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Review

Non-canonical ubiquitylation: Mechanisms and consequences

Gary S. McDowell[a,b,c], Anna Philpott[a,*]

[a] Department of Oncology, University of Cambridge, Hutchison/Medical Research Council (MRC) Research Centre, Cambridge CB2 0XZ, UK
[b] Proteomics Centre, Department of Pathology, Boston Children's Hospital, Boston, MA 02115, USA
[c] Department of Pathology, Harvard Medical School, Boston, MA 02115, USA

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ABSTRACT

Post-translational protein modifications initiate, regulate, propagate and terminate a wide variety of processes in cells, and in particular, ubiquitylation targets substrate proteins for degradation, subcellular translocation, cell signaling and multiple other cellular events. Modification of substrate proteins is widely observed to occur via covalent linkages of ubiquitin to the amine groups of lysine side-chains. However, in recent years several new modes of ubiquitin chain attachment have emerged. For instance, covalent modification of non-lysine sites in substrate proteins is theoretically possible according to basic chemical principles underlying the ubiquitylation process, and evidence is building that sites such as the N-terminal amine group of a protein, the hydroxyl group of serine and threonine residues and even the thiol groups of cysteine residues are all employed as sites of ubiquitylation. However, the potential importance of this "non-canonical ubiquitylation" of substrate proteins on sites other than lysine residues has been largely overlooked. This review aims to highlight the unusual features of the process of non-canonical ubiquitylation and the consequences of these events on the activity and fate of a protein.

Contents

1. Mechanisms of ubiquitylation

Ubiquitylation, the specific addition of ubiquitin groups to targeted proteins, regulates processes as diverse as proteasomal and lysosomal degradation, subcellular localization and DNA damage repair (Kerscher et al., 2006). Of these, our fullest understanding comes from many years of biochemical investigation of the degradation of ubiquitylated proteins by the proteasome (reviewed in (Hershko and Ciechanover, 1998; Varshavsky, 1997b), see Fig. 1).

The process of ubiquitylation begins with the adenylation of the C-terminus of ubiquitin (Ub), an 8.5 kDa, 76-residue protein, by the Ub-activating (E1) enzyme using energy from the hydrolysis of ATP (Hershko et al., 1981). This modification activates the

Abbreviations: Ub, ubiquitin; SCF, Skp-Cullin-F-box; HECT, homologous to E6 carboxyl terminus; RING, really Interesting New Gene; SUMO, small ubiquitin-like modifier; UPS, ubiquitin proteasome system; UFD, ubiquitin fusion degradation; ERAD, endoplasmic reticulum-associated degradation; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital.

* Corresponding author. Tel.: +44 1223 762675; fax: +44 1223 336902.
E-mail address: ap113@cam.ac.uk (A. Philpott).

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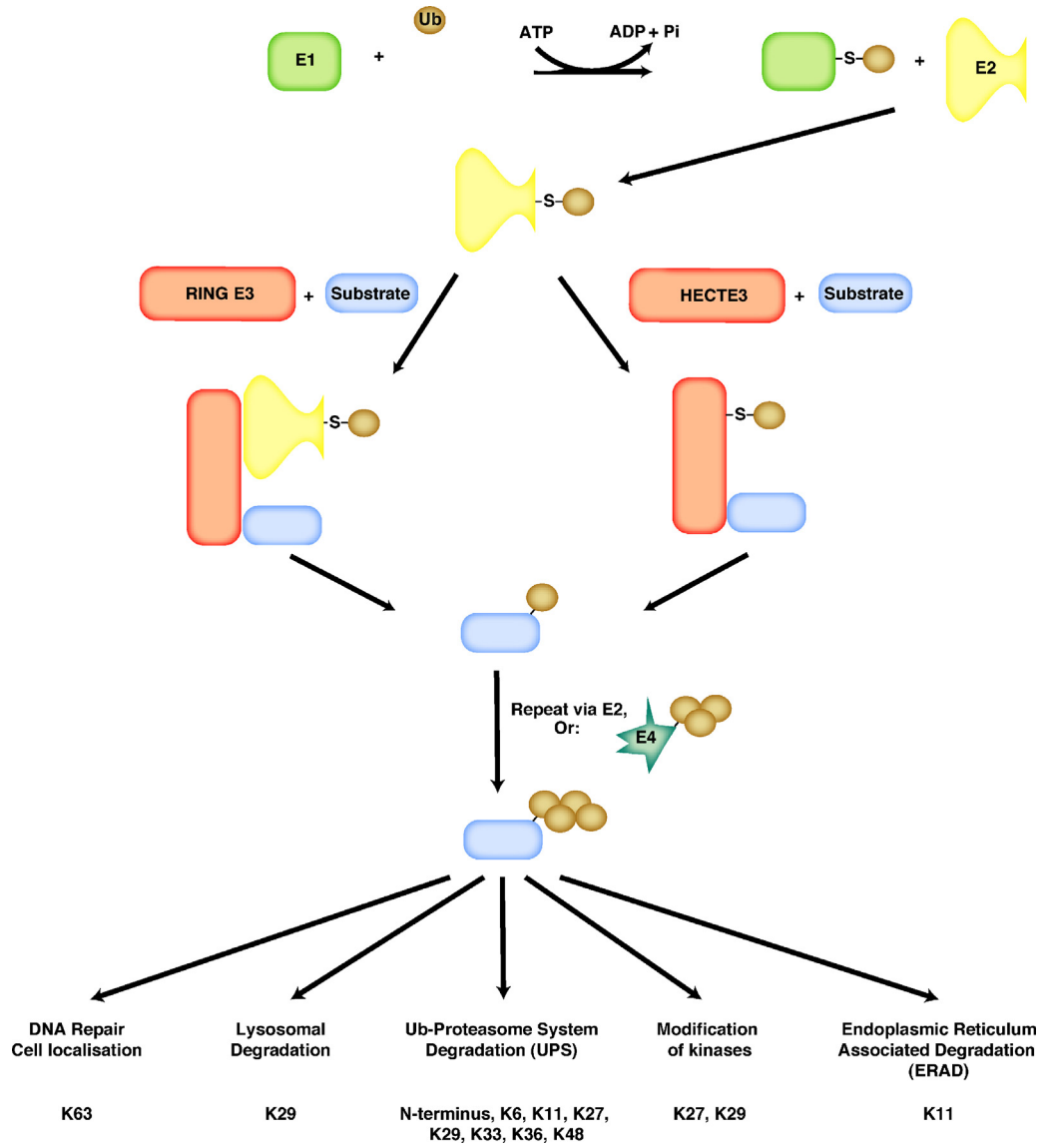

