiquitin can also be targeted to a protein's amino terminus (Breitschopf et al. 1998); to thiol or hydroxyl side chains in Cys, Ser, and Thr residues (Cadwell & Coscoy 2005, Pao et al. 2018, Shimizu et al. 2010, Wang et al. 2012); or in a phosphoribosylation-dependent manner to a substrate Ser (Bhogaraju et al. 2016, Qiu et al. 2016). Transfer of a single ubiquitin to one or multiple target residues is referred to as monoubiquitylation or multimonoubiquitylation, respectively, and often changes the interaction landscape of the modified protein (Yau & Rape 2016). Subsequent decoration of a substrate-attached ubiquitin with further ubiquitin molecules results in polymeric chains that can adopt distinct structures and functions. Functions have been assigned to five out of eight possible homotypic conjugates: K11- and K48-linked ubiquitin chains target proteins for degradation to the 26S proteasome (Chau et al. 1989, Jin et al. 2008); M1- and K63-linked polymers allow for assembly of large protein complexes during DNA repair, NF-κB transcription factor activation, or protein synthesis (Tokunaga et al. 2009, Wang et al. 2001); and K6-linked chains play important roles in mitophagy (Michel et al. 2017). In addition, roles of heterotypic ubiquitin chains that contain more than one type of linkage are beginning to emerge. For example, as described in more detail below, K11/K48-branched chains constitute proteasomal priority signals for efficient elimination of aggregation-prone and cytotoxic proteins (Meyer & Rape 2014, Yau et al. 2017).

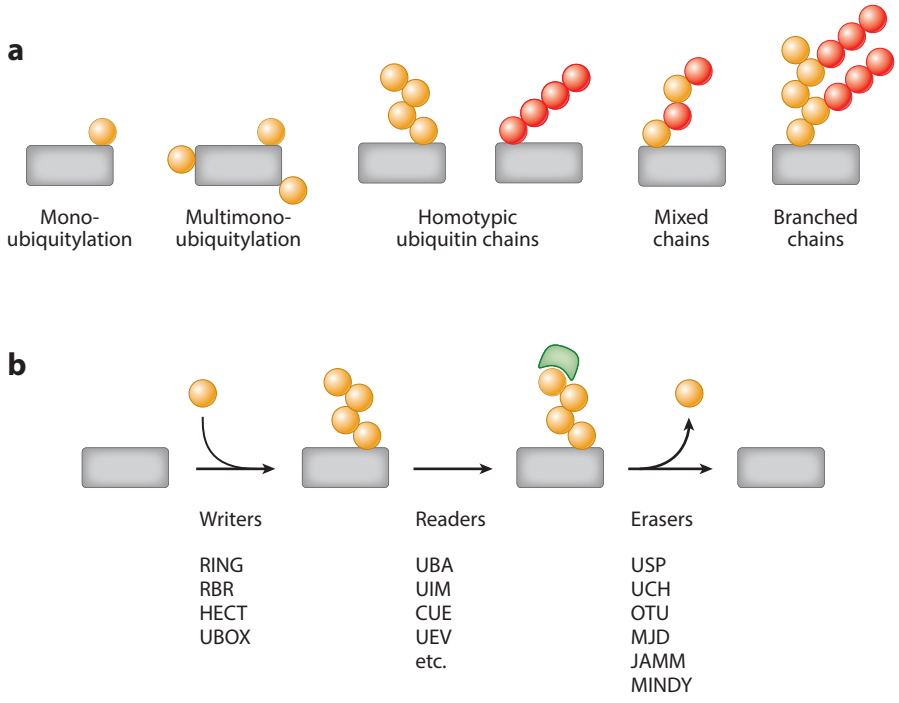

Figure 1

The components of ubiquitin-dependent signaling. (*a*) Ubiquitin can be attached to substrates as single subunits (monoubiquitylation or multimonoubiquitylation) or in the form of polymeric ubiquitin chains. Homotypic chains contain a single linkage, while heterotypic conjugates with multiple linkages can be of either a mixed or a branched nature. (*b*) The basic players in ubiquitin-dependent signaling are E3 ligases (writers), which transfer ubiquitin to specific substrates; ubiquitin-binding effectors (readers), which couple the ubiquitin modification to downstream signaling events; and deubiquitylases (erasers), which cleave ubiquitin off proteins.

Assembly and removal of the diverse ubiquitin signals depend on tightly regulated enzymes (**Figure 1*b***). E3 ligases with characteristic RING, UBOX, RBR, or HECT domains recruit select substrates as well as an activated form of ubiquitin to catalyze specific ubiquitin transfer (Deshaies & Joazeiro 2009, Scheffner et al. 1993, Wenzel et al. 2011). Conversely, deubiquitylases (DUBs) with signature USP, UCH, OTU, MJD, JAMM, or MINDY domains process ubiquitin precursors, edit chain topologies, or cleave ubiquitin off substrates to terminate signaling (Harrigan et al. 2018, Mevissen & Komander 2017). The human genome encodes ~600 E3 ligases and ~100 DUBs, many of which are signal transducers essential for development or are mutated in disease (Rape 2017). In between attaching and cleaving off ubiquitin are dozens of effector proteins that recognize the ubiquitin tag through motifs, including UBA, UIM, or UEV domains, and couple substrate modification to a downstream biological output. Together, the combination of distinct ubiquitin marks with writers (E3 ligases), readers (effectors), and erasers (DUBs) constitutes a powerful signaling language that is essential for development and homeostasis in every eukaryote (Yau & Rape 2016).

As implied by the thousands of ubiquitylation sites reported in metazoan cells, ubiquitin-dependent signaling is very prevalent (Emanuele et al. 2011, Kim et al. 2011), and the ability of ubiquitylation to impose specific, quantitative, and reversible regulation onto biological pathways

places this modification at the hubs of many essential signaling networks. In this review, we discuss conserved and recently elucidated principles of ubiquitin-dependent signaling. To illustrate the core functions of this modification, we follow the typical life cycle of a protein, from transcription to function to degradation, and describe how attachment of ubiquitin affects substrate behavior and cellular information flow. While our approach does not allow us to mention all of the many functions of ubiquitylation, focusing on the recurrent and essential roles of ubiquitin-dependent signaling highlights how this modification has emerged as a powerful language to direct cellular and organismal behavior.

SPECIFICITY AND REVERSIBILITY OF UBIQUITIN-DEPENDENT SIGNALING

The large number of human E3 ligases and DUBs immediately suggests that ubiquitin-dependent signaling is highly specific and reversible. Both of these properties are illustrated by the essential role of ubiquitylation in controlling chromatin architecture and gene-specific transcription, two events that are critical for accurate protein synthesis and metazoan development. Key to chromatin organization are posttranslational modifications of histone proteins, and histone H2A was the first protein to be identified as a substrate for ubiquitylation (Goldknopf et al. 1977). In interphase cells, roughly 10% of H2A molecules are monoubiquitylated on a specific Lys residue, K119 (referring to the human amino acid sequence), by a family of multisubunit E3 ligases referred to as Polycomb repressive complex 1 (PRC1). All PRC1 complexes are built around two RING domain proteins, RING1A and RING1B, but differ in the noncatalytic subunits that target these machineries to their proper sites on chromatin (Gao et al. 2012, Levine et al. 2002, McGinty et al. 2014, Wu et al. 2013).

