The increasing complexity of the ubiquitin code

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Ubiquitylation is essential for signal transduction as well as cell division and differentiation in all eukaryotes. Substrate modifications range from a single ubiquitin molecule to complex polymeric chains, with different types of ubiquitylation often eliciting distinct outcomes. The recent identification of novel chain topologies has improved our understanding of how ubiquitylation establishes precise communication within cells. Here, we discuss how the increasing complexity of ubiquitylation is employed to ensure robust and faithful signal transduction in eukaryotic cells.

Communication allows human societies to thrive. The basis for such interactions is our ability to articulate thoughts into words and sentences that can trigger particular responses from other individuals. Similarly, precise communication between and within cells is needed to support the development or homeostasis of a complex metazoan organism. The failure of cells to translate cues from their environment into proper action, be it division, differentiation or survival, can cause many diseases — a dramatic example is cancer resulting from unabated growth factor signalling.

Post-translational modification with the highly conserved 76-residue protein ubiquitin provides cells with an essential mechanism to establish precise communication. Ubiquitylation is brought about by a cascade of three enzymes : E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme, and E3 ubiquitin protein ligase[1–4]. In the simplest version of the process, monoubiquitylation, a single ubiquitin molecule is covalently attached to the ε-amino group of a lysine residue in a target protein. This reaction can also occur on multiple lysine residues of the substrate to yield a modification referred to as multi-monoubiquitylation (Fig. 1a). The very efficient monoubiquitylation of histone H2A, which is mediated by the Polycomb repressive complex and can account for up to 10% of the total histone H2A population, allowed the discovery of the first substrate of the ubiquitin pathway[5]. Adding single ubiquitin subunits typically alters intra- or intermolecular interactions that in turn affect localization, complex formation or activity of the modified protein[6] (Fig. 1b). For example, monoubiquitylation mediates an interaction of the DNA polymerase processivity factor PCNA with translesion synthesis DNA polymerases[7,8], whereas the same modification on the SMAD4 transcription factor prevents it from binding its signalling partner SMAD2 (ref. 9).

Ubiquitin contains seven lysine residues that, together with its amino terminus, provide eight attachment sites for further ubiquitin molecules, thereby allowing the formation of polymeric chains (Fig. 2a). In homotypic ubiquitin conjugates, all building blocks of the chain are connected through the same lysine or methionine residue (Fig. 2b,c). These conjugates adopt distinct but often very dynamic conformational ensembles that can be recognized by effector proteins with linkage-specific ubiquitin-binding domains (UBDs)[10,11]. Recognition by UBDs couples the modification of a substrate to a downstream event, such as protein turnover, recruitment to a specific location or assembly of a signalling complex[1]. Thus, similarly to words or sentences that elicit actions, ubiquitin chains can encode information that is deciphered by UBDs to trigger specific biological outcomes.

Providing the classic example for signalling through homotypic ubiquitin conjugates, K48-linked chains are able to target proteins to the 26S proteasome for degradation (Fig. 2b). K48 linkages are the most abundant connection between ubiquitin molecules in cells, and their levels rapidly increase following proteasome inhibition[12]. K48-linked chains were originally discovered to promote the proteasomal turnover of short-lived model substrates[13], and they are now known to be the product of several E3 ligases associated with degradation, including the cell-cycle-associated E3 ligase SCF (ref. 14), the ER-associated degradation (ERAD) E3 gp78 (ref. 15), or E6-AP, an E3 that targets the tumour suppressor p53 for degradation following viral infection[16]. Conversely, removal of K48-linked chains from substrates by deubiquitylases (DUBs) often prevents degradation[17,18]. Consistent with these observations in cells, K48-linked chains efficiently trigger proteasomal degradation in purified systems[19,20]. However, even for this paradigmatic ubiquitin chain type, the substrate can sometimes dictate an alternative fate: for example, attachment of K48-linked chains to the yeast M4 protein inhibits its function as a transcription factor, but does not deliver the protein for proteasomal degradation[21].