Fig. 1. The ubiquitylation cascade. Ub is activated by an Ub-activating (E1) enzyme using energy from ATP hydrolysis and passed to an Ub-conjugating (E2) enzyme. Ub can then be passed to a substrate protein, specified by the distinct E3 ligase that binds both the substrate and the E2. Many E3 ligases, such as the Really Interesting New Gene (RING) E3 ligases, act as a scaffold to pass Ub from the E2 directly to the substrate protein. In contrast, HECT (homologous to E6-AP carboxy-terminus) E3 ligases form a covalent bond with Ub themselves. The Ub moiety, covalently bound to the substrate protein, is then itself ubiquitylated by either successive rounds of ubiquitylation or by addition of a pre-assembled polyUb chain by the action of an E4 ligase. Once ubiquitylated to the appropriate extent, the substrate is targeted for a variety of cellular processes. Ubs can be cleaved from the protein at all stages by the action of de-ubiquitylating enzymes (DUBs).

Ub moiety energetically throughout the ubiquitylation cascade for eventual transfer to the substrate protein. Ub is then covalently fused via a thioester linkage to a cysteine residue in the E1 by attack of the cysteine at the C-terminus of ubiquitin, releasing AMP (Ciechanover et al., 1981). The E1 cannot just take Ub straight to the substrate (though such a concept is possible, such as in non-ribosomal polypeptide synthesis (Cane and Walsh, 1999)). Instead, Ub is shuttled from the E1 onto a cysteine residue of an Ub-conjugating (E2) enzyme (Hershko et al., 1983), of which there are at least 50 in the mammalian genome (Wu et al., 2003). This E2 can then pass on this Ub moiety to protein targets in concert with an Ub (E3) ligase.

Ubiquitylation may involve the E2 passing the Ub directly onto the substrate protein using the E3 ligase as a scaffold, such as with the Skp-Cullin-F-box (SCF) class of E3 ligase (Jackson et al., 2000). Alternatively, the E2 passes the ubiquitin to a cysteine residue of a homologous to the E6-AP carboxyl terminus (HECT) E3 ligase, which then passes the ubiquitin to its protein target (Huibregtse et al., 1995). In either case, the Ub is "canonically" passed onto a lysine residue of the substrate protein via an isopeptide bond. The cycle can then be repeated to add further Ub moieties to the Ub already attached to the substrate. In some cases, E4 ligases can add a polyUb chain to a monoubiquitylated site: for instance p300 can carry out this function on a site monoubiquitylated by Mdm2 on p53 (Grossman et al., 2003). It is, however, the E3 ligase that confers specificity for the final ubiquitylation of the substrate protein (Hershko, 1988) and this class of enzymes show the greatest diversity within the ubiquitylation machinery, numbering several hundred in mammals (Huibregtse et al., 1995; Jackson et al., 2000; King et al., 1995; Sudakin et al., 1995).

The particular site of ubiquitin chain linkage between Ub molecules can be determined either by the E2 alone (David et al., 2010) or in some cases more specifically by the E3 ligase (David et al., 2011). Various linkages of polyUb chains may confer distinct properties. For example, K48-linked chains target proteins for proteasomal degradation (Thrower et al., 2000) as can K11-linked

chains (Matsumoto et al., 2010; Song and Rape, 2011), while in fact experimentally, it has been shown that all lysines except K63 are able to target proteins for proteasomal degradation (Xu et al., 2009). Several other Ub chain linkages have been described, and these can have a variety of other biological functions such as regulating subcellular localization (Pickart and Fushman, 2004), as described in Figure 1. Indeed, it is clear that many other combinations of homologous, heterologous and branched ubiquitin chains are possible and the functions of these are yet to be elucidated (Komander, 2009).

2. Canonical ubiquitylation

Canonical ubiquitylation sites on target proteins are described as lysine residues in the definition of the ubiquitin proteasome system (UPS) described above (Freiman and Tjian, 2003). However, unlike many post-translational modifications, and even unlike Ub-like modifiers such as small Ub-like modifier (SUMO), there is no known consensus site for identifying an ubiquitylation site (Peng et al., 2003), although there are examples of protein ubiquitylation occurring on specified lysines, such as lysines 21 and 22 in IκBα (Baldi et al., 1996). Conversely there are proteins where ubiquitylation can occur on any lysine, for example c-Jun (Treier et al., 1994) and cyclin B (King et al., 1996). Therefore determination of which lysine residues in a protein are ubiquitylated must be undertaken on a case-by-case basis.

If the canonical description of ubiquitylation is correct (Freiman and Tjian, 2003), removal of all lysine residues in a protein should completely stabilize against Ub-mediated proteasomal degradation. Indeed there are examples of proteins that appear completely stabilized by removal of lysines, most commonly by mutation of lysines to arginines or by chemical lysine modification (Hershko et al., 1986). However many investigators observed that proteins lacking lysines were still being polyubiquitylated and that these polyUb chains could still target the substrate protein for proteasomal degradation. Where could this ubiquitylation be occurring?

