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Site-specific ubiquitination: Deconstructing the degradation tag

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Abstract

Ubiquitin is a small eukaryotic protein so named for its cellular abundance and originally recognized for its role as the post-translational modification (PTM) "tag" condemning substrates to degradation by the 26S proteasome. Since its discovery in the 1970s, protein ubiquitination has also been identified as a key regulatory feature in dozens of non-degradative cellular processes. This myriad of roles illustrates the versatility of ubiquitin as a PTM; however, understanding the cellular and molecular factors that enable discrimination between degradative versus non-degradative ubiquitination events has been a persistent challenge. Here, we discuss recent advances in uncovering how site-specificity — the exact residue that gets modified — modulates distinct protein fates and cellular outcomes with an emphasis on how ubiquitination site specificity regulates proteasomal degradation. We explore recent advances in structural biology, biophysics, and cell biology that have enabled a broader understanding of the role of ubiquitination in altering the dynamics of the target protein, including implications for the design of targeted protein degradation therapeutics.

Introduction

Ubiquitin is an 8.6 kDa protein covalently appended as a posttranslational modification (PTM) to target proteins (substrates), most frequently via an isopeptide bond between ubiquitin's C terminus and the epsilon amino group of a lysine side chain on the substrate. Less commonly, the primary amino group of a protein's N terminus or the side chain of cysteine, serine, and threonine can serve as sites of ubiquitin attachment [1,2]. Additionally, ubiquitination targets can be nonproteinaceous; a recent study identified ubiquitin-modified

lipopolysaccharides incurred during *Salmonella* bacteria infection [3]. Attachment of ubiquitin to target proteins proceeds via a highly regulated, three-enzyme cascade (Figure 1a). The ubiquitin modification is traditionally conceptualized as a "degradation tag", identifying and delivering condemned substrates for degradation via binding to the 26S proteasome. It is also now widely recognized that ubiquitination is also involved in a rich array of other non-degradative cellular processes (Figure 1b).

Lysine is a relatively abundant amino acid, accounting for 6% of the total proteome [4], and, thus, the typical protein contains numerous theoretical sites of canonical ubiquitin attachment. Additionally, ubiquitin itself contains seven lysines (at positions 6, 11, 27, 29, 33, 48, and 63), allowing formation of polyubiquitin chains with diverse lengths, linkages, and topologies. As such, ubiquitin modifications exist in a vast combinatorial space [5] that must be carefully regulated to ensure accurate cellular signal transduction and prevent aberrant degradation.

Together, the site of ubiquitin attachment and the ubiquitin chain identity/length form a 'ubiquitin code' [6,7] that can be read out by cellular factors. Basic chemical constraints present a molecular recognition problem: what controls which lysines are modified, and how are these modifications read or translated into the correct cellular response? Moreover, how can this system be harnessed for the degradation of disease-causing proteins?

How does ubiquitination site selection arise?

Theoretically, all surface-exposed lysines are equally probable and competent for ubiquitination; in practice, the 'writers' of the ubiquitin code determine which substrate lysines are actually modified. E3 ubiquitin ligases are the terminal enzymes of the ubiquitination machinery cascade, downstream of the E1 ubiquitin-activating enzymes and E2 ubiquitin-conjugating enzymes. E3s are complex and highly regulated enzymes [7,8] that all share a "matchmaker" role. In the case of RING E3s, the E3 brings the substrate and ubiquitin-charged E2 together to facilitate ubiquitin transfer. By contrast, homologous to the E6AP carboxyl terminus (HECT) and RING-between-RING (RBR) E3s participate in the enzymatic transfer of ubiquitin to substrate themselves via a covalent E3-ubiquitin intermediate (a thioester linkage between ubiquitin's C terminus and a catalytic E3 cysteine residue).