Monoubiquitylation of K119 of H2A at transcription start sites results in silencing of downstream genes (Wang et al. 2004) (**Figure 2**). The ubiquitin tag on H2A functions by recruiting enzymes that add further repressive histone modifications or by preventing interactions with proteins that drive transcription elongation. To provide an intriguing example, H2A monoubiquitylation by PRC1 attracts the PRC2 complex, which then implements repressive histone H3 K27 trimethylation; this modification in turn recruits more PRC1 complexes (Blackledge et al. 2014, Cao et al. 2002, Cooper et al. 2014, Kalb et al. 2014). The cooperation between the enzymatic machineries catalyzing H2A monoubiquitylation and H3 trimethylation allows H2A ubiquitylation to spread along whole chromosomes, as demonstrated during X chromosome inactivation in females (Almeida et al. 2017). However, despite the high abundance of H2A monoubiquitylation and the frequent dysregulation of PRC1 in disease, the catalytic activity of RING1B as well as K119 in H2A is not essential for gene regulation during fly or mouse development (Cao et al. 2005, Illingworth et al. 2015, Pengelly et al. 2015).

While monoubiquitylation of H2A K119 restricts gene expression, attachment of ubiquitin to distinct histone Lys residues, in either H2A or other nucleosome components, results in different outcomes (**Figure 2**). Rather than inhibiting transcription, monoubiquitylation of histone H2B on K120 by an E3 composed of RNF20 and RNF40 stimulates gene expression by recruiting methyltransferases that install activating histone H3 methylation marks (Kim et al. 2005, Pavri et al. 2006). Monoubiquitylation of proximal Lys residues in histone H3 by UHRF1 attracts the DMNT1 DNA methyltransferase and enables epigenetic inheritance of DNA methylation status (Ishiyama et al. 2017, Nishiyama et al. 2013, Qin et al. 2015). Conversely, monoubiquitylation of K91 of histone H4 by the E3 BBAP is important for DNA damage signaling and metazoan development (Tessadori et al. 2017, Yan et al. 2009). Monoubiquitylation of H2A, H2B, H3, and H4 therefore documents the importance of where a ubiquitin moiety is attached: Even though

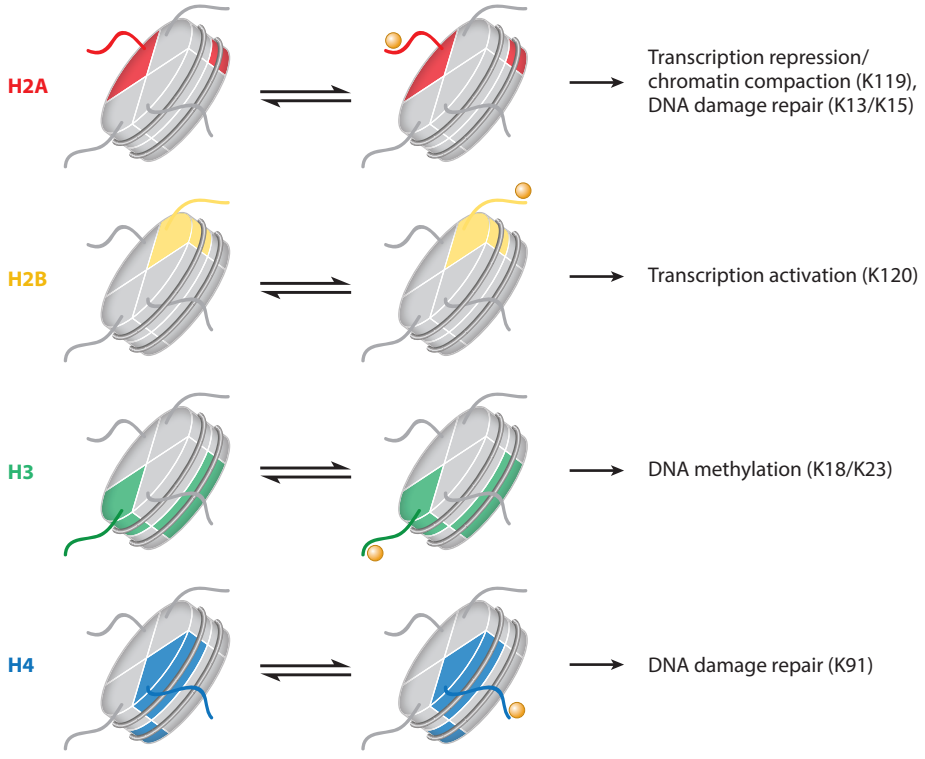

Figure 2

Dynamic regulation by site-specific and reversible monoubiquitylation. Each histone present in core nucleosomes can be ubiquitylated at specific Lys residues by E3 ligases and deubiquitylated by counteracting deubiquitylases. Depending on the attachment site of ubiquitin and the corresponding histone protein, ubiquitylation has different biological consequences, including gene silencing, transcription activation, and initiation of DNA repair.

these histone proteins are located in close proximity to each other, their site-specific ubiquitylation has divergent consequences in controlling gene expression, DNA methylation, or DNA repair.

In addition to shedding light onto specificity, histone modification also illustrates how reversibility shapes ubiquitin-dependent signaling. As ubiquitylated histones control gene expression, the swift removal of ubiquitin is often important for the rapid changes in transcriptional programs observed during early metazoan development. H2A can be deubiquitylated by MYSM1, BAP1, USP16, USP21, or USP48, whereas DUBs for H2B include USP7, USP27X, USP42, USP44, USP49, and USP51; moreover, USP3 and USP22 have been proposed to act on both ubiquitylated H2A and H2B (Adorno et al. 2013, Atanassov et al. 2016, Harrigan et al. 2018, Mevissen & Komander 2017, Scheuermann et al. 2010, Uckelmann et al. 2018). The large number of histone-directed DUBs likely reflects the need for fine-tuned transcription at distinct chromatin locations, at particular times during the cell cycle, or in certain cell types. To ensure that deubiquitylation occurs at the right time and place, most histone-directed DUBs require cofactors that establish substrate specificity or modulate catalytic activity (Mevissen & Komander 2017). For example, the adaptor proteins ATXN7L3 and ENY2 recruit three different DUBs—USP22, USP27X, and USP51—to monoubiquitylated H2B across all chromosomes (Atanassov et al. 2016). Conversely, association of USP44 with the NCoR repressor allows this DUB to specifically target

H2B at promoter sequences (Fuchs et al. 2012, Lan et al. 2016). If histone deubiquitylation is disturbed, an organism faces aberrant differentiation or disease. For example, overexpression of USP22, a subunit of the 1.8-MDa SAGA complex targeting H2A, is part of an 11-gene signature associated with metastasis and chemotherapy resistance (Glinsky 2006), whereas overexpression of the chromosome 21–encoded USP16 accounts for the self-renewal defects of hematopoietic stem cells that are observed in Down syndrome (Adorno et al. 2013).

Reversibility is a central theme of ubiquitin-dependent signaling. In most cases, we think of E3 ligases and DUBs as acting subsequent to each other to first turn on and then switch off a signaling event. However, these counteracting enzymes can produce more complex signaling outputs: By acting in direct opposition, DUBs can generate thresholds of E3 activities that need to be surpassed for downstream signaling, as seen during cell division or endoplasmic reticulum–associated degradation (ERAD) (Rape et al. 2006, Zhang et al. 2013). If E3 activation occurs at the same time as DUB inactivation, switch-like changes in a signaling output can be generated. This has been observed during mitophagy, when simultaneous activation of the E3 PARKIN and inhibition of the counteracting USP30 lead to rapid tagging of a defective mitochondrion with a dense ubiquitin signal (Cunningham et al. 2015, Gersch et al. 2017, Kane et al. 2014, Kazlauskaite et al. 2014, Koyano et al. 2014, Wauer et al. 2015a). Conversely, spatial separation of E3s and DUBs can restrict ubiquitylation to particular locales, as documented by the local ubiquitylation of growth factor receptors at the plasma membrane and their deubiquitylation on endosomes (Levkowitz et al. 1998, McCullough et al. 2004). More complex regulation emerges when DUBs act on E3s as well as E3 substrates: For example, USP7 deubiquitylates the E3 MDM2 and the MDM2 substrate p53 and thereby establishes context-specific p53 activation that can be exploited for therapeutic purposes (Kategaya et al. 2017, Turnbull et al. 2017). Underscoring the importance of reversibility for ubiquitin-dependent signaling, E3s and their opposing DUBs are often found in the same protein complex (Elliott et al. 2014, 2016; Sowa et al. 2009).