3. Ubiquitin isopeptide linkages to lysines and peptide linkages to the N-terminus

Lysine residues form a covalent linkage to Ub by formation of an isopeptide bond to the carboxy terminus of Ub using the lysine amine group (see Fig. 2A). Lysine-less proteins are missing these side-chains but all proteins contain another amine group; polypeptides consist of a backbone created by peptide bond formation between amino acid residues, so in a linear chain there is always a free amine group (at the N-terminus) and a free carboxylate group

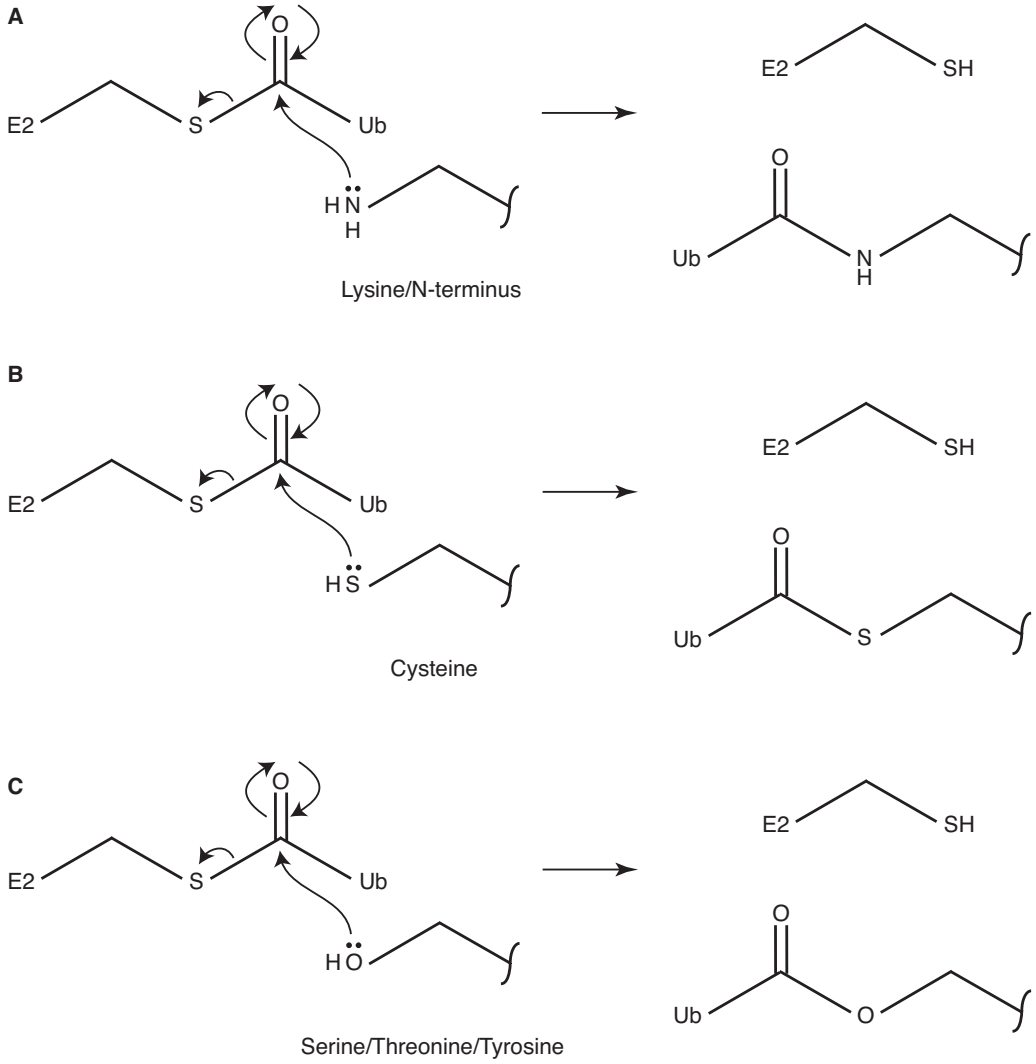

Fig. 2. Nucleophilic sites of ubiquitylation. Proteins contain many nucleophilic sites capable of attacking an E2-Ub thioester linkage and undergoing ubiquitylation. The best-described sites are (A) the amine-containing internal lysine residues and the free amine of the N-terminus of the polypeptide backbone. However (B) cysteine thiols and (C) hydroxyls on serines, threonines and tyrosines could also potentially be ubiquitylated by an identical mechanism.

(at the C-terminus). Thus, the N-terminus presents a reactive amine group that could be fused to Ub in the same way as lysine. Such N-terminal ubiquitylation was first identified in MyoD (Breitschopf et al., 1998). Preventing ubiquitylation of all amine groups by reductive dimethylation, and of the N-terminal amine group of MyoD specifically by carbamylation (Hershko et al., 1984) not only stabilized the protein, but also demonstrated that MyoD lysines are not ubiquitylated when the N-terminal amine group is blocked. Hence, N-terminal ubiquitylation may be required as a 'priming' ubiquitylation (Breitschopf et al., 1998).

It is important to note that ubiquitylation of the N-terminal amine group itself is distinct from ubiquitylation by the 'N-end rule' (Wang et al., 2008, reviewed in Varshavsky, 2011). A destabilizing N-terminal residue in a protein can act as a degron to facilitate ubiquitylation and proteasomal degradation. However the Ub moiety in N-end rule-targeted ubiquitylation is fused to an internal lysine residue and not to the N-terminal amine group. Therefore the N-terminus acts as a signal but not as an acceptor for ubiquitylation.