Following early work on K48-linked chains, mutation of an additional residue, K63, was found to impede formation of polymeric ubiquitin conjugates[22]. Rather than triggering degradation, K63-linked chains serve as molecular glue that allows for rapid and reversible formation of pivotal signalling complexes (Fig. 2c). K63 linkages are now known to regulate activation of the NF-κB transcription factor[23–25], DNA repair[7,26,27], innate immune responses[28], clearance of damaged

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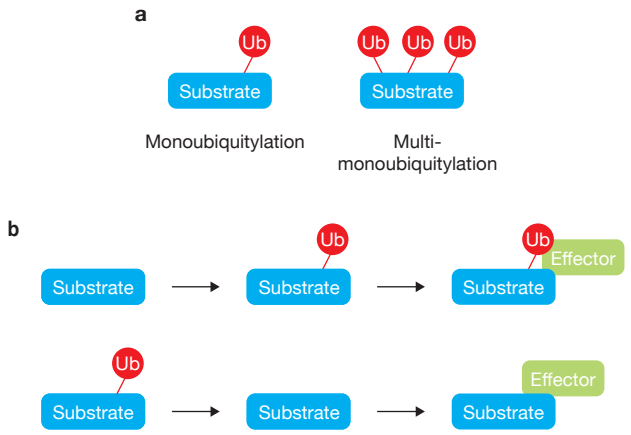

Figure 1 Signalling by monoubiquitylation. (**a**) A schematic representation of monoubiquitylation and multi-monoubiquitylation. (**b**) Monoubiquitylation can recruit effector proteins to elicit a biological outcome (top), as illustrated with PCNA[7,8]. Monoubiquitylation can also prevent protein interactions, requiring removal of the ubiquitin tag for signalling to occur (bottom). An example is provided by SMAD4, which depends on deubiquitylation to engage its signalling partner SMAD2[9].

mitochondria[29,30], and protein sorting[31,32] — and they can also guide assembly of large protein complexes that drive mRNA splicing[33] or translation[34,35]. Reminiscent of second messengers in signalling cascades, K63-linked chains are able to perform their functions even if they are not attached to substrate proteins[36,37]. In agreement with these observations, E2s, E3s and UBDs involved in signal transduction, rather than protein degradation, produce and read K63-linked conjugates[1,38,39], whereas K63-specific DUBs terminate signalling events brought about by this chain topology[40,41].

A series of proteomic and immunological approaches revealed that ubiquitin chains composed of linkages involving the remaining five lysine residues as well as the α-amino group of the first methionine (M1) of ubiquitin also exist in cells[12,42–45]. Depending on the cellular context, several of these 'atypical chains' are able to rival in abundance the prototypical K48 or K63 linkages[12,44,45]. Accordingly, enzymes that can produce or disassemble such ubiquitin chain topologies, frequently with astonishing specificity, have been discovered[17,29,46,47]. Recent progress in understanding how atypical chains are assembled and recognized has revealed a ubiquitin code that is much more complex than originally anticipated. We have learned new 'words' in the ubiquitin signalling 'vocabulary', how they can be combined and how ubiquitin signals can be modified to provoke particular responses in the cell. Rather than being a simple code that assigns one specific output to each signal, ubiquitylation has emerged as a cellular language in which information transfer depends both on the nature of the signal and on the context in which it is sent. In this Review, we highlight these developments that have refined our appreciation of ubiquitin-dependent signalling as an essential communication module in eukaryotic cells.

New words in the ubiquitin vocabulary

The first atypical conjugates with assigned roles in signalling were K11- and M1-linked ubiquitin chains (also referred to as linear chains). K11-linked chains are abundant in asynchronously growing yeast, whereas in human cells they are preferentially produced during mitosis and early G1[25,45,48]. Conversely, M1-linked conjugates are rapidly synthesized following activation of inflammatory signalling cascades[46,49–51]. M1 linkages are also observed on pathogenic bacteria that have escaped vacuolar compartments and face ubiquitin-dependent autophagy in the cytoplasm[52,53]. Both chain types are produced by linkage-specific enzymes: K11-linked chains depend on an essential E3, the anaphase-promoting complex (APC/C) and its specific chain-elongating E2, Ube2S[47,54–58], whereas M1-linked chains are the product of LUBAC, a heterotrimeric RBR-family E3 ligase that is composed of HOIP, HOIL-1L and Sharpin[46,49–51,59]. Each chain type can also be disassembled by OTU-family DUBs with strong linkage preference: the K11-specific Cezanne and the M1-specific OTULIN[17,60,61]. Interestingly, LUBAC interacts with its antagonist OTULIN, underscoring the dynamic nature and tight regulation of ubiquitin-dependent signaling[62–64].