COREGULATION: SPECIFICITY VERSUS PLASTICITY OF UBIQUITIN-DEPENDENT SIGNALING

Following gene expression and mRNA maturation, protein synthesis relies on accurate mRNA translation on cytoplasmic ribosomes. While ubiquitylation is known to control ribosome function or specificity (Higgins et al. 2015, Werner et al. 2015), we focus our discussion on its role in monitoring the accuracy of protein synthesis through quality control (QC). Misfolded proteins that fail to attain their functional conformation need to be rapidly degraded to prevent them from interfering with cell function or survival. To accomplish this task, cells command proteasomes that eliminate soluble QC substrates decorated with K11-linked, K48-linked, or K11/K48-branched chains (Chau et al. 1989, Jin et al. 2008, Meyer & Rape 2014). Conversely, larger protein aggregates or dysfunctional organelles are degraded through the autophagy-lysosome pathway, which often relies on K6- or K63-linked conjugates (Ordureau et al. 2014, Wurzer et al. 2015). These degradation routes are tightly interconnected, as the load capacity of both proteasome- and autophagy-mediated degradation is controlled by the mTORC1 kinase (Pandey et al. 2007, Rousseau & Bertolotti 2016, Singh & Cuervo 2011, Zhang et al. 2014). Moreover, while proteasome inhibition upregulates autophagy, proteasomal degradation of cargo regulators helps terminate the autophagy process (Antonioli et al. 2014, Liu et al. 2016). The coordination of proteasome- and autophagy-dependent degradation likely reflects the importance of eliminating misfolded proteins before they can wreak havoc in the cell.

QC poses interesting regulatory challenges for the ubiquitylation machinery. As rampant degradation would deplete proteins required for cellular function and survival, it is critical that QC

E3 ligases retain specificity to eliminate only misfolded proteins while leaving their folded counterparts intact. At the same time, QC enzymes typically need to target a large number of similarly misfolded and potentially cytotoxic factors. Despite the delicate balancing act between specificity and plasticity, QC E3 ligases also have to be highly efficient in labeling their targets with ubiquitin, as slow degradation and the ensuing accumulation of aggregation-prone polypeptides are common features of aging and neurodegeneration. Highlighting a principle of ubiquitin-dependent signaling, the ability of QC E3 ligases to bridge specificity, plasticity, and efficiency allows these enzymes to coregulate large sets of related, in this case misfolded, proteins.

The E3 Listerin exemplifies how ubiquitylation enzymes can bridge specificity and plasticity by detecting aberrant cellular structures and targeting all proteins associated with these (**Figure 3*a***). Listerin specifically associates with 60S ribosomal subunits that are still engaged with nascent chains but have been released from 40S subunits by ribosomal splitting factors as a result of ribosomal stalling and defective protein synthesis (Bengtson & Joazeiro 2010, Brandman

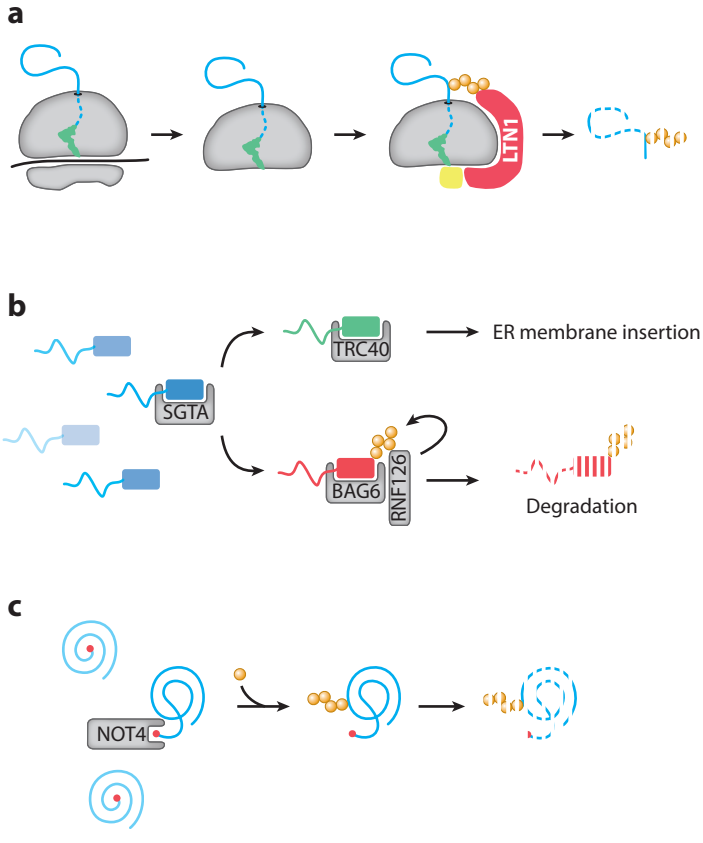

Figure 3

Ubiquitin-dependent coregulation of related proteins. (*a*) E3 ligases can target related substrates by binding to cellular structures or common proteins associated with these targets. This is illustrated by the E3 Listerin, which binds stalled ribosomes indicative of aberrant protein synthesis. (*b*) E3 ligases can also cooperate with chaperones that recognize related misfolded or mislocalized proteins and hand these proteins over to E3 ligases for ubiquitylation and degradation. (*c*) Recognition of related substrates can also occur through binding of E3 ligases to a degenerate degron that is present in related proteins, such as those that expose an acetylated amino terminus upon misfolding.

et al. 2012, Chu et al. 2009, Shao et al. 2013). Listerin interacts with 60S subunits by positioning its catalytic RING domain in the proximity of the ribosomal exit tunnel, thereby allowing the E3 ligase to transfer a ubiquitin chain to any emerging polypeptide (Lyumkis et al. 2014, Shao et al. 2015). To increase the probability that Listerin encounters a Lys residue in the substrate, cells command the RQC2 protein to extend the emerging nascent chain with carboxy-terminal Ala and Thr residues, referred to as CAT tails (Shen et al. 2015). This pushes the faulty polypeptide further out of the ribosomal channel and thus increases the likelihood that previously shielded Lys residues are exposed to Listerin's active site for ubiquitylation (Kostova et al. 2017).