To check that N-terminal amine ubiquitylation was truly responsible for destabilizing MyoD (rather than representing targeting via the N-end rule), a number of other possible processes were also investigated. Conversion of the N-terminal residue of MyoD into a lysine residue (as occurs with conversion of some acidic residues to arginine to target for ubiquitylation via the N-end rule (Ferber and Ciechanover, 1987)) did not occur and so does not account for the destabilizing effect of the N-terminal residue. Moreover, the effects of the N-end rule on non-canonical ubiquitylation of MyoD were dismissed as N-end rule inhibitors had no effect on the stability of MyoD (Breitschopf et al., 1998). Fusion of a Myc-tag to the N-terminus to block the ubiquitylation of the N-terminal amine of MyoD did stabilize the protein (Breitschopf et al., 1998) (although we would note that a C-terminal fusion control was not attempted, see below). Overall the evidence clearly indicates that N-terminal amine ubiquitylation, and not the N-end rule, targets MyoD for proteasomal degradation.

N-terminal ubiquitylation is apparent in a number of proteins (Breitschopf et al., 1998; Chen et al., 2004; Ciechanover and Ben-Saadon, 2004; Kuo et al., 2004; Mayer, 2005; Sadeh et al., 2008); for instance recent evidence has shown a role in cell cycle-regulating proteins such as cyclin G1 (Li et al., 2009). It has also been demonstrated that there are naturally occurring lysineless proteins such as p16INK4a and HPV-58 E7 where this non-canonical N-terminal ubiquitylation must be essential for UPS-mediated degradation (Ben-Saadon et al., 2004). In addition, the Ub fusion degradation (UFD) pathway can target proteins for degradation where Ub is fused linearly via its C-terminus to the N-terminus of a protein (Johnson et al., 1995).

The most direct verification of N-terminal ubiquitylation would be by mass spectrometry (MS) and this technique has been used to demonstrate N-terminal ubiquitylation of ERK3 and p21 (Coulombe et al., 2004). However, mass spectrometry may not always be suitable for such analysis if the N-terminus does not yield a suitably-sized peptide, or if the peptide is too unstable to reach tandem MS analysis. In lieu of MS evidence, biochemical analyses are required.

Deletion of a portion of the N-terminus, for example the first 20 residues, could demonstrate the importance of the N-terminus in targeting the substrate for polyubiquitylation. However this may not allow distinction of N-terminal ubiquitylation from ubiquitylation targeted by the N-end Rule (Varshavsky, 1997a); both may require a particular sequence at the N-terminus to target for ubiquitylation but the modification will occur at different sites in the two processes. Moreover, an obvious caveat to this approach is that there is still a free amine group at the truncated N-terminus. Therefore preventing ubiquitylation of the N-terminus of a protein more directly is necessary to unequivocally demonstrate native N-terminal ubiquitylation.

A common strategy is to add a bulky tag to the N-terminus of the protein (Trausch-Azar et al., 2010) under the rationale that such a group will physically block the normal N-terminal site from ubiquitylation. In many cases this does seem to result in protein stabilization, but there are many factors to consider when designing such an approach. Firstly, there is some debate as to what defines a "bulky" tag (Trausch-Azar et al., 2010). Secondly, once a tag is established as suitable and stabilizes the protein when fused to the N-terminus, it is important to determine that fusion of the same tag to the C-terminus does not also bring about protein stabilization; this control is often not included but can be very informative (Vosper et al., 2009). Moreover, in the case of intrinsically disordered proteins it may well be the case that a tag, particularly a folded tag such as GFP, may stabilize the protein through blockage of unfolding initiation sites also required for proteasomal degradation (Prakash et al., 2004). This was the case with the proneural protein Ngn2 (Vosper et al., 2009), where N-terminal fusion of GFP stabilizes Ngn2, but so does the C-terminal fusion.

A third approach for identifying N-terminal ubiquitylation could be by using chemical methods to "block" the N-terminus i.e. directly preventing its ubiquitylation. Methods to prevent ubiquitylation include reductive methylation with sodium cyanoborohydride, guanidination with O-methylisourea or carbamylation (Breitschopf et al., 1998). Due to similarity of the lysine and N-terminal amine groups, reductive methylation will also result in blocking of all lysine residue side-chains as well as the N-terminus. However guanidination is specific to lysine residues whilst carbamylation is specific to the N-terminal amine group only.

There is also a fourth method to specifically block the N-terminus alone. Particular amino acid residues at the N-terminus are associated with up- or down-regulation of co-translational N-terminal acetylation (Bradshaw et al., 1998; Polevoda and Sherman, 2000, 2003a,b). It has been suggested that N-terminal co-translational acetylation occurs in 75% of eukaryotic proteins (Polevoda and Sherman, 2003b) although an investigation of N-terminal processing disputes this and places the figure at 30% (Meinnel et al., 2005). Notwithstanding, the process of N-terminal acetylation is well characterized, including identification of N-terminal consensus sites for the regulation of acetylation (Bradshaw et al., 1998; Polevoda and Sherman, 2002, 2003a,b; Utsumi et al., 2001), and studies of protein degradation in N-terminally acetylated proteins have been undertaken (Polevoda and Sherman, 2002). Importantly, N-terminal co-translational acetylation has been shown to be incompatible with N-terminal ubiquitylation (Caron et al., 2005). The specificity of the effect of N-terminal acetylation on preventing ubiquitylation, and hence UPS-mediated degradation, can be verified by blocking acetylation using the Palmiter method (Palmiter, 1977). Using this method Vosper and colleagues found that Ngn2 is targeted for degradation by N-terminal ubiquitylation (Vosper et al., 2009).