The terminal E3 enzyme plays a crucial role in this matchmaking process (Figure 1c). The human proteome encodes hundreds of E3s, compared to only ~60 E2s and 2 E1s, illustrating the importance of E3s in controlling substrate and site selectivity [9–12]. As such, it is tempting to speculate that the decision of which proteins/sites to ubiquitinate is at the discretion of the E3-substrate pairing, but this cannot explain the whole story. In fact, while some E3s have dedicated sites and substrates, others interact with hundreds of targets in response to cellular stressors [13,14]. Moreover, mutation of preferred ubiquitination sites does not deter certain E3s from interacting with and ubiquitinating another site on the substrate [1], suggesting that, to a certain extent, ubiquitin site selection occurs stochastically (Figure 1c). Additionally, a class of enzymes known as E4 ubiquitin chain-elongation factors facilitate ubiquitin monomer addition to previously conjugated ubiquitin

chains, expanding upon the modification written by the E3 (Figure 1a) [15]. Thus, future studies are needed to parse cellular and molecular determinants of ubiquitin site selection.

In contrast to these E1, E2, and E3 ubiquitin code 'writers', deubiquitinases serve as 'erasers'. The ~80 human deubiquitinases work in opposition to the conjugation machinery to remove and recycle ubiquitin modifications that are no longer needed or erroneous. Deubiquitinases are also carefully regulated, playing a key role in which substrates and sites remain ubiquitinated for interaction with the cellular 'readers' of site-specific ubiquitination [16]. Moreover, a suite of other ubiquitin-like proteins, known as ubiquitin-like modifiers (UBLs), share structural homology with ubiquitin but have non-overlapping regulatory roles [17]. Interestingly, one of the best characterized UBLs, SUMO, is frequently directed to target lysines via a consensus motif recognized by SUMO's dedicated E2, Ubc9, [18]. Though other non-canonical SUMOylation sites have been observed, this Ubc9-directed consensus motif suggests distinct mechanisms for site selection.

Competition with other lysine PTMs, such as lysine acetylation, at sites of ubiquitination imparts an additional dimension to the site-specificity question. Indeed, recent studies have shown that acetylation of the lysine residues on ubiquitin itself can alter the intra-ubiquitin chain linkages built by multiple E3s *in vitro*, fundamentally altering the "ubiquitin code" message contained in the modification [19]. Furthermore, crosstalk between ubiquitin and other non-lysine-based modifications expands the ubiquitin code. For example, recent work shows that caspase cleavage near the C terminus of target proteins exposes cryptic binding sites for the E3 ligase CHIP (carboxy-terminus of Hsp70-interacting protein), promoting ubiquitination events that shuttle the cleaved substrate to the cellular quality control and degradation machinery [20]. C-terminal proteolysis has also recently been shown to expose masked proteasomal initiation regions, termed degrons. For example, calpain protease cleavage of a transcription factor liberates a proteasome initiation region that triggers ubiquitination and degradation of the transcription factor upon viral infection [21].

Here we explore the molecular mechanisms for reading the ubiquitin code. While it is clear there are a host of ubiquitin receptors and binding partners, the mechanisms by which they can discriminate among different ubiquitinated substrates is less clear. We focus on recent work exploring how the fundamental biophysical changes resulting from the attachment of ubiquitin to the substrate connects to proteasomal degradation.

Molecular and thermodynamic consequences of site-specific ubiquitination

Molecular recognition of a ubiquitinated protein signal is nontrivial and multifaceted. The site of modification, the ubiquitin chain length, and intra-ubiquitin chain lysine connectivity all have the potential to play a role in modulating which downstream cellular factors interact with the ubiquitinated substrate. These observations, however, are frequently insufficient to fully describe ubiquitin signaling; for example, proteasomal ubiquitin receptors have similar affinities for K63-linked ubiquitin chains as they do for the more canonically degradation-associated K48-linked ubiquitin chains [22]. In addition, the 26S proteasome readily degrades K63-linked ubiquitin-modified substrates *in vitro* [23–26].