Consistent with the production of K11-linked chains during cell division, these conjugates target cell cycle regulators for degradation[20,47,65,66] (Fig. 2b). K11 linkages are recognized by the proteasome[48,67], and they increase in abundance in response to proteasome inhibition[12,42]. In biophysical studies using proteasomes stripped of substrate delivery factors, single K11-linked chains trigger proteasome binding and substrate degradation almost as efficiently as K48-linked chains[20]. K11 linkages are also found in conjugates that mediate cell-cycle-independent functions, including regulation of the Hif1α transcription factor[68], ERAD in yeast[12], or the innate anti-viral immune response[69]. As K11-specific ubiquitin-binding domains have not yet been discovered, the molecular function of these chains in cell-cycle-independent reactions remains poorly understood.

M1-linked conjugates are recognized by linkage-specific UBDs, such as the UBAN domain[70,71], which couple modification of a substrate to integration into larger protein assemblies (Fig. 2c). Adding to a growing list of cellular functions, M1-linked chains play pivotal roles in immune signalling and NF-κB activation[49–51,72], regulation of interferon production[73], and the control of Wnt signalling during blood vessel formation[62]. It is interesting to note that M1- and K11-linked chains seem to encode information similar to canonical counterparts: whereas both M1- and K63-linked conjugates orchestrate the assembly of protein complexes, K11- and K48-linked chains can drive proteasomal degradation (Fig. 2b,c). How distinct chain topologies with similar functions are employed to fine-tune the consequences of ubiquitylation will be discussed below.

More recently, additional linkages have been assigned potential functions in ubiquitin-dependent signalling. K6 linkages were observed during removal of damaged mitochondria from cells[29], K27 linkages have been implicated in regulating DNA repair and autoimmunity[74,75], K29 linkages have been attributed roles in proteasomal degradation[76], and K33-linked chains were proposed to regulate trafficking through the trans-Golgi network[77]. In contrast to M1-, K11-, K48- and K63-linked chains, for which linkage-specific ubiquitylation enzymes are known, these alternative linkages seem to be assembled by enzymes with mixed specificity. K6 linkages mediate functions of the E3 ligase Parkin, but this E3 also connects ubiquitin chains through K11, K48 and K63[29,78,79]. The bacterial E3 NleL produces both K6 and K48 linkages[80]. UBR5 (a tumour suppressor E3 associated with the degradation of RORγt transcription factors[81], inhibition of telomeric RNF168 (ref. 82) and restriction to DNA damage signalling[83]) assembles K11 and K29 linkages, whereas a poorly studied E3, KIAA10, connects ubiquitin molecules through both K29 and K48[84]. Moreover,

the HECT-family E3 APEL1 produces K33 linkages as well as K11 and K48 linkages[85], and CUL3, which was proposed to produce K33-linked chains[77], also often monoubiquitylates substrates[86,87]. This broader specificity of E3s is reflected by their opposing DUBs, which target multiple atypical linkages with similar efficiency. TRABID, for example, preferentially cleaves both K29 and K33 linkages[85,88–90]; OTUD3, a DUB that has been implicated in stabilizing the tumour suppressor PTEN, takes on K6 and K11 linkages[17,91]; and OTUD2, which binds the ubiquitin-selective segregase p97 (also known as Cdc48 or VCP, valosin-containing protein) and probably acts in ERAD[92], targets K11, K27, K29 and K33 linkages[17]. These observations indicate that atypical linkages between ubiquitin molecules might frequently be incorporated into heterotypic (rather than homotypic) conjugates, a notion that was recently supported by experimental evidence: both K6 and K63 linkages contribute to Parkin-dependent removal of damaged mitochondria[29,30,93,94], and both K11 and K63 linkages are involved in the efficient endocytosis of MHC class I molecules[95]. Moreover, K29 linkages are incorporated into chains that also contain ubiquitin molecules connected through K48[90].