4. Ubiquitylation on additional non-canonical sites

As an additional site for ubiquitylation to the established lysines, the N-terminal amine group still resembles them chemically, but it is possible to generalize the mechanism of ubiquitylation further. Lysines are able to form isopeptide bonds due to the presence of a nitrogen atom that can attack the thioester linkage between ubiquitin and the ubiquitin-conjugating (E2) enzyme. In this manner the nitrogen is acting as a nucleophile, attracted to electropositive centers by virtue of its lone pair of electrons, and this can attack the thioester linkage at the electron-deficient carbonyl carbon, which acts therefore as an electrophile. In principle, the same mechanism

might be employed but using other nucleophiles aside from nitrogen. There are several amino acid residues with the potential to attack electron-deficient carbonyl carbons as nucleophiles; serines, threonines and tyrosines all contain electron-rich oxygen atoms capable of forming hydroxyester bonds, while cysteine residues have the potential to act as nucleophiles using lone pairs of electrons on sulfur (illustrated in Fig. 2). Two key questions must be asked with respect to these types of non-canonical ubiquitylation: does it occur; and is it physiologically relevant?

5. Thioester linkage of ubiquitin to cysteine

That cysteine residues might undergo modification by ubiquitylation may seem at first counterintuitive as thioester linkages (in a manner similar to disulphide bonds) can be broken under reducing conditions and the intracellular environment is often viewed as "reducing". However, as described above and in Fig. 1, at each stage of the ubiquitylation process, before loading ubiquitin onto the substrate the E1/2/3 enzymes are all able to carry ubiquitin via a thioester linkage, which is used to allow energetically favorable attack of the substrate nucleophile. The stability of these E1/2/3 intermediates was recently discussed (Song et al., 2009). The reactivity of E2/3 thiol residues and thioester bonds is affected by the E2 and E3 enzymes, mainly by non-covalent protein–protein interactions. In fact, the Ub–cysteine interaction is so stable, it has been suggested that thioesterases may be required to control any unwanted cysteine ubiquitylation on E2 and E3 enzymes (Song et al., 2009). Indeed, exposed cysteine residues on the surface of proteins have the closest pKa to physiological pH of any amino acid residue and so very small changes in pH can control their nucleophilicity greatly (Marino and Gladyshev, 2011), making them potential sites for ubiquitylation of multiple proteins.

A role for cysteine ubiquitylation was first identified in the peroxisomal import factor Pxp5 where this modification plays a signaling role (Carvalho et al., 2007; Grou et al., 2008; Kragt et al., 2005; Leon and Subramani, 2007; Williams et al., 2007). Lysineless Pxp5 was still ubiquitylated whilst ubiquitylation of the N-terminal amine group was prevented by co-translational acetylation as shown by mass spectrometry (Williams et al., 2007). Ubiquitylation on cysteines was postulated and such a possibility was investigated by comparing SDS-PAGE in reducing and non-reducing conditions (Carvalho et al., 2007) (as the thioester linkage is broken by the presence of reducing agents). This approach did indeed demonstrate that reducing agent-dependent ubiquitylation of Pxp5 occurred on a conserved cysteine residue (Williams et al., 2007). The related protein Pex20p also undergoes cysteine ubiquitylation to recycle the protein to the cytosol from the peroxisome (Leon and Subramani, 2007). In all other confirmed cases of non-canonical ubiquitylation, the modified protein is targeted for degradation, (see Table 1). Overall, many more non-canonical ubiquitylation events and their functions will have to be elucidated before we can conclude that ubiquitylation predominantly controls protein stability or whether it has a wider role in cellular signaling.

This cysteine monoubiquitylation on Pxp5 could be considered a transient modification in which a weak thioester linkage might provide for a more dynamic system. Indeed, it might seem unlikely that thioester linkages could be maintained long enough to allow development of a polyUb chain (canonically, a K48-linked ubiquitin tetramer or longer (Thrower et al., 2000)) targeting for degradation. However, the first evidence for poly-ubiquitylation via thioester linkages to cysteines came from viral E3 ligase systems (Cadwell and Coscoy, 2005, 2008). A cysteine residue was identified as a potential acceptor of Ub in one form of major histocompatibility complex (MHC) I whereas a form of the protein with a serine at the same position was not ubiquitylated (Cadwell and Coscoy, 2005). Introduction of cysteines to additional positions could also target for ubiquitylation leading to lysozomal degradation (Cadwell and Coscoy, 2008). The authors speculated that E3 ligases, such as the modulators of immune recognition (MIR) E3 ligases which ubiquitylate MHC I, may broaden their range of substrates through the ability to ubiquitylate on non-canonical sites (Cadwell and Coscoy, 2005).

Cysteine ubiquitylation has thus been demonstrated as capable of targeting cellular proteins for proteasomal-mediated degradation in vertebrate systems. However, such observations are hampered by the fact that cysteine ubiquitylation is more difficult to identify than ubiquitylation of amine groups chiefly, due to the relative instability of the thioester bond particularly in

Table 1
Non-canonically ubiquitylated proteins. NT: N-terminus; C: cysteine; S: serine; T: threonine.