A major hurdle in biochemical and biophysical studies of the effects of ubiquitination at individual sites is the difficulty in purifying homogenously ubiquitinated proteins with native isopeptide linkages [27]. A diverse array of chemical biology approaches for site specifically conjugating ubiquitin to target proteins of interest have enabled substantial gains in this area [28], but such approaches frequently come with tradeoffs in the chemical properties of the ubiquitin-substrate bond, deubiquitinase sensitivity, and proteasomal recognition. As such, purification of ubiquitinated proteins with native isopeptide linkages typically relies upon biochemical reconstitution of ubiquitination enzymatic machinery [29–31], isolation directly from cells [32], or total chemical synthesis [33].

Recent studies have revealed that the biophysical consequences of ubiquitination are site-specific and can serve as a key mechanism to regulate signaling and function (Figure 2). For example, we showed that ubiquitination can affect the energy landscape of a protein in a site-specific manner [30,31], allowing the substrate to access high-energy, partially unfolded states only when modified at certain sites. Moreover, we found that the proteasome selectively recognizes and degrades substrates ubiquitinated at these destabilizing sites, uncovering a new layer of regulation for proteasomal degradation. We also determined that the exact thermodynamic mechanism driving this destabilization depends on the site of ubiquitination (Figure 2a), with entropic vs. enthalpic mechanisms driving destabilization at different sites within the same substrate [31].

Interestingly, the observed ubiquitin-induced destabilization was rarely of sufficient magnitude to unfold the substrate at equilibrium; ubiquitin-destabilized substrates largely retained the ability to recognize binding partners and did not yield drastically different HSQC signatures compared to their unmodified counterparts by NMR [30,31]. Instead, fluctuations from the native state resulting in population of partially unfolded states on the landscape appear to be responsible for the observed differences in proteasomal degradation. Another possibility is that the fate of ubiquitin-destabilized substrates may be under kinetic control, with ubiquitin attachment modulating the height of the barriers to these partially unfolded, proteasome engageable states. Beyond proteasomal recognition, these ubiquitin-induced energetic changes likely also direct molecular recognition by other cellular factors, ubiquitin-regulated signaling pathways, and nodes in the proteostasis network.

Furthermore, site-specific ubiquitination can impart large conformational changes to key signaling proteins (e.g., Ras, kinases) [34] that affect protein function [35,36], enzymatic activity [37], protein—protein interactions mediated via the ubiquitin moiety [34,38], and even the conformation of the proteasome components itself [39,40]. In addition, the removal of the positive charge from the lysine side chain upon ubiquitination imparts specific molecular recognition and cellular signaling consequences. Recent work using chemical biology approaches to screen multiple aliphatic side chain geometries for the amine that is conjugated to ubiquitin found that the lysine amine geometry is crucial for site determination by E2s/E3s, explaining why these ubiquitination enzymes frequently have canonical acceptor lysines [41].

Site-specificity in 26S proteasomal degradation

On a broad level, rewiring of cellular ubiquitin networks — targeting different proteins for degradation by the proteasome — can direct cell fates, such as the aging process in *C. elegans* [42] or erythrocyte maturation [43]. These examples highlight the critical role of ubiquitination site-specificity in tuning the flux through degradative pathways. Numerous challenges, however, have prevented a cell- and system-wide understanding of how ubiquitinated protein fate is regulated. Thus, we do not yet have a full understanding of the molecular rules that govern these fates, nor do we have a complete map of what proteins are ubiquitinated under a given condition.

Historically, ubiquitin chain length and intra-ubiquitin linkage identity were posited to act as a "switch" between degradation and other cellular fates, with K48-linked chains of four or more ubiquitin moieties believed to confer proteasomal degradation. However, recent work has challenged this paradigm, with multiple ubiquitin chain linkages shown to be robustly degraded both *in vitro* and *in vivo* [24,44,45]; even monoubiquitin can act as a degradation signal [46]. Furthermore, mechanistic studies have demonstrated that diverse ubiquitin chain types have similar affinities for the proteasomal ubiquitin receptors and that the proteasome can recognize and degrade multiple ubiquitin chain architectures [47]. Therefore, the code for degradation cannot be as simple as the type of ubiquitination.