Combining signals with mixed and branched ubiquitin chains

In contrast to homotypic chains, where ubiquitin subunits are connected by a single linkage type, heterotypic conjugates contain multiple linkages and typically come in two types: mixed and branched. Mixed chains are composed of different linkages, but each ubiquitin is modified with only one other ubiquitin molecule (Fig. 3a). If, instead, a single ubiquitin subunit within a chain is modified with two or more ubiquitin molecules at a time, branched chain structures are generated (Fig. 3b). A cellular function for heterotypic chains was already suggested in studies of the yeast ubiquitin-fusion degradation (UFD) pathway, a system that has revealed many features of ubiquitin-dependent signalling[76]. In model substrates of the UFD pathway, a ubiquitin molecule fused to the amino terminus of various proteins serves as the site of chain formation by two enzymes, the HECT E3 UFD4 and the U-box-containing UFD2[76,96]. Whereas UFD4 preferentially assembles K29-linked chains[97], UFD2 connects ubiquitin molecules through K48[98]. Interestingly, mutation of either K29 or K48 in the fused ubiquitin of the model substrate interfered with ubiquitin chain formation and prevented proteasomal degradation, suggesting that this chain moiety is modified on both K29 and K48[96]. UFD2 can also cooperate with the K63-specific HECT E3 RSP5 to promote degradation of the yeast transcription factor SPT23[99], whereas UFD4 collaborates with the E3 UBR1 to decorate a DNA repair protein with conjugates that contain both K29 and K48 linkages[100]. These studies, therefore, suggest that UFD2 and UFD4 can assemble heterotypic conjugates, but the exact structure of these chains and the precise signalling function of each linkage remain poorly understood.

Reminiscent of the synthesis of heterotypic conjugates by cooperating E3 ligases, the APC/C uses different E2 enzymes to assemble ubiquitin chains that drive the proteasomal degradation of cell cycle regulators. In human cells, chain formation is mostly initiated by the E2 Ube2C (also known as UbcH10), which binds the APC/C through a winged-helix domain in APC2 and a canonical E2-binding surface on the RING-domain of APC11[101–103]. Chain elongation proceeds through a different E2, Ube2S[54–56], which uses a unique C-terminal peptide to bind the APC/C at the same time as Ube2C[104]. These APC/C-specific E2s have distinct linkage specificity: whereas Ube2C assembles mixed chains that

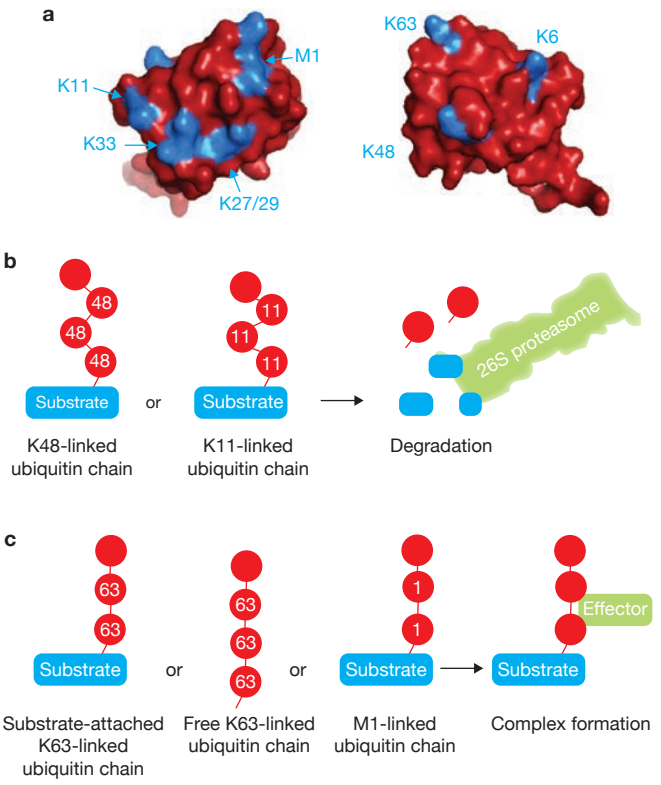

Figure 2 Signalling by homotypic ubiquitin chains. (**a**) The structure of ubiquitin, with the eight potential attachment sites for chain formation shown in blue. (**b**) Homotypic K11- and K48-linked chains can drive proteasomal degradation. (**c**) Homotypic K63- or M1-linked ubiquitin chains mediate complex assembly.

contain K11, K48 and K63 linkages, Ube2S only produces K11-linked chains[54,56,104]. As Ube2S can attach K11-linked chains to internal moieties of conjugates assembled by Ube2C[48], the combination of Ube2C and Ube2S activity leads to the formation of K11/K48-branched ubiquitin chains[48,105]. These conjugates efficiently promote substrate recognition by the proteasome and the p97 segregase, allowing them to drive degradation of proteins that are engaged in stable interactions[48]. Interestingly, p97 also recognizes K11 and K48 linkages for extraction of proteins from the endoplasmic reticulum and outer mitochondrial membranes[106,107], and heterotypic conjugates also seem to be involved in proteasomal processing of the Cubitus interruptus (Ci) transcriptional regulator[108]. Thus, whereas rampant formation of randomly branched ubiquitin chains can prevent proteasomal degradation of certain proteins[109], formation of K11/K48-branched conjugates seems to constitute a strong proteolytic signal.