This mode of substrate selection is conceptually similar to cases in which chaperones recognize groups of misfolded proteins and then hand their clients over to E3 ligases for ubiquitylation and degradation. This was first shown for the E3 CHIP, whose binding to the HSP70 and HSP90 chaperones allows it access to misfolded polypeptides (Connell et al. 2001). Similarly, transmembrane proteins that fail to be delivered to their proper destination are kept soluble by the BAG6 or Ubiquilin chaperones before being handed over to RNF126 for ubiquitylation (Hessa et al. 2011, Itakura et al. 2016, Rodrigo-Brenni et al. 2014, Shao et al. 2017) (**Figure 3*b***). Both BAG6 and Ubiquilin also participate in the recognition of nascent cytoplasmic polypeptides that fail to achieve their native conformation (Hjerpe et al. 2016, Yau et al. 2017), yet in this case, they cooperate with HSP70 and HSP90 to deliver substrates to the E3s UBR4 and UBR5 (Yau et al. 2017). The latter QC pathway is very important: ∼10–15% of all newly synthesized proteins are subject to immediate ubiquitin-proteasome-dependent degradation (Wang et al. 2013), and mutations in enzymes of K11/K48-specific QC, including UBR4 and UBR5, cause a broad range of neurodegenerative diseases (Yau et al. 2017).

As an intrinsic mode of substrate selection, several QC E3 ligases detect degenerate motifs, so-called degrons, that are presented by misfolded polypeptides yet are buried in proteins that have attained a functional conformation. Such degrons could be as small as an acetylated amino terminus that is commonly exposed only upon misfolding or failure to engage a critical partner (Mischerikow & Heck 2011, Shemorry et al. 2013) (**Figure 3*c***). Misfolding can also interfere with protein processing by cotranslational Met aminopeptidases, which leads to recognition of incompletely matured proteins by other QC E3s such as UBR1 and UBR2 (Heck et al. 2010, Kim et al. 2014). Similar principles of substrate selection account for recognition of truncated proteins that emerge due to a limitation in the availability of selenocysteine (Lin et al. 2015); misfolded nuclear proteins whose exposed core hydrophobic residues are recognized by a disordered domain in the yeast E3 SAN1 (Gardner et al. 2005, Geffen et al. 2016, Rosenbaum et al. 2011); or proteins that miss a constitutive binding partner and likely present unoccupied hydrophobic binding surfaces, as seen for orphan ribosomal proteins recognized by HUWE1 or UBE2O (Nguyen et al. 2017, Sung et al. 2016, Yanagitani et al. 2017).

Although ubiquitin chain formation can occur on a millisecond timescale and with high processivity (Pierce et al. 2009, Wickliffe et al. 2011), QC networks further improve efficiency of ubiquitin-dependent signaling by employing multiple E3 ligases. Such pathways include the UBR4/UBR5-dependent QC pathway, which targets newly synthesized proteins; the ubiquitin-fusion degradation pathway; and N-end-rule QC centered on UBR1 (Hwang et al. 2010, Liu et al. 2017, Yau et al. 2017). Cooperating E3 ligases can assemble branched ubiquitin conjugates that allow for higher affinity toward the p97/VCP segregase and provide stronger degradation signals than do homotypic polymers (Blythe et al. 2017, Bodnar & Rapoport 2017, Meyer & Rape 2014, Yau et al. 2017). The AAA-ATPase p97/VCP is able to unfold proteins (Bodnar & Rapoport 2017) and mobilize ubiquitylated targets out of stable complexes (Rape et al. 2001) and is thus well suited to degrade proteins en route to aggregation. By providing a ubiquitin signal to recruit a powerful

segregase, QC E3 ligases improve the efficiency of protein degradation to ensure cellular function and survival.

QC E3 ligases illustrate how balancing specificity and plasticity of substrate selection allows for coregulation of many proteins with shared, in this case biophysical, characteristics. To extend this concept, degenerate degrons also enable coregulation of functionally related proteins: The E3 $SCF^{\beta TrCP}$ targets many proteins involved in cell growth and division that contain a short phosphodegron (Skaar et al. 2013), whereas the E3 anaphase-promoting complex (APC/C) triggers the degradation of many cell cycle regulators with low-complexity degrons, referred to as D box, KEN box, or ABBA motif (Davey & Morgan 2016). Interestingly, the APC/C also produces K11/K48-branched chains (Meyer & Rape 2014, Yau et al. 2017), which likely allows it to drive the large changes in the eukaryotic proteome observed during mitotic exit.

LOCALIZED UBIQUITIN-DEPENDENT SIGNALING

Either during or after synthesis, proteins need to be delivered to their cellular sites of action. The role of ubiquitylation in controlling protein trafficking illustrates how this modification can be implemented with high spatial precision. By acting at defined locations, ubiquitylation can impose directionality onto signaling cascades but also ensure the function of organelles that in turn establish cellular architecture and homeostasis.

The role of ubiquitylation in protein transport was first recognized during inactivation of epidermal growth factor receptor (EGFR) that had engaged its ligand, EGF (Levkowitz et al. 1998, Yoon et al. 1995). Following ligand binding and receptor activation by phosphorylation, EGFR is modified on multiple Lys residues by E3s of the Cbl family and is thus directed toward internalization by endocytosis (Levkowitz et al. 1998, Yoon et al. 1995) (**Figure 4*a***). Cbl E3s also target other receptors, such as the Tyro3, Axl, and Mer receptors implicated in the natural killer cell response toward metastases (Paolino et al. 2014), and additional E3s, such as IDOL, CHIP, and Nedd4-family E3s, further broaden the substrate spectrum of ubiquitin-dependent endocytosis (Bachofner et al. 2017, Tawo et al. 2017, Zelcer et al. 2009). The recruitment of these enzymes to their plasma membrane–localized targets involves either E3 domains or adaptor proteins, which engage only the activated and phosphorylated membrane receptors (Lin et al. 2008, MacGurn et al. 2011). As discussed for chromatin-localized ubiquitylation, these membrane-localized ubiquitylation events are reversible, and up to 15 distinct DUBs modulate the efficiency of ubiquitin-dependent endocytosis of EGFR (Savio et al. 2016).

Internalized receptors are often recognized by the ESCRT-0 complex with its HRS and STAM subunits. Both HRS and STAM contain two ubiquitin-binding domains, which associate with multiple ubiquitin subunits attached to the cargo (Hirano et al. 2006, Mizuno et al. 2003). ESCRT-0 then hands the substrate over to the ESCRT-I complex, which in turn funnels the substrate to ESCRT-II and later to ESCRT-III for budding of cargo vesicles into the lumen of multivesicular bodies. The controlled handover of cargo is established by a combination of ubiquitin-binding domains present in all ESCRTs and binary interactions between the different complexes (Frankel & Audhya 2018). This localized ubiquitylation coupled to sequential ubiquitin-dependent interactions establishes directionality of signaling. Moreover, by remaining bound to the ubiquitylated protein, substrate handover shields cargo from deubiquitylation, which would impede its lysosomal degradation and instead allow for recycling to the plasma membrane (McCullough et al. 2004, Savio et al. 2016).