The substrate itself can also play a role in regulating proteasomal degradation. For instance, engagement with the proteasome requires an unstructured region [48] (Figure 2b). On a proteome-wide level, degradation-promoting ubiquitination sites appear only loosely correlated with particular ground-state secondary or tertiary structural features on the substrate [31,49,50]. Biophysical studies evaluating the effect of ubiquitination at different sites on the same protein demonstrated that at some sites ubiquitination can alter the energetics, regulating whether a substrate is degraded by the 26S proteasome *in vitro* [30]. Importantly, these modifications increase the population of partially unfolded states on the energy landscape but do not alter the ground-state, or native, protein structure [31]. The exact cellular pathways that respond to such ubiquitin-mediated changes in energetics (e.g. proteostasis systems) have yet to be identified. For example, a destabilized substrate could be recognized and transformed by a chaperone [51,52] or an unfoldase such as p97 [53–56], prior to delivery to the 26S proteasome.

One hurdle to identifying degradative vs. regulatory ubiquitination sites throughout the proteome is the technical difficulty of mapping ubiquitination sites in general. Recent advancements in mass spectrometry and proteomic profiling techniques have enabled considerable progress in this area [5]. New native mass spectrometry methods allow interrogation of E3:substrate:small-molecule ternary complexes to understand how individual ubiquitination sites are chosen in targeted protein degradation systems [57]. Advances in analysis pipelines [58] and sample preparation workflows [59] have increased the coverage and resolution possible from ubiquitin proteomics experiments. Beyond mass spectrometry, development of activity-based chemical probes for RING E3s [60] have allowed for direct profiling of E3-substrate interactions in cell lysates. These new methods will facilitate a more complete understanding of what sites in the proteome are

ubiquitin-modified, which E3s 'write' a given modification, and under what conditions these modifications occur, providing a foundation for broader profiling, or even predictive algorithms, of degradative vs. non-degradative ubiquitination sites.

Site-specific ubiquitination in targeted protein degradation and therapeutics

While it is increasingly clear that site-specificity is important for cellular 'readers' to correctly translate the ubiquitin code, we still lack a "dictionary" for what associates a certain site with a particular fate (or what cellular factors cause a site to switch from one fate to another). Elaborating this dictionary with new technologies is particularly important in light of new approaches for small molecule-directed targeted protein degradation therapeutics. Specifically, proteolysis-targeting chimeras (PROTACs) are divalent molecules containing target-binding and E3-recruiting moieties [61]. Typically, they are directed to disease-associated targets, such as oncogenes overexpressed in a cancer state, to induce ubiquitination and degradation of pathogenic proteins. Although PROTAC development has historically focused on cancer therapeutics [62], there is increasing interest in developing targeted degraders of viral, metabolic, and cardiovascular disease-associated proteins and neurodegeneration-associated aggregates [63].

Advances in PROTAC technologies have been thoroughly reviewed elsewhere [61,64,65], but it is clear that the basic science discoveries of the roles of site-specific ubiquitination have important implications for their future. First, the evolving role of ubiquitin chain topology/length in 26S proteasome recognition for degradation expands the repertoire of E3s/E3-recruiting moieties that may successfully induce target degradation. Additionally, previous work indicates that PROTAC linker length (between the target-binding and E3-recruiting sites) can determine whether or not degradation occurs, potentially by mediating which target protein lysine residues are accessible for ubiquitination [66,67]. The understanding of the specific molecular effects of ubiquitination at individual sites gained from the studies discussed herein, coupled to future predictive methods for the structural, energetic, and biological consequences of ubiquitination at given sites, empowers rational design of degradation-inducing therapeutics. It is clear that continued studies are crucial to fully realize this therapeutic potential of degradation-directed site-specific ubiquitination.

Beyond the proteasome – site specificity in regulation of cellular processes

Beyond proteasomal degradation, site-specific ubiquitination is crucial for driving biological signaling and regulation of non-degradative processes (Table 1). By definition, the decision of whether to degrade a given substrate ubiquitinated at a distinct site is intricately linked to all of the other options. As such, a comparison with non-degradative site-specific ubiquitination is fundamental to understanding cellular decision making for proteasomal degradation. Although outside the scope of this review, Table I summarizes some recent advancements in this area.