Modification of substrates with heterotypic ubiquitin chains also plays important roles in non-proteolytic signalling. Binding of interleukin-1 (IL-1) to its cognate receptor triggers a signalling cascade dependent on TRAF6, an E3 ligase that assembles K63-linked chains[23,110], and the M1-linkage-specific E3 LUBAC[46]. Whereas K63-linked chains recruit the TAK1 kinase to ligand-engaged IL-1 receptor (IL-1R) complexes[24,25,36], M1-linked chains are recognized by NEMO, a subunit of the IKK kinase that is activated by TAK1[71]. As shown by digestion with linkage-specific DUBs — an approach referred to as UbiCrest[17] — the large majority of M1-linked chains produced after IL-1 stimulation were attached to

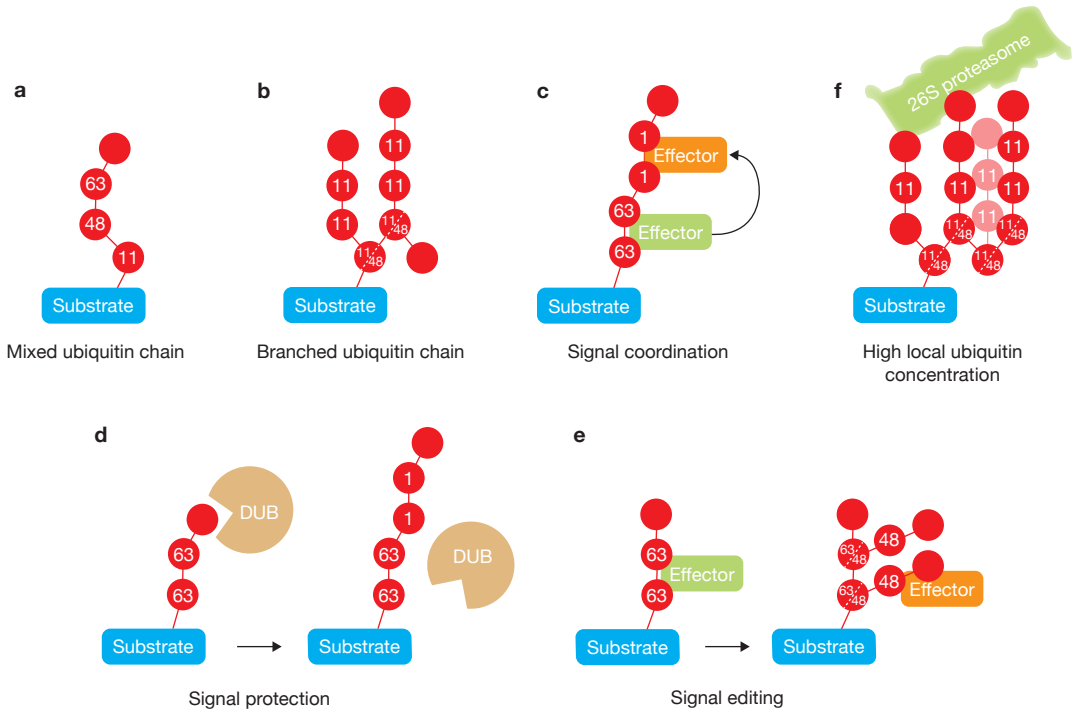

Figure 3 Signalling by heterotypic ubiquitin chains. (**a**) A schematic representation of mixed ubiquitin chains. The different linkages were chosen arbitrarily. (**b**) A schematic representation of K11/K48-branched ubiquitin chains. (**c**) Signal coordination as a potential function of mixed or branched ubiquitin chains, which could recruit multiple UBDs with distinct linkage specificity to bring components of signal transduction pathways into proximity. (**d**) Mixed or branched chains could be protected from disassembly by linkage-specific DUBs. (**e**) Branching distinct ubiquitin chains off an initial conjugate could alter signalling, for example if proteolytic K48-linked chains would be branched off non-proteolytic K63-linked conjugates. (**f**) Branching could provide a means to increase the local concentration of ubiquitin on substrates, a feature that should help recruit 26S proteasomes for degradation[20].