Localized ubiquitin-dependent signaling occurs at many cellular locations. Another well-documented example is ERAD, which eliminates defective membrane or luminal proteins of the ER. Substrates of ERAD are ubiquitylated by ER-localized E3s and are delivered to the

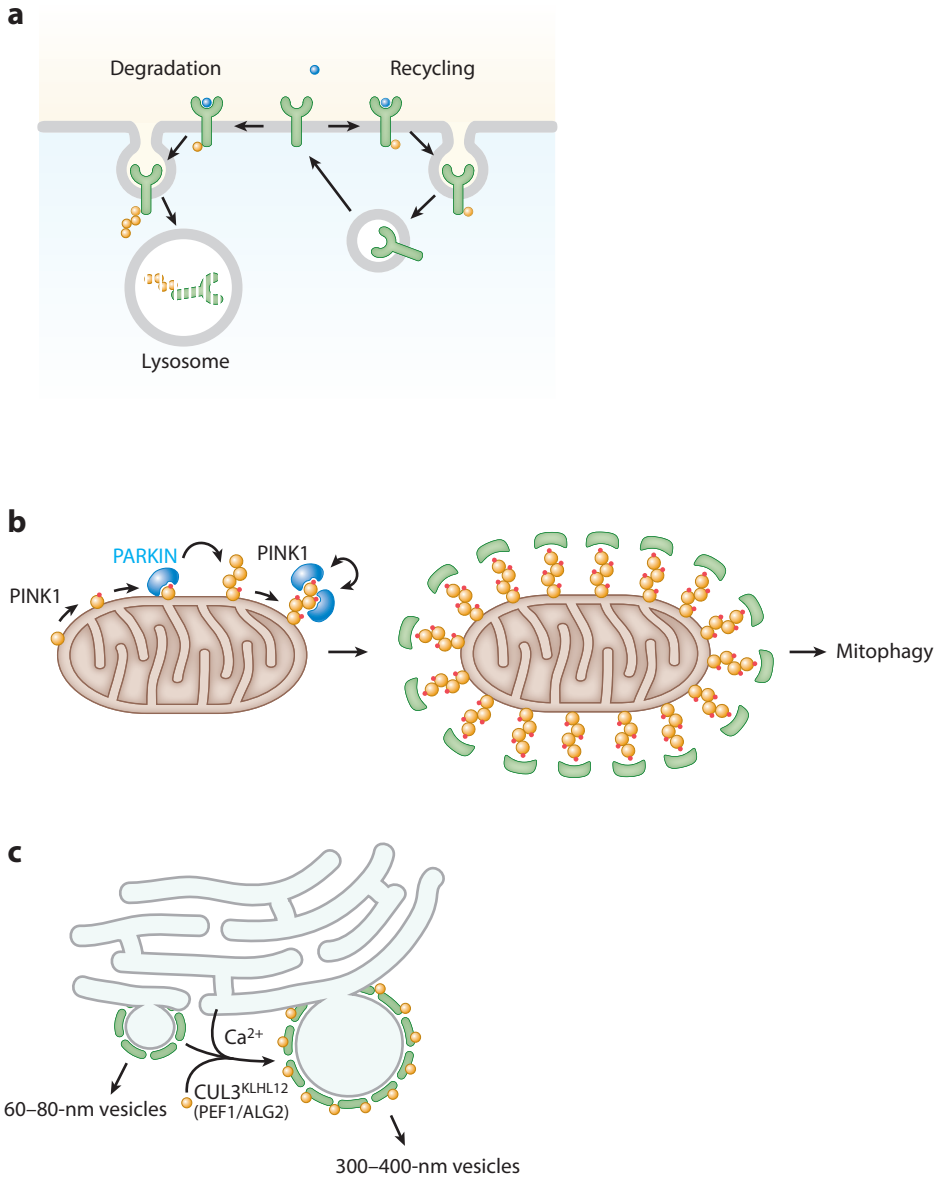

Figure 4

Spatial regulation by localized ubiquitylation. (*a*) Local separation of ubiquitylation at the plasma membrane and deubiquitylation at endosomes controls trafficking of internalized membrane receptors to the lysosome or their recycling to the plasma membrane. (*b*) Localized ubiquitylation allows for selective clearance of damaged mitochondria, while intact organelles are left intact. (*c*) Release of calcium from the ER allows for localized ubiquitylation of COPII vesicle coat proteins to regulate vesicle size.

proteasome by a combination of p97/VCP, ubiquitin-binding proteins, and chaperones (Berner et al. 2018). Membrane-localized E3 ligases are also encountered at the Golgi apparatus, mitochondria, or peroxisomes (Dobzinski et al. 2015, Narendra et al. 2008, Williams et al. 2008), and in several cases, the targeting of E3s requires specific cofactors that localize predominantly to particular organelles (Yang et al. 2018, Zhu et al. 2017).

A dramatic intersection of localized ubiquitylation and protein trafficking is seen during mitophagy, when cells eliminate defective mitochondria that would otherwise interfere with energy production or produce toxic reactive oxygen species (**Figure 4*b***). Mitophagy is initiated when the PINK1 kinase starts to accumulate on the outer membrane of a damaged mitochondrion (Narendra et al. 2010, Yamano & Youle 2013), where PINK1 phosphorylates both the ubiquitin-like domain of the E3 PARKIN and ubiquitin itself (Kane et al. 2014, Kazlauskaite et al. 2014, Koyano et al. 2014, Schubert et al. 2017). Binding of phosphorylated ubiquitin to PARKIN drastically increases the activity of this E3 (Wauer et al. 2015a,b), allowing PARKIN to decorate outer mitochondrial membrane proteins with ubiquitin conjugates containing K6 and K63 linkages (Ordureau et al. 2014, 2015; Sarraf et al. 2013). By labeling more mitochondrial proteins with ubiquitin, PARKIN creates more phosphorylation sites for PINK and thus triggers a signal amplification loop that selectively labels defective mitochondria with a dense ubiquitin mark (Harper et al. 2018). This localized signal is recognized by autophagy receptors that initiate lysosomal removal of the damaged mitochondrion, while functional organelles are left intact (Lazarou et al. 2015).

Localized ubiquitylation also directly controls the trafficking machinery, which has to be assembled at specific sites in the cell. Ubiquitylation appears to directly control secretory protein capture into vesicles at the level of the COPII coat protein, which is responsible for the transfer of membrane cargo from the ER to the Golgi apparatus (**Figure 4*c***). To accommodate large cargo, such as collagen and apolipoprotein particles, cells recruit the E3 $CUL3^{KLHL12}$ to the ER surface, where this E3 monoubiquitylates the COPII coat protein SEC31 and increases COPII vesicle size (Butkinaree et al. 2014, Gorur et al. 2017, Jin et al. 2012). This reaction is supported by a transcriptional circuit that increases KLHL12 levels in response to collagen II expression (Ishikawa et al. 2017). In addition, $CUL3^{KLHL12}$ is activated at the cytosolic face of the ER membrane, which is dependent upon two penta-EF-hand coadaptors that translate the release of calcium from the ER into local substrate binding by $CUL3^{KLHL12}$ (McGourty et al. 2016). Similar regulation of the trafficking machinery is encountered in endocytosis, during which monoubiquitylation of adaptor proteins at the plasma membrane connects cargo molecules with the internalization machinery (Polo et al. 2002, Weinberg & Drubin 2014, Woelk et al. 2006), and such regulation likely plays a role in the packaging of protein aggregates into unconventional vesicles at the surface of the ER membrane (Lee et al. 2016). Together, these examples document how localized ubiquitylation can be implemented to control eukaryotic signal transduction with high spatial precision.

UBIQUITIN-DEPENDENT CONTROL OF SIGNAL TRANSDUCTION

Following successful trafficking, proteins need to fulfill their assigned functions. Out of many possibilities, we focus our discussion on the ability of ubiquitylation to monitor essential signal transducers. Ubiquitylation, through its reversible nature, its rapid kinetics, and the versatility of outcomes encoded in distinct conjugates, helps stabilize signaling platforms, integrate signals into convergent outputs, and ensure proper signaling dynamics. We stress that these examples do not include all functions of ubiquitylation in controlling protein activity but rather were chosen to highlight recurrent principles of ubiquitin-dependent signaling that are important for metazoan development and disease.