Protein	Function	Non-canonical site	Notable features	References
MyoD	Transcription factor	NT	N-terminus necessary and sufficient for ubiquitylation targeting for degradation	Breitschopf, Bengal (1998)
Erk3	Cell cycle – kinase	NT	Direct evidence from mass spectrometry	Coulombe, Rodier (2004)
p21	Cell cycle – cyclin dependent kinase inhibitor	NT		Bloom et al. (2003), Coulombe, Rodier (2004)
HPV16 E7	Viral protein	NT		Reinstein et al. (2000)
EBV LMP1	Viral protein	NT		Aviel et al. (2000)
EBV LMP2A	Viral protein	NT		Ikeda et al. (2002)
Id2	Transcriptional regulation	NT		Fajerman et al. (2004)
ARF	p53 antagonist – tumor suppressor	NT	No lysines in human $p14^{ARF}$; only one lysine in mouse $p19^{ARF}$ with no effect on ubiquitylation	Kuo, den Besten (2004)
Pex5p	Protein translocation	C	Monoubiquitylation required for translocation	Carvalho, Pinto (2007)
Pex20p	Protein targeting and translocation	C	For recycling of protein to cytosol, cysteine ubiquitylation required	Leon and Subramani (2007)
TCRα	Immunity	S	ERAD	Ishikura, Weissman (2010)
NS-1	Immunoglobulin	S, T	pH dependence of ubiquitylation for ERAD	Shimizu, Okuda-Shimizu (2010)
BST-2/tetherin	Antiviral restriction factor	C, S, T	HIV Vpu2 protein stimulates non-canonical ubiquitylation for ERAD	Tokarev, Munguia (2011)
CD4	Immunity	C, S, T	HIV Vpu2 protein stimulates non-canonical ubiquitylation for ERAD	Magadan, Perez-Victoria (2010)
Bid	Apoptosis	C, S, T	Proteasomally degraded	Tait, de Vries (2007)
MHCI	Immunity	C, S, T	Recognized by E3 ligases from Kaposi's Sarcoma-associated Herpes virus for lysosomal degradation	Cadwell and Coscoy (2005, 2008), Wang, Herr (2007)
Ngn2	Transcription factor	NT, C, S, T	Vertebrate protein targeted for proteasomal degradation	McDowell, Kucerova (2010), Vosper et al. (2007), Vosper, McDowell (2009)

mass spectrometry analysis (Tait et al., 2007) or under the reducing conditions of SDS-PAGE. Instead, it is important to adopt a biochemical approach that takes into account the possibility of these more labile ubiquitylation events. For instance, biochemical analysis has been undertaken to investigate the degradation of the proneural transcription factor Ngn2 using *Xenopus* frog egg extract, which contains all the components required for rapid UPS-mediated degradation of this protein (Vosper et al., 2009). Wild-type Ngn2 is ubiquitylated on cysteines as well as lysines and the N-terminus. While cysteine ubiquitylation alone is sufficient to target for UPS-mediated degradation, mutation of cysteines to alanines in an otherwise wild-type protein has no effect on stability of Ngn2 in interphase egg extract. However, mutation of cysteines alone conferred as much stabilization as mutation of all lysines to arginines in mitotic extract, indicating a cell cycle-regulated role for non-canonical ubiquitylation in this case. The half-life of Ngn2 is significantly shorter in mitosis than in interphase, and cysteine ubiquitylation may contribute to this enhanced rate of turnover (Vosper et al., 2009). Similar ubiquitylation of Ngn2 via thioester linkages to cysteines were also seen in the *Mus musculus* P19 embryonal carcinoma cell line, which responds to Ngn2 over-expression by undergoing neuronal differentiation, indicating that this mode of ubiquitylation is conserved between vertebrate species (McDowell et al., 2010). Moreover, the related proneural protein Ngn3 can also be ubiquitylated on cysteines (Roark et al., 2012).

6. Hydroxyester linkages to serines, threonines, tyrosines

Ubiquitins could also be attached to serines, threonines and tyrosines via hydroxyester linkages which are more thermodynamically stable than thioester bonds but form less easily, and so are conversely less kinetically stable (see Section 8). Evidence for Ub–hydroxyester linkages is suggested for the protein Bid, produced as part of the apoptotic pathway (Tait et al., 2007). However, in this study structural properties such as folding stability (as distinct from non-canonical ubiquitylation) could not be ruled out as targeting Bid for degradation, due to extensive mutation of the protein.

The strongest evidence for Ub–hydroxyester linkages comes from research into the endoplasmic reticulum-associated degradation (ERAD) pathway, where serines and threonines are sufficient for ubiquitylation of major histocompatibility complex I heavy chain by the viral E3 ligase mK3 targeting for ERAD (Wang et al., 2007). In this case lysines are also present in the cytoplasmic tail of the heavy chain but mutation of all lysines and cysteines to arginines and alanines has no effect on stability; however mutation of serines and threonines to alanines is stabilizing. Moreover, an absolute requirement for serine residues in targeting a protein for ERAD was demonstrated in TCRα. The cytoplasmic tail of TCRα consists of the residues RLWSS and is not only ubiquitylated and degraded but replacement of serines with alanines reduces this ubiquitylation, while mutation of serines to cysteines, threonines or lysines maintains ubiquitylation and degradation (Ishikura et al., 2010). In this case, the exact position of the serines within the tail is not as important as the nature of the surrounding residues, where less hydrophobic residues enhance ubiquitylation on serine.

Non-canonical ubiquitylation for ERAD is upregulated in many viral infection settings. The HIV protein Vpu interacts with the $SCF^{\beta\text{-TRCP}}$ E3 ligase to promote ubiquitylation of BST-2 on cysteines, serines and threonines (Tokarev et al., 2011). BST-2 acts as a regulator of immunity and so targeting it for rapid degradation is advantageous to the survival of the HIV virus. Vpu has a similar role in targeting CD4 for ERAD (Magadan et al., 2010). High pH-dependent ubiquitylation was also observed on the immunoglobulin protein NS-1 but mutation of serines and threonines did not ablate ubiquitylation, which still occurred on lysine residues (Shimizu et al., 2010). These examples again illustrate the wide range of viral non-canonical ubiquitylation.

In targeting for proteasomal degradation, serines and threonines are also implicated in the degradation of the proneural protein Ngn2 (Vosper et al., 2009). A form of the protein where the N-terminal amine group is blocked by acetylation, and all lysines and cysteines are mutated to non-functional replacements, is still ubiquitylated in *Xenopus* egg extract and is still stabilized in the presence of proteasome inhibitors. It is, perhaps, interesting to compare Ngn2 to the related protein, Ngn3, a transcription factor also implicated in the regulation of cell cycle exit (Johansson et al., 2007) but instead best known for its role in directing endocrine cell fate in the gut and developing pancreas (Bertrand et al., 2002; Gu et al., 2002; Jenny et al., 2002; Schwitzgebel et al., 2000). Biochemical approaches indicate that, similar to Ngn2, Ngn3 undergoes non-canonical ubiquitylation. However, mutation of cysteines to alanines has no effect on the stability of Ngn3 even in a mitotic setting (Roark et al., 2012) whereas Ngn2 is significantly stabilized by mutation of its cysteines to alanines at least in mitosis (McDowell et al., 2010). In fact there is little evidence that cysteines residues are ubiquitylated in Ngn3 but instead the evidence points to a role for serines and threonines, which are clearly ubiquitylated and can direct destabilization of Ngn3 when all lysine residues are mutated to arginines (Roark et al., 2012). The differences exposed by comparison of these closely related proteins highlight the importance of investigating ubiquitylation and degradation on a protein by protein basis rather than relying on extrapolation between family members.