K63-linked conjugates[111]. By combining linkages that can be recognized by distinct UBDs, these M1/K63-linked chains might recruit both TAK1 and IKK to the same receptors (Fig. 3c), which could accelerate activation of IKK by TAK1[111]. Alternatively, decoration of K63-linked chains with M1-linked conjugates could prevent deubiquitylation by linkage-specific DUBs (Fig. 3d), thereby extending the time that the signalling pathway is active[112].

It should be noted that the concept of heterotypic conjugates is not limited to ubiquitin chains with multiple linkages, but could also include conjugates that are composed of distinct ubiquitin-like modifiers. The SUMO-targeted ubiquitin ligases (STUBLs) add ubiquitin chains to proteins that are already decorated with the ubiquitin-like SUMO[113–115]. At least *in vitro*, the STUBLs can directly ubiquitylate the previously attached SUMO tag[116]. Appending ubiquitin to SUMOylated proteins tremendously alters the properties of the modified substrate: whereas SUMO usually signals through mediating protein interactions, addition of a ubiquitin chain by STUBLs frequently triggers complex disassembly or proteasomal degradation. The change in fate brought about by decorating SUMOylated proteins with proteolytic ubiquitin chains is thus reminiscent to chain editing, in which a non-proteolytic ubiquitin chain type is replaced by a K48-linked chain[117]. STUBLs have now been implicated in several signalling pathways, including the cellular responses to arsenic poisoning and DNA damage, when SUMOylated proteins involved in handling chromatin need to be eliminated at various stages of the process[118–122].

Akin to sentences crafted from multiple words, the results described above have ascribed biological meaning to combinations of different ubiquitin chain topologies. As work in this area is still in its infancy, the mechanisms underlying assembly, recognition and function of heterotypic conjugates are not yet fully understood. Biophysical studies have implied that the distinct connections of a mixed or branched conjugate can signal independently of each other[123], and so branching K48-linked chains off a K63-linked conjugate might provide a means to terminate signalling events without the need to remove the activating signal (Fig. 3e). Moreover, branching allows E3 ligases to add multiple copies of a chain type to the same lysine residue in a substrate (Fig. 3f), and we hypothesize that the ensuing increase in the local ubiquitin concentration could explain the strong proteolytic signal derived from K11/K48-branched chains[20,48]. It is also possible that UBDs (or specific combinations of them) preferentially recognize branched conjugates, and that these conjugates might provoke unique reactions. So far, the analysis of heterotypic chains has been limited to indirect methods, such as digestion of conjugates in cell lysates with different combinations of linkage-specific DUBs or expression of ubiquitin variants that allowed for detection of branch signatures after treatment with site-specific proteases[17,48,90,111]. To understand the physiological relevance of the increasingly complex ubiquitin code, it will be important to develop new technologies that allow detection of mixed or branched ubiquitin chains in cells without expressing mutant forms of ubiquitin (Box 1).

Post-translational modification of ubiquitin

Further expanding the signalling repertoire of ubiquitylation, ubiquitin has recently been found to be subject to post-translational modifications, including phosphorylation (Fig. 4a). As seen in large-scale proteomic

studies, ubiquitin can be phosphorylated on most of its serine, threonine and tyrosine residues[124–126]. Under normal growth conditions, the overall concentration of phosphorylated ubiquitin seems to be low; in yeast cells, less than 0.5% of ubiquitin is phosphorylated at S65, and only 1% of the ubiquitin molecules in human cells carry a phosphate group at this site[78,127]. This situation can change drastically during signalling, and ubiquitin molecules phosphorylated at S65 can make up ~20% of the ubiquitin pool attached to damaged mitochondria that are destined for removal by mitophagy[78].

Our understanding of the consequences of ubiquitin phosphorylation for signal transduction greatly increased with the identification of the ubiquitin kinase encoded by the Parkinson's disease gene *PINK1*. In healthy cells, PINK1 is imported into mitochondria, a reaction that results in the proteolytic release and cytoplasmic degradation of its kinase domain[128]. If, however, a mitochondrion is damaged, import of PINK1 is prevented and the kinase accumulates on the outer membrane of the damaged organelle[129,130], where it phosphorylates S65 of ubiquitin as well as its counterpart in the ubiquitin-like domain of the RBR-family E3 ligase Parkin[78,79,131–134]. The phosphorylated ubiquitin attached to mitochondrial membrane proteins helps to recruit and activate Parkin, and allows it to ubiquitylate more proteins on the damaged organelle that in turn are substrates for PINK1. Phosphorylated ubiquitin also establishes a robust landing platform for the autophagy adaptors OPTN and NDP52[135,136], which, when phosphorylated by TBK1, seem to recognize ubiquitin chains of multiple topologies[135–137]. Thus, phosphorylation of ubiquitin sets in motion intertwined feedback loops that robustly decorate damaged mitochondria with ubiquitin chains to elicit their selective removal from cells.