Conclusions and outlook

Rather than acting as a passive 'tag', site-specific ubiquitination imparts a myriad of consequences to target proteins that drive regulation of proteasomal degradation and other cellular processes. Recent detailed mechanistic studies have revealed how, and under what conditions, individual ubiquitin sites are selected, illuminating everything from basic biological signaling pathways to new therapeutic strategies. Although mapping ubiquitination sites has historically been challenging, new methods in structural biology, mass spectrometry, and chemical biology have enabled unprecedented resolution of molecular mechanisms in site-specific ubiquitination associated with proteasomal degradation and beyond. Future studies will expand and refine these mechanisms towards predictive models of the biophysical and cellular effects of ubiquitination at individual ubiquitination sites. Ultimately, to understand ubiquitination site-specificity on a protein-by-protein and proteome-wide level is to have the power to engineer cellular ubiquitination pathways, tipping the cellular scales towards or against degradative outcomes.

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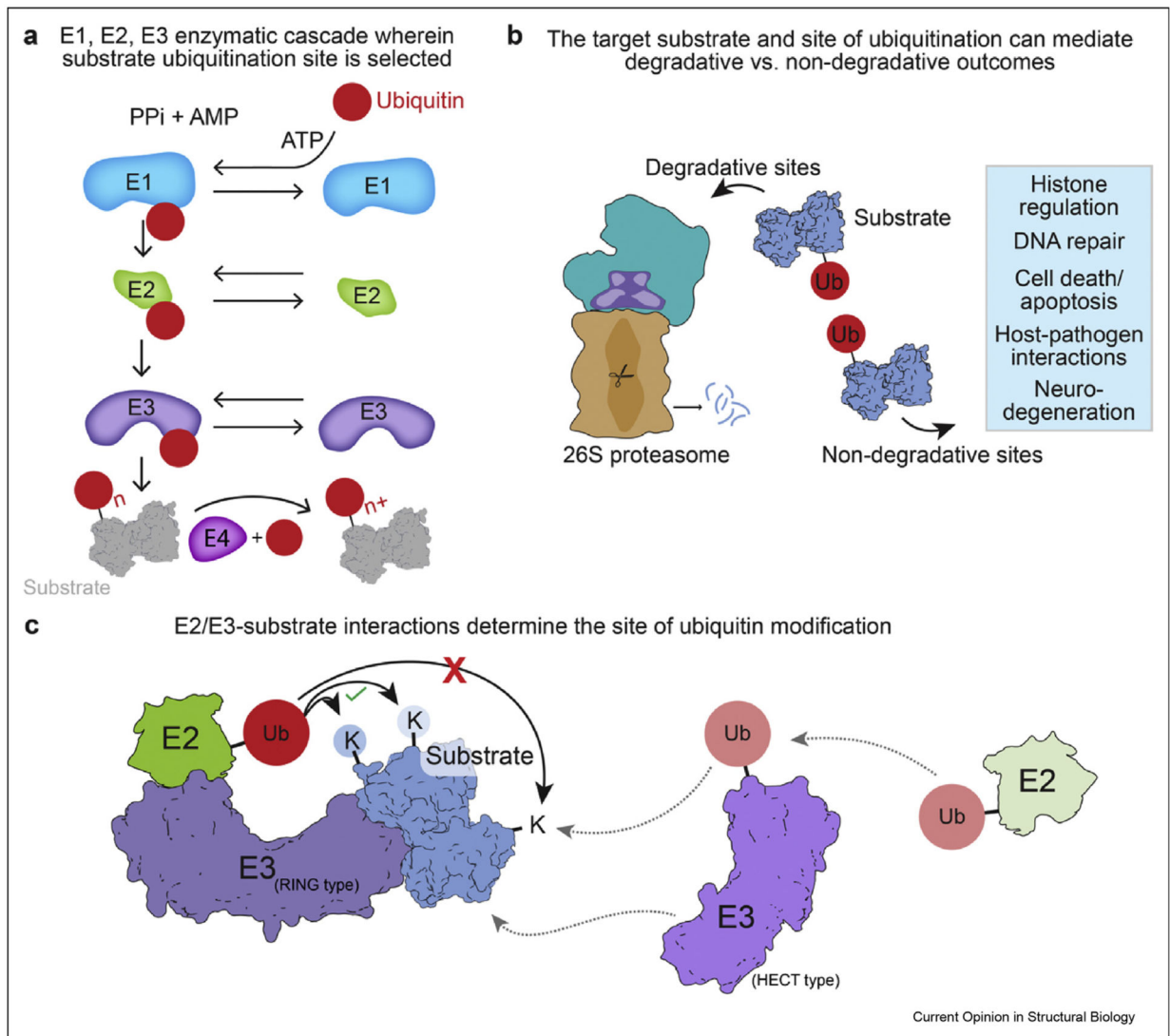
Figure 1.