Tyrosines may offer a final potential site of non-canonical ubiquitylation. However, whilst containing a similar nucleophilic hydroxyl group to serines and threonines, tyrosines consist of a phenolic group, which may be less reactive than the aliphatic serine and threonine residues. The presence of an aromatic ring in this residue may result in delocalization of electron density around the aromatic system giving a less reactive lone pair of electrons on the hydroxyl group. As yet, no direct evidence for tyrosines as non-canonical sites of ubiquitylation has been demonstrated.

7. Deubiquitylation

Deubiquitylating enzymes (DUBs) catalyze the removal of ubiquitin moieties from proteins, nd use nucleophilic cysteine residues to attack the carbonyl group of the protein–ubiquitin linkage to liberate the substrate protein, leaving the ubiquitin moiety attached to the DUB (Amerik and Hochstrasser, 2004). Deubiquitylation can be used to cancel ubiquitylation signals, as a quality control for regulating ubiquitylation, and to remove ubiquitin chains from substrate proteins prior to proteasomal degradation. At present there is no evidence for a role of DUBs in deubiquitylation of non-canonical residues. DUBs rely on nucleophilic cysteines themselves and, as discussed below, the exchange of ubiquitin moieties to and from cysteine residue to cysteine residue in both ubiquitylation and deubiquitylation is likely to be a very rapid exchange process that could provide an extremely dynamic ubiquitylation response.

The possibility also exists that deubiquitylation could occur through other channels. Cysteines are very sensitive to the redox environment of a cell (Wang et al., 2012) and ubiquitylated serines and threonines could be sensitive to pKa changes leading to ester hydrolysis. It is possible that non-enzymatic deubiquitylation could occur depending on the local environment of the residue such as its pKa as determined by local protein structure and its subcellular location. However, this remains to be explored.

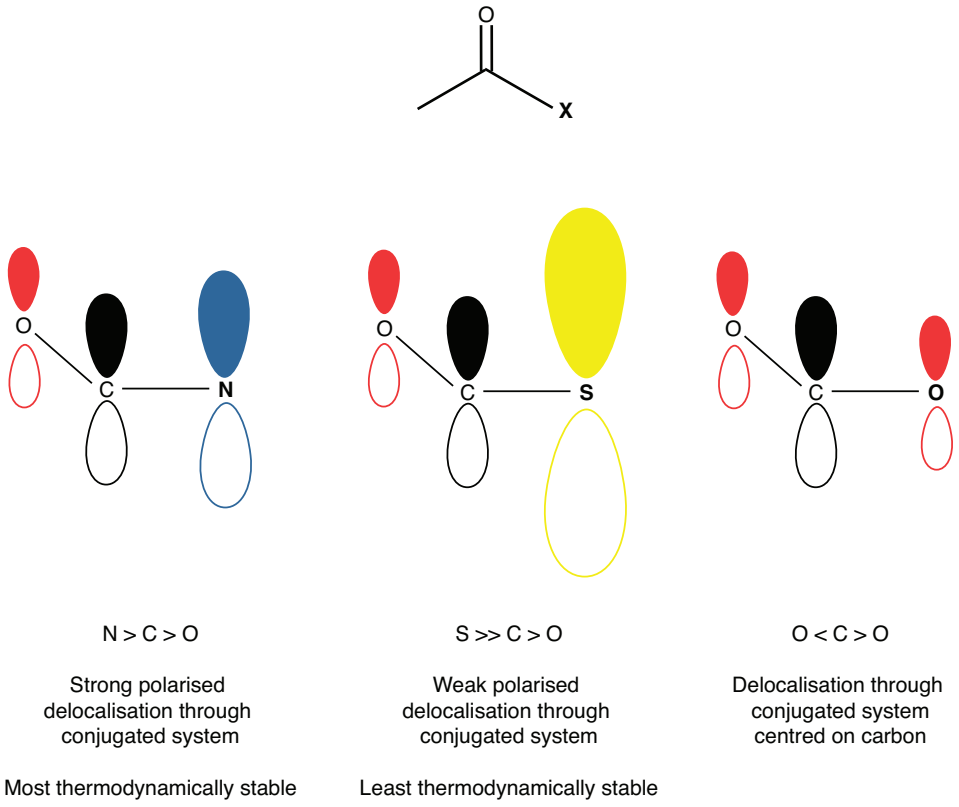

Fig. 3. Extent of orbital overlap in linkages to ubiquitin. In an ester-like linkage between the amino acid residue and the C-terminus of ubiquitin, the extent of orbital overlap relates to the size, symmetry and energy of the orbitals involved, how closely these properties match, and whether the bond is polarized. Hence amide bonds involving nitrogen are the strongest and thioester linkages using sulfur are the weakest.

