

N-terminal ubiquitination: more protein substrates join in

Aaron Ciechanover and Ronen Ben-Saadon

Department of Biochemistry and the Rappaport Family Institute for Research in the Medical Sciences, the Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096, Israel

The ubiquitin–proteasome system (UPS) is involved in selective targeting of innumerable cellular proteins through a complex pathway that plays important roles in a broad array of processes. An important step in the proteolytic cascade is specific recognition of the substrate by one of many ubiquitin ligases, E3s, which is followed by generation of the polyubiquitin degradation signal. For most substrates, it is believed that the first ubiquitin moiety is conjugated, through its C-terminal Gly76 residue, to an ϵ -NH₂ group of an internal Lys residue. Recent findings indicate that, for several proteins, the first ubiquitin moiety is fused linearly to the α -NH₂ group of the N-terminal residue. An important biological question relates to the evolutionary requirement for an alternative mode of ubiquitination.

Conjugation of ubiquitin to the protein target proceeds through a three-step cascade mechanism. Following activation by the ubiquitin-activating enzyme, E1, the activated ubiquitin moiety is transferred by one of several E2 enzymes (ubiquitin-carrier proteins or ubiquitin-conjugating enzymes, UBCs) to the substrate that is specifically bound to a member of the ubiquitin-protein ligase family of proteins, E3. There are several distinct families of E3s, based on specific, commonly shared structural motifs. For the E3s containing HECT (homologous to the E6-AP C-terminus) domains, the ubiquitin moiety is transferred from the E2 to the E3 before its conjugation to the substrate. By contrast, RING finger-containing E3s catalyze direct transfer of the activated ubiquitin from E2 to the E3-bound substrate. As the specific substrate-recognizing components of the ubiquitin–proteasome system (UPS), E3s play a key role in the system. It is not surprising therefore that the human genome contains ~1000 different E3s required to endow the UPS with its high specificity.

In most cases, it is believed, despite being shown for only a few proteins, that the first ubiquitin moiety is transferred to an ϵ -NH₂ group of an internal Lys residue in the substrate to generate a covalent isopeptide bond with the carboxyl group of the C-terminal Gly76 of ubiquitin. Successive addition of activated ubiquitin moieties to internal Lys48 of the previously conjugated moiety leads to generation of the polyubiquitin chain that is the degradation signal recognized by the 26S proteasome. Generation of polyubiquitin chains on other Lys residues in the

ubiquitin molecule or modification of proteins by a single ubiquitin moiety serve other functions such as activation of transcriptional regulators or routing of proteins to the lysosome/vacuole.

Degradation of polyubiquitinated substrates is carried out by the 26S proteasome complex, which does not generally recognize non-modified substrates. Short peptides and free and reusable ubiquitin are released during the degradative process (for recent reviews on the UPS, see for example Refs [1–5]; for a scheme of the UPS, see Figure 1).

The mechanistic details involved in polyubiquitin chain synthesis have remained elusive. It is not clear how the chain is elongated and how the more remote ubiquitin moieties are added to the ‘tip’ of the growing ‘tree’. It is also not clear how multiple lysines on the same substrate can be conjugated by the same E3. An important advance in this respect is a recent study [6] demonstrating that ubiquitin-charged E2 must be released from the E3 to allow polyubiquitination to occur. This freedom of the intermediate is probably necessary to allow catalysis. It is clear however that, for ubiquitination, each protein substrate must have a specific E3 recognition motif and one or more ubiquitin-anchoring groups. Surprisingly, only in a few substrates has a specific Lys been identified. In I κ B α , for example, Lys21 and Lys22 have been shown to be important [7]. By contrast, in cyclin B [8] and in the α chain of the T cell receptor cytosolic tail [9], any single Lys residue, even if artificially inserted, can serve as a ubiquitin acceptor. Moreover, in only a handful of cases, it has been shown directly, via chromatographic/Edman or mass spectrometric analyses, that ubiquitin is indeed anchored to a specific Lys residue (see for example Refs [10,11]). In most cases studied, and there are not particularly many, the assumption that an internal Lys serves as the polyubiquitin chain anchor has been based on mutational analyses.