(a) Enzymatic cascade for site-specific ubiquitination. Given that any surface exposed
lysine (or alternative residues) is theoretically competent for ubiquitination, the modification
site is a product of the identity of the E2 ubiquitin conjugating and E3 ubiquitin ligase
enzymes as well as cellular environmental conditions and the presence of cofactors.
E4 ubiquitin chain-elongation enzymes may add additional ubiquitin subunits to existing
substrate-anchored ubiquitin chains. **(b) Hypothetical example of a substrate protein with
two potential ubiquitination sites.** One site directs the substrate to the 26S proteasome for
degradation (the 'degradative' site), while the other is a 'non-degradative' site that regulates
one of many known cellular processes. **(c) Cartoon example of a hypothetical E2/RING
E3-substrate-ubiquitin ternary complex illustrating steric constraints that lead to
preferential substrate lysine ubiquitination (Left).** By contrast, a hypothetical alternative
E3 (here displayed as a HECT-type E3 charged with ubiquitin) exhibits differential lysine
site specificity with the same substrate based on conformational and topological properties
of the E3-substrate interaction (Right). Recent advances in structural biology have enabled
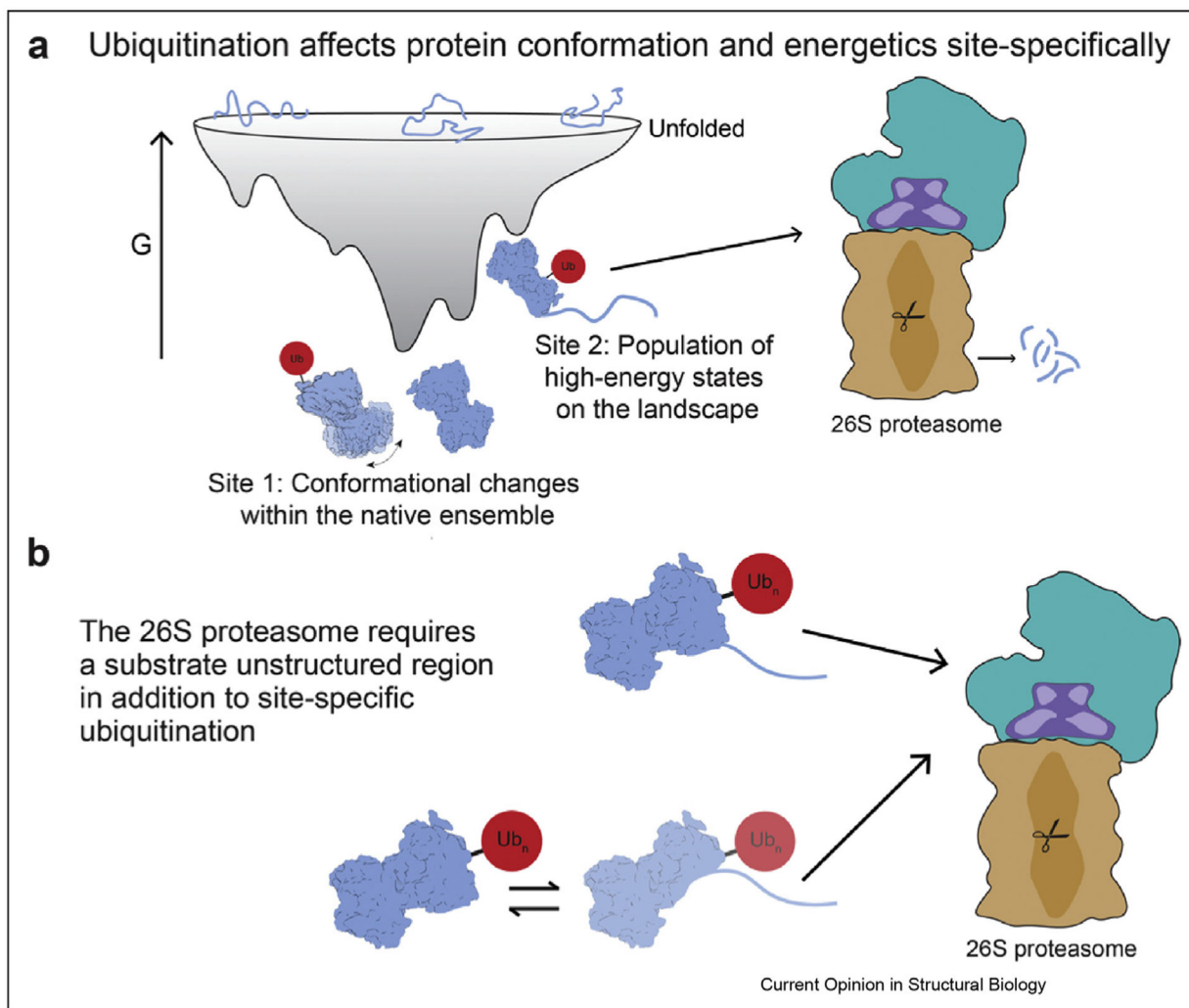
direct inspection of such complexes to illustrate how site specificity arises and subsequent molecular effects.