8. Perspectives: why does non-canonical ubiquitylation occur?

The current evidence for non-canonical ubiquitylation is summarized in Table 1. Having established that non-canonical ubiquitylation can occur, the question remains as to its physiological importance. As illustrated in Fig. 3, thioester linkages to cysteine are actually the weakest bonds a protein could form with Ub (Clayden et al., 2000), while ester linkages with hydroxyls have an intermediate stability and isopeptide or amide bonds with amines form the most stable linkages. Whilst an unstable link is perhaps useful in a highly dynamic signaling system, are cysteines not simply too weak to be relevant as a nucleophile for ubiquitylation? In terms of thermodynamics, cysteines indeed would be a poor choice for stable ubiquitylation. However, the biochemical environment of the cell rarely ventures into the realm of stable thermodynamic equilibrium; rather, such biochemical reactions are governed by the kinetics of reactions.

Fig. 4 illustrates why cysteines may play a significant role particularly in ubiquitin modification for signaling. Ub is shuttled along the ubiquitylation cascade from an Ub-activating enzyme, or E1, to an Ub-conjugating enzyme, or E2, and in the case of HECT domains, onto an E3 ligase, and in all these cases, Ub is conjugated to a cysteine residue on the enzyme. Therefore a weak thioester

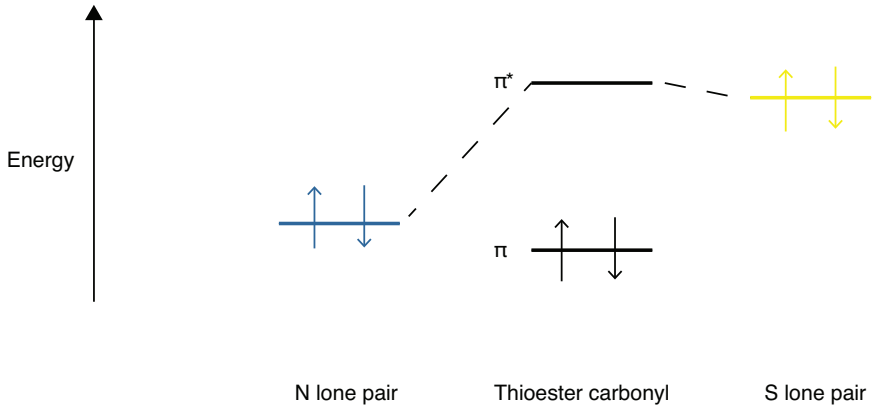

Fig. 4. Kinetics of protein–ubiquitin covalent bond formation. As a soft, high-energy nucleophile, the lone pair of electrons on sulfur form a better energy overlap with the lowest unoccupied molecular orbital (LUMO) of the E2–ubiquitin linkage, which is a soft electrophile, than the hard nucleophile presented by the nitrogen lone pair. Hence reaction with a sulfur nucleophile will occur more swiftly than with nitrogen.

linkage is always attacked by a nucleophilic group when ubiquitylating a substrate protein. As this thioester link consists of diffuse orbitals, particularly from the large sulfur atom, and there is not a large polarization of charge, this is classed as a soft electrophile (Clayden et al., 2000). Soft electrophiles react preferentially with nucleophiles which are also soft, that is with diffuse large orbitals and high energy highest occupied molecular orbitals (HOMOs) that overlap well with the lowest unoccupied molecular orbital (LUMOs) of the thioester linkage (which is the carbonyl π^* MO). In this respect, sulfur thiols will undergo the fastest reaction with a thioester carbonyl, followed by hydroxyls, followed by amines, which become harder, more charge-dominated nucleophiles. So whilst an Ub–cysteine linkage may be labile, it is formed more quickly than the more stable isopeptide bond and could potentially provide a greater range for dynamic signaling behavior by ubiquitylation.

If a variety of different sites in a protein could target for UPS-mediated degradation, this could provide a highly dynamic system for swift ubiquitylation and deubiquitylation events. Such a system may be required to maintain short-lived proteins in a dynamic range of concentrations that can be rapidly altered in response to changing environmental and developmental cues. A variety of different ubiquitylating systems could also be employed in rapid signaling events or where a labile response is required.

9. Non-canonical ubiquitylation: future prospects

The evidence for non-canonical ubiquitylation is easily missed by current methods used to investigate ubiquitin-mediated proteolysis due to the similarity in the chemistry of the N-terminal amine group to lysine, or the labile nature of thioester and hydroxyester linkages. Indeed, ubiquitylation assays using both reducing and non-reducing gel conditions highlight how simple it can be to overlook non-canonical ubiquitylation by typical SDS-PAGE methods (McDowell et al., 2010; Vosper et al., 2009). Moreover, mass spectrometry provides the most accurate method of directly observing post-translational modifications on proteins but is not ideally suited to detecting these non-canonical modifications. Firstly, statistical analyses of spectra must be sophisticated enough to be able to identify post-translational modification such as ubiquitylation (for a review, see Serang and Noble, 2012). Secondly, even when ubiquitylation is specifically investigated, problems with efficient protein digestion, chemical artifacts in sample preparation and enrichment strategies using either anti-ubiquitin or anti-diglycine antibodies (for pre- or post-trypsin digestion enrichment, respectively) can hinder efficient identification, and especially quantitation, of ubiquitylation events (reviewed in further detail by Sylvestersen et al., 2013 and discussed in particular reference to human disease by Kessler, 2013). Finally, even taking into account all these other challenges, ubiquitylation on non-canonical sites, particularly cysteines, serines and threonines, will not be observed using regular sample preparation methods due to high-temperature and reducing agent-dependent sample preparation. It is possible that a method determined for palmitoylation to selectively cap all free cysteines and selectively cleave only those with a thioester modification could be used (Drisdel and Green, 2004; Roth et al., 2006) but this will of course be unable to differentiate between ubiquitylation, palmitoylation and other thioester modifications (Tom and Martin, 2013). It is important to be sensitive to the potential presence of non-canonical ubiquitylation, which may be much more widespread than appreciated at present, as well as to develop new methodology to detect distinct non-canonical ubiquitylation events if we are to truly understand the emerging complexity of protein regulation by ubiquitylation.

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