A recent study by Bloom and colleagues [12] demonstrates that the cell-cycle regulator p21 is targeted for degradation through a ‘non-traditional’ mode of ubiquitination that occurs on the N-terminal residue of the protein. The study is also important as it attempts to resolve a basic problem concerning whether regulated degradation of p21 requires prior ubiquitination or whether it is mediated by the proteasome without modification (see Refs [13,14]). While the second problem still requires further studies, N-terminal ubiquitination emerges as an important novel mode of modification.

Corresponding author: Aaron Ciechanover (c_tzachy@netvision.net.il).

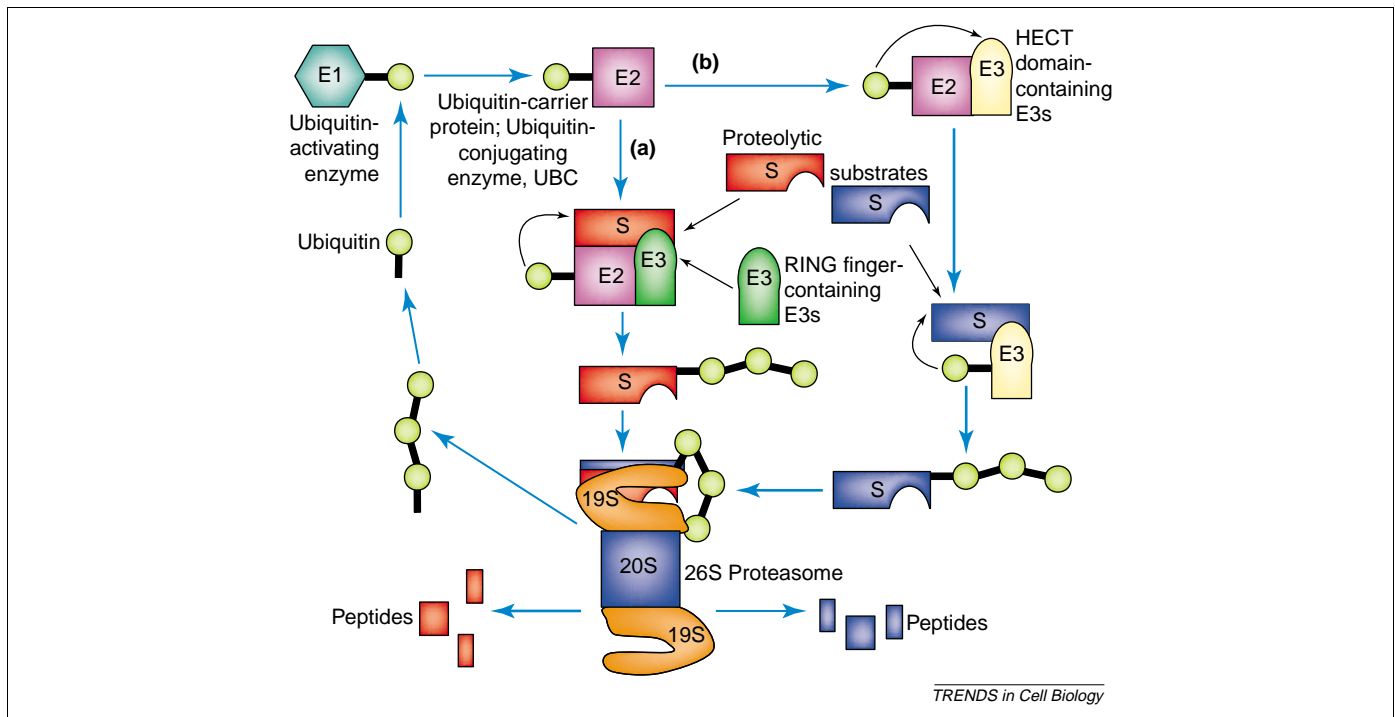


Figure 1. The ubiquitin–proteasome system (UPS). Ubiquitin is first activated to a high-energy intermediate by E1. It is then transferred to a member of the E2 family of enzymes. From E2 it can be transferred directly to the substrate (S, red) that is bound specifically to a member of the ubiquitin ligase family of proteins, E3 (a). This occurs when the E3 belongs to the RING finger family of ligases. In the case of a HECT-domain-containing ligase (b), the activated ubiquitin is transferred first to the E3 before it is conjugated to the E3-bound substrate (S, blue). Additional ubiquitin moieties are added successively to the previously conjugated moiety to generate a polyubiquitin chain. The polyubiquitinated substrate binds to the 26S proteasome complex (comprising 19S and 20S sub-complexes): the substrate is degraded to short peptides, and free and reusable ubiquitin is released through the activity of de-ubiquitinating enzymes (DUBs).