Signal Amplification

The role of ubiquitylation in stabilizing signaling platforms is illustrated by cells that need to repair DNA double-strand breaks. Such DNA damage poses a severe threat to organismal survival, as it can lead to the gross chromosomal rearrangements that are frequently encountered during tumorigenesis. Robust signaling by one or few DNA breaks among the 3 Mb of human genomic information requires that cells rapidly detect the damage and then stabilize a signaling platform that orchestrates subsequent cell cycle arrest, transcription pausing, and DNA repair. Ubiquitylation performs an essential role in this process.

Early during the DNA damage response, the E3 RNF8 is recruited to sites of damage through its interaction with MDC1, a scaffold protein that is attracted to DNA breaks by binding to phosphorylated histone H2AX (Huen et al. 2007, Kolas et al. 2007, Mailand et al. 2007) (**Figure 5*a***). RNF8 decorates the linker histone H1 with K63-linked ubiquitin chains (Thorslund et al. 2015), which provides a recognition element for a second E3, RNF168 (Doil et al. 2009, Stewart et al. 2009). RNF168 then ubiquitylates K13 and K15 of histone H2A in a proximal nucleosome (Mattiroli et al. 2012). Notably, RNF168 itself binds monoubiquitylated $H2A^{K15}$, which enables this E3 to spread histone ubiquitylation signals at sites of DNA damage (Panier et al. 2012). Similar to phosphorylation of histone H2AX by the ATM kinase, ubiquitylation of H2A by RNF168 therefore triggers positive feedback control, which establishes a stable signaling platform of ubiquitylated and phosphorylated histones that extends to ~1 Mb beyond the site of damage.

As with most positive feedback loops, these reactions need to be under tight control: To prevent uncontrolled spreading of the ubiquitin signal at inappropriate cell cycle stages or wrong locations, cells install inhibitory phosphorylation marks on RNF8 or use the E3s TRIP12 and UBR5 to target RNF168 for degradation (Gudjonsson et al. 2012, Okamoto et al. 2013, Orthwein et al. 2014). Moreover, cells command several DUBs to cleave ubiquitin marks off H2A (Harrigan et al. 2018); among these DUBs, OTUB1 has a nonconventional role in limiting signaling by inhibiting E2 enzymes that assemble critical ubiquitylation tags at the site of DNA damage (Nakada et al. 2010).

Having established a robust signaling domain, ubiquitylation also helps cells negotiate which repair pathway is to be engaged. Metazoan cells use two major pathways to repair double-strand breaks: error-prone nonhomologous end joining (NHEJ) and error-free homologous recombination (HR). K63-linked ubiquitin chains, as attached to H1, recruit the E3 BRCA1 through its binding partner, RAP80 (Kim et al. 2007, Sobhian et al. 2007, Wang et al. 2007), which together inhibit the DNA end resection that is required for HR (Hu et al. 2011). At the same time, H2A monoubiquitylated on K15 is recognized by 53BP1 (Fradet-Turcotte et al. 2013, Wilson et al. 2016), which attracts further proteins that displace effectors of HR from sites of DNA damage (Boersma et al. 2015, Djuzenova et al. 2015, Xu et al. 2015). During G1, when no replicated sister chromatids are available, HR-dependent repair is additionally suppressed by $CUL3^{KEAP1}$-dependent ubiquitylation, which interferes with the formation of critical repair complexes (Orthwein et al. 2015). The positive feedback centered around RNF8 and RNF168 thus promotes DNA repair by NHEJ.

DNA repair by HR also relies on ubiquitylation but utilizes distinct E3 ligases: When BRCA1 and its partner, BARD1, are in complex with proteins other than RAP80, they promote HR through ubiquitylating substrates such as the resection factor CtIP (Zhao et al. 2014). The E3 RNF138 further stimulates DNA end resection by displacing an end-protection factor from sites of DNA damage (Ismail et al. 2015, Schmidt et al. 2015), and the E3s PRP19 and RFWD3 promote downstream signaling by ubiquitylating the RPA complex that binds to the single-stranded DNA produced by end resection (Elia et al. 2015, Marechal et al. 2014). While more needs to be learned

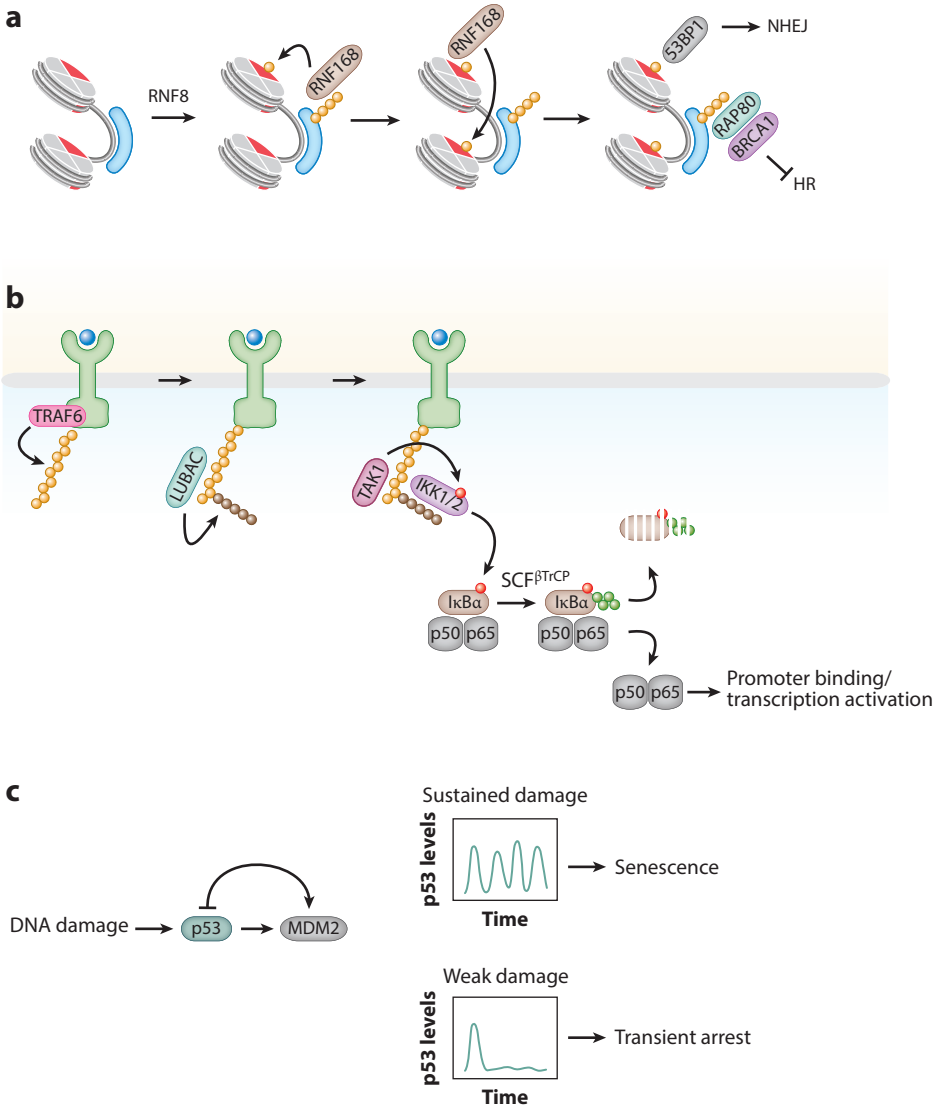

Figure 5

Temporal control of signaling by ubiquitylation. (*a*) Signal amplification by ubiquitylation. A positive feedback loop centered around RNF168-dependent histone ubiquitylation stabilizes a signaling platform for DNA damage repair by nonhomologous end joining (NHEJ) while inhibiting repair by homologous recombination (HR). (*b*) Combinatorial signaling by heterotypic conjugates. M1/K63-hybrid ubiquitin chains allow for efficient activation of the kinases TAK1 and IKK. IKK then catalyzes the phosphorylation and subsequent degradation of the IκBα inhibitor to allow for nuclear translocation and DNA binding of NF-κB. (*c*) Negative feedback control by ubiquitylation. Activation of the p53 transcription factor induces expression of the E3 MDM2, which in turn triggers p53 degradation. Recurrent pulsatile activation and degradation of p53 allow cells to decide whether to induce transient cell cycle arrest and senescence, respectively.

about substrates and effectors of DNA damage–induced ubiquitylation, these findings document how this modification can coordinate complex signaling networks by first establishing a stable signaling platform and later orchestrating downstream signaling events.