Phosphorylation of ubiquitin can affect its structure as well as its interactions with conjugating enzymes, DUBs or ubiquitin-binding domains[127,134,135,138]. As illustrated with Parkin, phosphorylation of ubiquitin can be a double-edged sword: whereas phospho-ubiquitin is specifically recognized by an allosteric site in Parkin to evoke striking structural rearrangements and catalytic activation of the E3 (Fig. 4b)[138], the catalytic domains of Parkin use phospho-ubiquitin less efficiently than unmodified ubiquitin and are unable to build ubiquitin chains that are composed entirely of phospho-ubiquitin[29,134]. Phospho-ubiquitin also impairs recognition by USP8 and USP30, two DUBs that have been implicated in preventing mitophagy[30,94,134], suggesting a mechanism that maintains a signal that triggers removal of the damaged organelle (Fig. 4c).

How other post-translational modifications of ubiquitin impinge on cellular signalling events is less understood. Ubiquitin can be phosphorylated at other residues, with S57 being the most prominently modified site[124,126], yet the responsible kinases remain unknown. Both yeast cells that naturally lack a PINK1 homologue and human cells that were engineered to live without PINK1 contain ubiquitin phosphorylated at S65[78,127], suggesting that additional kinases can target this residue. Ubiquitin can also be acetylated at K6 and K48, which *in vitro* can affect the build-up of K11-, K48- or K63-linked chains[139]. However, less than 0.03% of ubiquitin molecules are modified in this way, and the responsible acetylation enzyme is not known. Finally, effector proteins of pathogenic bacteria can convert Gln40 of ubiquitin into a glutamate residue, which can profoundly inhibit ubiquitin chain synthesis[140]. Whether similar modifications can occur on ubiquitin conjugates and alter recognition by cellular UBDs is not yet known.

BOX 1 New technologies to decipher a complex ubiquitin code

Understanding the connectivity between substrate-linked ubiquitin molecules in cells is critical to our ability to decipher the ubiquitin code. Current strategies that avoid expression of mutant ubiquitin variants are based on mass spectrometry[12,78,124], linkage-specific antibodies that can detect M1, K11, K48 or K63 linkages[44,45,149], and ubiquitin sensors that couple a linkage-specific ubiquitin-binding domain to a fluorescent reporter[52]. These approaches are able to recognize particular linkages within a substrate-attached conjugate or at a particular cellular location, yet they cannot report on the presence of multiple linkages within the same chain or on the same ubiquitin molecule. Providing a step towards sequencing ubiquitin chains, combinatorial treatment of ubiquitin conjugates with linkage-specific DUBs — a powerful approach referred to as UbiCrest[17] — allows for analysis of more complex chain topologies. To fully understand the wide range of different heterotypic ubiquitin chains, researchers will need to develop additional technologies. Such strategies could include: UbiCrest approaches that employ DUBs with specificity for branched conjugates or even proteases with different target sites on ubiquitin; linkage-specific antibodies that either recognize branched structures or act as coincidence detectors for the presence of multiple linkages within one conjugate; or middle-down mass spectrometry approaches that avoid complete digestion of ubiquitin to retain information about multiple attachment sites on a single chain building block[150]. When complemented with synthetic biology approaches to re-engineer the linkage specificity of ubiquitin ligases in cells[48], such a toolkit would put researchers in a position to unravel the remaining mysteries of ubiquitin-dependent signal transfer.

Dense information in a ubiquitin cloud?

The attachment of branched chains to cell cycle regulators or conjugation of heterotypic chains to outer mitochondrial membrane proteins results in a high local concentration of ubiquitin on the modified substrate or organelle. In the case of SUMOylation, clustered modifications on multiple subunits of protein complexes have been described as a SUMO 'cloud' that provides a high-avidity signal for SUMO-binding proteins[141]. Is it possible that some ubiquitin signals similarly depend on the local concentration, rather than the connectivity of ubiquitin molecules?