N-terminal ubiquitination

Surprisingly, substitution of all nine internal Lys residues in the myogenic transcriptional switch protein MyoD did not affect significantly its stability both *in vivo* and *in vitro* [15]. Degradation of the lysine-less (LL) protein in cells was inhibited by proteasome inhibitors and was accompanied by accumulation of ubiquitin conjugates. *In vitro*, LL MyoD was ubiquitinated and its degradation required polyubiquitin chain formation. Selective chemical modification of the N-terminal group but not of internal lysines of MyoD blocked degradation almost completely. Fusion of a Myc tag to the N-terminal but not to the C-terminal residue of wild-type MyoD stabilized the protein both *in vivo* and *in vitro* ([15]; K. Breitschopf and A. Ciechanover, unpublished). This finding ruled out the possibility that substitution of all the internal Lys residues ‘forced’ ubiquitination at the N-terminal residue that would not have occurred otherwise. Taken together, these results strongly suggested that the first event in ubiquitin-mediated degradation of MyoD involves fusion of ubiquitin to the N-terminal residue. Additional ubiquitin moieties are then conjugated to Lys48 of the previously conjugated moiety (Figure 2). N-terminal tagging stabilizes the protein, most probably by blocking access of ubiquitin to the specific ubiquitination site at the N-terminus. Using a similar set of experiments, five other proteins have been identified that traverse the N-terminal ubiquitination pathway: (i) the human papillomavirus 16 (HPV 16) oncoprotein E7 [16], (ii) latent membrane protein 1 (LMP1) [17] and (iii) 2A (LMP2A) [18] of the Epstein Barr virus (EBV), (iv) the cell-cycle-dependent kinase (CDK)

inhibitor p21 [12] and (v) the Id2 (Inhibitor of differentiation 2) developmental regulator [19]. It is important to note that, for some of these proteins, substitution of the internal lysines inhibited slightly both conjugation and degradation, suggesting that these residues, probably by also serving as ubiquitin anchors, can modulate the stability of the protein. It is not impossible to assume that N-terminal ubiquitination and modification of internal Lys residues are catalyzed by different ligases, and, for transcriptional regulators, possibly occur in different subcellular compartments (e.g. the nucleus and cytosol).

For E7–16 [16], LMP1 [17], Id2 [19] and MyoD (K. Breitschopf and A. Ciechanover, unpublished), it has been shown that truncation of a short N-terminal segment stabilized the proteins, suggesting that the entire domain, beyond the single N-terminal residue, plays a role in governing the stability of the proteins. This segment might be necessary to allow the mobility/flexibility necessary for the N-terminal residue to serve as a ubiquitin acceptor. It can also serve as a recognition domain for the E3. There is no homology between the N-terminal domains of these four proteins, suggesting that, if the four N-terminal domains serve as recognition motifs, they must be recognized by different components of the UPS. Interestingly, the LMP2A E3 was identified as AIP4 and/or WWP2, members of the NEDD4 family of the HECT-domain-containing ligases [18]. The PY tyrosine-based protein–protein interaction motif of LMP2A recognized by this E3 resides in the N-terminal domain of the molecule, supporting the hypothesis that, in these proteins, the E3-binding