Combinatorial Signaling

As documented by the ability of phosphorylation, glycosylation, and SUMOylation to mark substrates for recognition by E3 ligases or ubiquitin-binding effectors, different types of posttranslational modifications affect each other to coordinate multiple signal transduction events (Kaelin 2007, Prudden et al. 2007, Skaar et al. 2013, Wild et al. 2011, Xie et al. 2007). In addition, heterotypic ubiquitin chains also enable another level of cross talk: As distinct topologies can have different outputs, heterotypic conjugates containing multiple linkages potentially allow for combinatorial control of signaling. The formation of hybrid or branched chains typically requires separate enzymatic activities for chain initiation and branching, which introduces the possibility that independent regulation of the cooperating enzymes could introduce new signaling modalities.

These principles are highlighted by the pathways that lead to activation of the NF-κB transcription factor, a process initiated by binding of cytokines, antibodies, pathogens, or similar ligands to cognate membrane receptors (**Figure 5*b***). Ligand engagement results in the formation of receptor complexes that contain the cIAP1/2, TRAF6, or Pellino E3 ligases, which in turn decorate receptor complex subunits with K63-linked chains (Hrdinka & Gyrd-Hansen 2017). The K63-linked conjugates recruit LUBAC, a trimeric E3 ligase that specifically produces M1-ubiquitin linkages (Tokunaga et al. 2009). While LUBAC was initially proposed to decorate Lys residues in receptor components with M1-linked chains, recent evidence suggests that its major substrates are the already attached K63-linked conjugates (Emmerich et al. 2013, 2016; Wertz et al. 2015). Capping of K63-linked conjugates with M1 linkages leads to the formation of M1/K63-hybrid ubiquitin chains that trigger downstream NF-κB activation.

What is the potential function of M1/K63-hybrid chains? K63-linked conjugates activate the TAB-TAK1 kinase, whose TAB2 or TAB3 subunits bind specifically to K63 linkages (Kanayama et al. 2004, Wang et al. 2001, Xu et al. 2009). Conversely, M1-linked conjugates recruit the IKK kinase through their specific recognition by the UBAN domain of the IKK subunit NEMO (Rahighi et al. 2009). K63/M1-hybrid chains could therefore bring TAK1 and IKK into proximity to increase the efficiency with which TAK1 activates IKK for downstream phosphorylation of the NF-κB inhibitor IκBα (Deng et al. 2000). In addition to a UBAN domain, NEMO also contains a carboxy-terminal zinc finger that interacts with K63-linked conjugates (Laplantine et al. 2009); K63/M1-linked chains may therefore provide a mode for bivalent, and thus more efficient, recruitment of IKK to activated receptor complexes. Finally, by capping K63 conjugates, M1-linked subunits could prevent deubiquitylation of receptor complexes by the DUB A20 and thus extend signal duration for robust NF-kB activation (Wertz et al. 2015). In either case, heterotypic chains would coordinate enzymatic activities to promote downstream signal transduction.

Signaling through M1/K63-hybrid chains is modulated by multiple DUBs that can disassemble such conjugates with high specificity. The OTU domain–containing OTULIN specifically cleaves M1 linkages (Keusekotten et al. 2013, Rivkin et al. 2013), and its mutation results in an inflammatory and autoimmunity syndrome caused by aberrant NF-κB activation (Damgaard et al. 2016). While OTULIN has not been found to associate with activated receptor complexes, it stably engages LUBAC and may therefore control NF-κB activation by restricting LUBAC autoubiquitylation (Elliott et al. 2014). Conversely, the tumor suppressor CYLD cleaves M1 linkages as well as K63 linkages (Komander et al. 2009). Although CYLD associates with LUBAC through an intermediary factor (Elliott et al. 2016, Kupka et al. 2016), it appears to limit neither

LUBAC autoubiquitylation nor the overall abundance of M1 linkages. Instead, CYLD is recruited to activated receptor complexes, where it has been suggested to edit ubiquitin conjugates to enable efficient formation of hybrid chains (Hrdinka & Gyrd-Hansen 2017). Finally, the tumor suppressor A20 can bind M1 linkages and K63 linkages through distinct zinc finger domains but disassembles only K63-linked, and not M1-linked, conjugates (Bosanac et al. 2010, Skaug et al. 2011, Wertz et al. 2015). This finding suggests that A20 opposes the most upstream signal that sets NF-κB activation in motion.

The complex regulation of the NF-κB pathway illustrates how heterotypic ubiquitin conjugates control the efficiency and timing of signal transduction. Other heterotypic chains modulate the strength of a ubiquitin tag, as documented by the aforementioned K11/K48-branched polymers that function as proteasomal priority signals during cell cycle and protein QC (Meyer & Rape 2014, Yau et al. 2017). Combining different linkages might also qualitatively alter the output of the ubiquitylation reaction: While K63-linked chains activate immune receptor signaling, they also provide seeds for branching off of proteolytic, and thus inhibitory, K48-linked conjugates (Ohtake et al. 2016, 2018). Although more work is needed, the emerging area of heterotypic chain formation and detection will likely be rich in new paradigms for ubiquitin-dependent signaling.

Signaling Dynamics

We discuss above the ability of ubiquitylation to trigger the degradation of misfolded and cytotoxic proteins, but proteolysis also plays a critical role in imposing signaling dynamics. Ubiquitin-dependent degradation, in its simplest iteration, limits the abundance of signal transducers, which allows cells to produce a significant change in output when required. Such regulation is often seen for transcription factors that are kept at low levels by continuous degradation but that rapidly rise in abundance when their ubiquitin-dependent degradation is inhibited due to a change in the cellular state. Underscoring the importance of these regulatory circuits, mutations in degron motifs that result in high basal levels of the transcription factors c-Myc, β-catenin, and Notch are tightly associated with tumorigenesis (Rape 2017, Welcker et al. 2004, Yada et al. 2004).

In addition, ubiquitin-dependent degradation is a recurrent component of negative feedback loops that limit the duration of signaling and thereby ensure that cells remain responsive to their environment. For example, the transcription factor HIF1α controls the expression of genes that allow an organism to improve oxygen delivery to tissues by inducing angiogenesis. Once proper oxygen supply of a tissue has been accomplished, oxidation of HIF1α at specific Pro residues leads to its recognition by the E3 $CUL2^{VHL}$ and to subsequent proteasomal degradation (Kaelin 2007). Negative feedback does not merely control transcription factors, as the kinase cyclin B1/CDK1 sows the seeds of its own destruction by activating the E3 APC/C (Qiao et al. 2016). Recurrent ubiquitin-dependent negative feedback can also generate signaling pulses that carry specific information. Under conditions of persistent DNA damage, feedback between p53 and its E3, MDM2, leads to recurrent accumulation and degradation of p53, with the number of pulses defining whether cells respond to the DNA damage by activating repair pathways or undergoing senescence (Purvis & Lahav 2013). Thus, ubiquitylation can encode information by establishing the proper dynamics of signal transduction cascades.