In a surprising twist to the story of ubiquitin-dependent signalling, it was the prototypical function of linkage-specific ubiquitylation, proteasomal degradation, that provided evidence for a role of the local ubiquitin concentration in signal transduction. Ground-breaking work had established that 3–4 molecules linked through K48 of ubiquitin drive efficient recognition by the 26S proteasome[13,19], yet it has become difficult to rationalize this observation with recent structures of the 26S proteasome bound to a substrate[142]. The large distance between ubiquitin receptors and the dynamic interactions of substrate delivery factors with the proteasome raised the possibility that multiple chain topologies could mediate degradation. Indeed, alternative chain types have been shown to mediate proteasomal turnover, including K11-linked, K63-linked and heterotypic conjugates[47,143,144]. In some cases, short polypeptides or proteins with stretches of low structural complexity

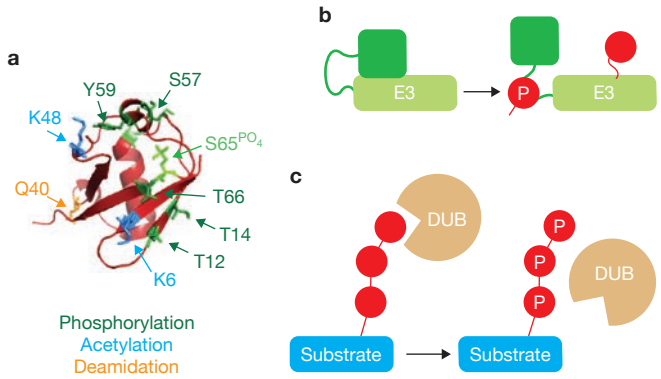

Figure 4 Signalling by modified ubiquitin. (**a**) Sites for ubiquitin phosphorylation, acetylation and deamidation that were detected by mass spectrometry are mapped onto the structure of ubiquitin phosphorylated at S65[134]. (**b**) Specific recognition of phosphorylated ubiquitin. As illustrated by the E3 ligase Parkin, binding of phospho-ubiquitin to an allosteric site can induce conformational changes that ultimately activate the E3 ligase[138]. (**c**) Inhibition of ubiquitin recognition by phosphorylation can prevent DUBs from terminating signal transduction[134].

can be delivered to the proteasome by monoubiquitylation or multi-monoubiquitylation[145,146].

In line with these observations, single-molecule studies recently showed that multiple K48-linked di-ubiquitin conjugates provide a stronger degradation signal than the canonical tetra-ubiquitin chain[20]. Initially, two di-ubiquitin entities cooperatively engage the proteasomal ubiquitin receptors Rpn10 and Rpn13, before addition of more di-ubiquitin signals accelerates turnover by increasing the local concentration of ubiquitin on the substrate. These findings are consistent with the notion that a large number of ubiquitylated proteins contain more than one attachment site for ubiquitin[42,43,124], and that increasing the number of lysine residues available for ubiquitylation can accelerate the turnover of model substrates[147]. The proteasome itself seems to regulate the local ubiquitin concentration, as the proteasome-associated E3 HUL5 adds additional K63-linked chains to proteins that are already bound to the degradative machine[148]. Thus, reminiscent of a ubiquitin cloud, the local concentration of substrate-attached ubiquitin molecules, rather than the linkage between them, might be an important determinant of proteasomal delivery.

Conclusion

Many historic examples illustrate the importance of precise communication, but few phrase it as pointedly as a line from a poem by Henry Wadsworth Longfellow about the ride of Paul Revere at the beginning of the American Revolutionary War: "One if by land, and two if by sea." The number of lanterns hung from the tower of the Old North Church in Boston's North End informed Revere about the British approach on Boston and allowed him to mount an effective response. Although we are beginning to realize that ubiquitylation establishes a much more complex code than originally thought, the principles of information transfer are the same: distinct types of modification can trigger specific cellular responses that are at the heart of essential reactions, such as cell division, differentiation and survival. By deepening our understanding of the ubiquitin code in all its complexity — the temporal and structural dynamics, the messages derived from mixed or branched chains, and the modification of its central player, ubiquitin — we stand to learn much about the fundamental organization of a cell. Given the central role of ubiquitylation in cellular communication pathways relevant for human development and disease, such knowledge should ultimately result in innovative and effective therapeutic approaches.

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COMPETING FINANCIAL INTERESTS

M.R. is co-founder and consultant to Nurix, a biotechnology company that operates in the ubiquitin space.

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