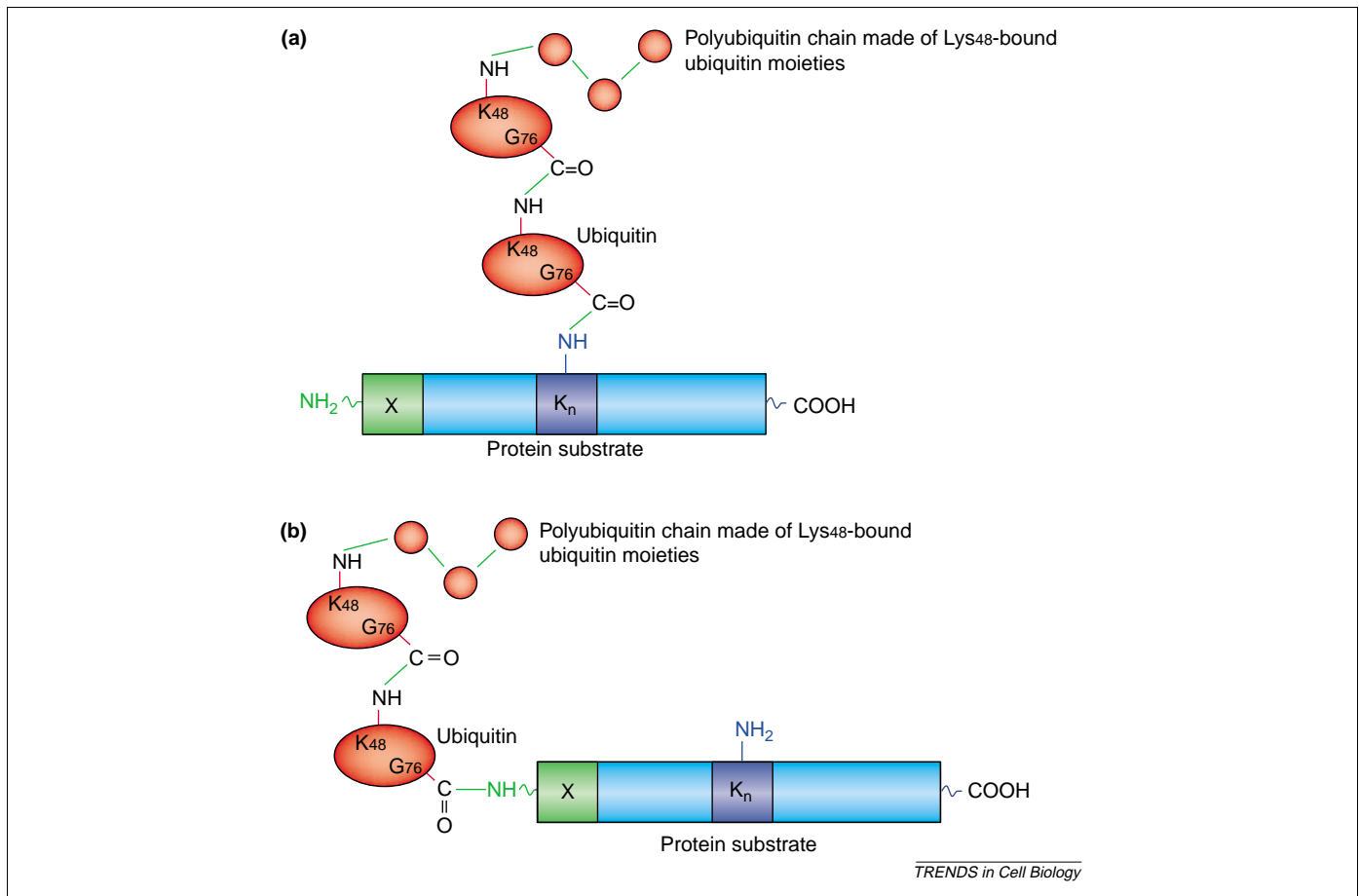


Figure 2. Ubiquitination on an internal lysine and on the N-terminal residue of the target substrate. **(a)** The first ubiquitin moiety is conjugated, through its C-terminal Gly76 residue, to the ϵ -NH₂ group of an internal lysine residue of the target substrate (K_n). **(b)** The first ubiquitin moiety is conjugated to a free α -NH₂ group of the N-terminal residue, X. In both cases, successive addition of activated ubiquitin moieties to internal Lys48 on the previously conjugated ubiquitin moiety leads to the synthesis of a polyubiquitin chain that serves as the degradation signal for the 26S proteasome.

domain might reside in close proximity to the ubiquitination site.

Is there direct evidence for N-terminal ubiquitination? Most probably not. All the different and independent lines of evidence in the various studies strongly suggest that ubiquitination occurs on the N-terminal residue, and any other scenario is highly unlikely. However, the only direct evidence will be demonstration of a fusion peptide between the C-terminal domain of ubiquitin and the N-terminal domain of the target substrate: such a fusion fragment has not been identified thus far. In the absence of any Lys and with only one NH₂ group left in the protein – the α NH₂ group – the only alternative to N-terminal ubiquitination must be based on novel chemistry for ubiquitination, such as esterification of a hydroxyl group of Thr, Ser or Tyr, or thiol esterification of a Cys residue, a modification that appears to be, based on monitoring the stability of the ubiquitin–substrate bond, highly unlikely. Bloom and colleagues bring us a little closer [12]. They conjugated N-terminally His-tagged ubiquitin to N-terminally HA-tagged p21 that contained a Factor-X proteolytic site immediately after the HA-tag (His–ubiquitin conjugated to HA–Factor-X-site–p21). Treatment of the cell-generated ubiquitin conjugate with Factor-X released a smaller species of p21 (that lacks His–ubiquitin and the HA-tag–Factor-X-site) and His–ubiquitin–HA-tag–Factor-X-site,