RESHAPING UBIQUITIN-DEPENDENT SIGNALING NETWORKS

As aberrant signaling is often linked to disease, loss-of-function or gain-of-function mutations in genes encoding ubiquitylation enzymes are at the heart of cancer and many developmental, autoimmune, and neurodegenerative diseases (Rape 2017). In addition, pathogenic bacteria or

viruses hijack ubiquitin-dependent signaling to ensure proliferation or to evade detection by the host immune system. Pathogen-dependent reshaping of ubiquitylation networks provides a final example to illustrate the complexity and versatility of ubiquitin-dependent signaling.

Although many bacteria are segregated in host cells in a vacuole-like compartment that helps these bacteria evade detection by the immune system, they need to gain access to the host cytoplasm and its nutrient-rich conditions to accelerate replication. As part of the host response to pathogen infection, multiple E3 ligases decorate such cytoplasmic bacteria with a ubiquitin coat that includes the M1- and K63-linked chains and initiates NF-κB-dependent production of inflammatory cytokines (Noad et al. 2017; Polajnar et al. 2017; van Wijk et al. 2012, 2017). In addition to this immune response, ubiquitylation induces pathogen removal by xenophagy (Wild et al. 2011). The latter process is conceptually similar to mitophagy, which potentially explains why PARKIN not only promotes the removal of defective mitochondria but also helps eliminate *Mycobacterium tuberculosis* (Manzanillo et al. 2013). The host response to pathogens illustrates how a ubiquitin signal emerging from a single location can coregulate multiple processes in a cell to fight bacterial infection.

As it is important for pathogens to counteract this response, bacteria have evolved mechanisms to subvert ubiquitin-dependent signaling in the host. To prevent generation of M1-linked signals, some bacterial E3 ligases of the IpaH family trigger the degradation of HOIP, the catalytic subunit of the M1-specific LUBAC complex (de Jong et al. 2016), while other members of the IpaH family promote the degradation of NEMO (Ashida et al. 2010). Production of K63 linkages is impaired by *Shigella* OspI, which deamidates a residue in the K63-specific E2 enzyme UBC13 and thereby prevents the interaction of UBC13 with TRAF6 (Sanada et al. 2012). Moreover, an effector of pathogenic *Escherichia coli* inhibits K63 linkage–dependent activation of TAK1 by methylation of a Cys residue in the ubiquitin-binding TAB2 and TAB3 subunits (Zhang et al. 2011).

Even though pathogens impede ubiquitylation reactions important for NF-κB activation, they still induce a striking ubiquitylation response in host cells (Fiskin et al. 2016). This response is in part due to pathogens having acquired enzymes that are capable of catalyzing ubiquitin transfer or removal (Keszei & Sicheri 2017, Pruneda et al. 2016). While some bacterial E3s or DUBs operate similarly to their eukaryotic counterparts, recent findings also revealed different catalytic mechanisms: The SdeA effector of *Legionella pneumophila* attaches ubiquitin to targets in a manner independent of the canonical cascade of E1, E2, and E3 enzymes (Bhogaraju et al. 2016, Qiu et al. 2016). Using mono-ADP-ribosyltransferase and nucleotidase-phosphohydrolase domains, SdeA first ADP-ribosylates ubiquitin and then transfers it to a Ser residue in substrates. This mode of ubiquitylation targets Rab-GTPases and the ER protein reticulon and may affect trafficking or degradation of the pathogen-containing vacuole (Bhogaraju et al. 2016, Kotewicz et al. 2017, Qiu et al. 2016). In addition, phosphoribosylation prevents loading of ubiquitin onto the active sites of canonical ubiquitylation enzymes, thus broadly interfering with the host networks of ubiquitin-dependent signaling (Bhogaraju et al. 2016). The surprising findings on SidE mechanism and function imply that additional principles of ubiquitin-dependent signaling await discovery.

OUTLOOK

The principles of ubiquitin-dependent signaling discussed here—i.e., reversibility, specificity, coregulation of related proteins, localized signaling, and the ability of ubiquitylation to establish congruent and dynamic information transfer—play essential roles in eukaryotic cell division, differentiation, and survival. The power of ubiquitylation to control such diverse and important processes stems from the different nature of ubiquitin signals; from the large number of distinct ubiquitin writers, readers, and erasers; and from the complex interplay of ubiquitylation with other

posttranslational modifications. Demonstrating the fast pace of the ubiquitin field, most findings discussed in this review were reported only in recent years. While this progress has allowed for an improved understanding of ubiquitin-dependent signaling, it has raised important questions that need to be addressed in future work.

Although much has been learned about functions of particular linkages, we still need to understand more about the biochemical information that is encoded in ubiquitin tags. While the roles of M1, K11, K48, and K63 linkages have been described extensively, and those of K6 and K29 linkages are beginning to emerge (Kristariyanto et al. 2015, Michel et al. 2017), little is known about ubiquitin connections through K27 or K33. This issue is compounded by recent observations that posttranslational modification of ubiquitin itself, as described in our above discussion on mitophagy, may further shape cellular functions of ubiquitylation. Moreover, we have just started to decipher the task of heterotypic ubiquitin chains: Their important roles during mitosis, QC, and NF-κB activation demand that we learn how to dissect the precise architecture of mixed or branched chains, including the nature, function, and sequence of distinct linkages within the conjugate. While there are 28 possible combinations for branched chains containing two linkage types, there is an even larger spectrum of topologies once more than two distinct linkages are incorporated. To which extent such chains encode unique functions is an important question to be addressed for a better understanding of the cellular language of ubiquitylation.

The ability of ubiquitylation to control cell behavior largely depends on the specificity and reversibility of this modification. Given what we have learned about kinases and phosphatases, a more in-depth understanding of E3 ligases and DUBs—their modes of substrate selection, kinetics, spatial and temporal regulation, and interplay—will likely reveal recurrent network motifs that drive eukaryotic signal transduction. As substrate recognition by E3 ligases often requires prior substrate modification by, among others, phosphorylation, oxidation, or SUMOylation, these studies will also provide insight into the cross talk of the ubiquitin system with other signaling networks. Much of this work is currently performed in cell culture, but given the important role of ubiquitin-dependent signaling in development and disease, it will be exciting to take these analyses into animal systems. An improved understanding of ubiquitin-dependent signaling will likely help uncover fundamental reactions that either drive metazoan development or are at the heart of disease.

Many reviews end by stating that an improved understanding of a given pathway will allow for development of new therapeutic strategies. In the case of ubiquitin-dependent signaling, this has become an exciting reality: Both proteasome inhibitors and small molecules that hijack E3 ligases to degrade disease-causing proteins have proven to be efficient in the treatment of various cancers. There is a strong push toward a rationale design of compounds that bring together an E3 ligase of choice—dependent on which linkage is needed—and a target whose ubiquitylation could provide therapeutic benefit. A deeper understanding of the principles of ubiquitylation will at one point allow us to rewire signaling networks more precisely to better treat a broader range of pathologies, including cancer, autism, craniofacial disorders, and neurodegeneration. We therefore expect that the basic science of deciphering a cellular language will pay off with improved therapeutic approaches that will benefit many people, bringing them together as envisioned by Arthur H. Compton.

DISCLOSURE STATEMENT

M.R. is cofounder of and consultant to Nurix, a biotech company operating in the ubiquitin space. Aside from this, the authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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