thus demonstrating that the HA-tag, which was previously part of p21, has become now part of the Factor-X-cleaved ubiquitin. The most likely conclusion that can be derived from this experiment is that the ubiquitin moiety was fused to the N-terminal residue of the HA-tag of p21. However, it is still possible, although highly unlikely, as it is for the other N-terminally ubiquitinated proteins, that ubiquitin modifies a non-Lys residue(s), potentially in this case one of the residues of the HA-tag or the Factor-X site. The HA-tag contains, for example, three Tyr residues. The evidence provided by Bloom clearly limits an unlikely non-peptide bond ubiquitination, such as esterification, to a much narrower zone in the N-terminal domain of the tagged p21, thus providing the strongest, yet still indirect, evidence we have so far for N-terminal ubiquitination.

Concluding remarks

N-terminal ubiquitination is a novel pathway distinct from the N-end-rule pathway [20]. In the latter, the N-terminal residue serves as a recognition and binding motif for the ubiquitin ligase E3 α ; however, subsequent ubiquitination occurs on an internal lysine(s). By contrast, in the N-terminal ubiquitination pathway, modification occurs on the N-terminal residue, whereas recognition by the E3 probably involves a downstream motif. It should be

mentioned that, in yeast, using the model fusion protein ubiquitin-Pro- β -galactosidase, a new proteolytic pathway has been described, designated the ubiquitin fusion degradation (UFD) pathway [21]. The stably fused ubiquitin moiety (that in this exceptional case, where Pro is the linking residue, is not removed by ubiquitin C-terminal hydrolases) functions as a degradation signal, in which its Lys48 residue serves as an anchor for the synthesized polyubiquitin chain. This pathway involves several enzymes, UFD 1–5, some of which appear to be unique and are not part of the ‘canonical’ UPS. It is possible that N-terminal ubiquitination is the most upstream event in the UFD pathway that was discovered using an artificial chimeric ubiquitin–protein model substrate; N-terminal ubiquitination, by contrast, could be the native mode of ubiquitination that provides substrates to the UFD pathway.

The physiological significance of N-terminal ubiquitination is still obscure. Naturally occurring LL proteins that are degraded by the ubiquitin system must traverse this pathway. Many such proteins, mostly viral, can be found in the databases. For Lys-containing proteins, they must contain a free, unmodified N-terminal residue. Such proteins constitute approximately ~25% of all cellular proteins, while the remaining ~75% are N α -acetylated and therefore cannot be ubiquitinated at the N-terminal residue. Whether a protein will be acetylated is dependent on the sequence of the N-terminal domain of the mature protein. This is determined by the combined activity of methionine aminopeptidases (MAPs) and N-terminal acetyltransferases (NATs) that is dependent on the specific sequence of up to the first four N-terminal residues (reviewed in Ref. [22]). Thus, it is possible to predict which intact proteins will be potential substrates of the N-terminal ubiquitination pathway. C-terminal fragments of proteins, N α -acetylated and free N-termini alike, can also be modified by ubiquitin at their N-terminal residue following limited processing: many proteins, such as the NF- κ B precursors p105 and p100 or caspase substrates are processed initially in a limited manner, generating a C-terminal fragment with a newly exposed N-terminal residue. For some of these processed C-terminal fragments, it is possible that they are targeted by N-terminal ubiquitination. Interestingly, most of the substrates identified thus far have a few lysine residues that might not be accessible to the E3s: MyoD has nine (out of 319), E7 two (out of 97), LMP1 has a single Lys residue (out of 440), LMP2A has three (out of 497), Id2 nine (out of 134) and p21 six (out of 164). From the random discovery of six N-terminally ubiquitinated proteins, it appears that their number could well be larger, and many more will be discovered, which will help in the unraveling of the unique characteristics that distinguish this group of substrates.

Acknowledgements

Research in the laboratory of A.C. is supported by grants from Prostate Cancer Foundation Israel - Centers of Excellence Program, the Israel Science Foundation - Centers of Excellence Program, the German-Israeli Project Cooperation (DIP), and a Professorship funded by the Israel Cancer Research Fund (ICRF). Infrastructural equipment has been purchased with the support of the Wolfson Charitable Fund Center of

Excellence for studies on *Turnover of Cellular Proteins and its Implications to Human Diseases*.

References

- Glickman, M.H. and Ciechanover, A. (2002) The Ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol. Rev.* 82, 373–428
- Pickart, C.M. (2001) Mechanisms of ubiquitination. *Annu. Rev. Biochem.* 70, 503–533
- Hilt, W. and Wolf, D.H. eds (2000) *Proteasomes: The World of Regulatory Proteolysis*, Eurekah.com/Landes Bioscience Publishing Company, Texas
- Hicke, L. and Dunn, R. (2003) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu. Rev. Cell Dev. Biol.* 19, 141–172
- Muratani, M. and Tansey, W.P. (2003) How the ubiquitin-proteasome system controls transcription. *Nat. Rev. Mol. Cell Biol.* 4, 192–201
- Deffenbaugh, A.E. et al. (2003) Release of ubiquitin-charged Cdc34-S ~ Ub from the RING domain is essential for ubiquitination of the SCF^{Cdc4}-bound substrate Sic1. *Cell* 114, 611–622
- Scherer, D.C. et al. (1995) Signal-induced degradation of I κ B α requires site-specific ubiquitination. *Proc. Natl. Acad. Sci. U. S. A.* 92, 11259–11263
- King, R.W. et al. (1996) Mutagenic analysis of the destruction signal of mitotic cyclins and structural characterization of ubiquitinated intermediates. *Mol. Biol. Cell* 7, 1343–1357
- Hou, D. et al. (1994) Activation-dependent ubiquitination of a T cell antigen receptor subunit on multiple intracellular lysines. *J. Biol. Chem.* 269, 14244–14247
- Goldknopf, I.L. and Busch, H. (1977) Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate-protein A24. *Proc. Natl. Acad. Sci. U. S. A.* 74, 864–868
- Gronroos, E. et al. (2002) Control of Smad7 stability by competition between acetylation and ubiquitination. *Mol. Cell* 10, 483–493
- Bloom, J. et al. (2003) Proteasome-mediated degradation of p21 via N-terminal ubiquitylation. *Cell* 115, 1–20
- Sheaff, R.J. et al. (2000) Proteasomal turnover of p21^{Cip1} does not require p21^{Cip1} ubiquitination. *Mol. Cell* 5, 403–410
- Jin, Y. et al. (2003) MDM2 promotes p21^{Waf1/cip1} proteasomal turnover independently of ubiquitylation. *EMBO J.* 22, 6365–6377
- Breitschopf, K. et al. (1998) A novel site for ubiquitination: the N-terminal residue and not internal lysines of MyoD is essential for conjugation and degradation of the protein. *EMBO J.* 17, 5964–5973
- Reinstein, E. et al. (2000) Degradation of the E7 human papillomavirus oncoprotein by the ubiquitin-proteasome system: targeting via ubiquitination of the N-terminal residue. *Oncogene* 19, 5944–5950
- Aviel, S. et al. (2000) Degradation of the Epstein-Barr virus latent membrane protein 1 (LMP1) by the ubiquitin-proteasome pathway: targeting via ubiquitination of the N-terminal residue. *J. Biol. Chem.* 275, 23491–23499
- Ikeda, M. et al. (2002) Lysine-independent ubiquitination of the Epstein-Barr virus LMP2A. *Virology* 300, 153–159
- Fajerman, I. et al. (2004) Degradation of the Id2 developmental regulator: targeting via N-terminal ubiquitination. *Biochem. Biophys. Res. Commun.* 314, 505–512
- Varshavsky, A. (1996) The N-end rule: functions, mysteries, uses. *Proc. Natl. Acad. Sci. U. S. A.* 93, 12142–12149
- Johnson, E.S. et al. (1995) A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J. Biol. Chem.* 270, 17442–17456
- Polevoda, B. and Sherman, F. (2003) N-terminal acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins. *J. Mol. Biol.* 325, 595–622