Figure 2.
(a) Protein conformational energy landscape depicting the energetic and structural consequences of site-specific ubiquitination. Left: At one ubiquitination site, the hypothetical substrate undergoes a conformational change within the native well that alters enzymatic activity and/or interaction partners. Right: At a different ubiquitination site, the hypothetical substrate is energetically destabilized by the addition of ubiquitin, allowing population of a high-energy, partially unfolded state on the landscape that may contain a sufficient unstructured region for degradation by the 26S proteasome. **(b)** The 26S proteasome requires an obligate unstructured to engage the condemned substrate for degradation (top). Recent studies have shown that ubiquitin can destabilize substrate proteins site specifically, regulating whether or not a substrate is recognized for degradation (bottom).

Table 1

Highlighted recent studies of site-specific ubiquitination in non-degradative biological regulation and summary of findings. Advances in structural biology and mechanistic biochemistry have enabled detailed studies of individual proteins and ubiquitination sites of interest, leading to advancements and insights across broad areas of biology.

Biological Process		Studies		Summary
Histone regulation/gene expression	1	Höllmüller etal., 2021 [68]	1	Established regulatory role for ubiquitin and linker histone H1
	2	Witus et al., 2021 [69]	2	Structure of BRCA1/BARD1 E3 heterodimer with the E2 enzyme UbcH5c bound to its cellular target, the nucleosome
	3	Kruijsbergen et al., 2020 [70]	3	Development of site-specific ubiquitin antibodies using histones as a model system
DNA repair	1	Chaugule et al., 2020 [71]	1	Substrate FANCI rewires E2 Ube2T active site to promote ubiquitinations at certain substrate sites
	2	Kelliher et al., 2020 [72]	2	E3 RNF168 ubiquitinates non-canonical histone variants at their divergent N-terminal tail lysine residues
Apoptosis/cell death	1	Fennell et al., 2020 [73]	1	Site-specific ubiquitination of E3 ligase HOIP activates apoptosis
	2	Li et al., 2020 [74]	2	RIPK1 (kinase that regulates apoptosis) is regulated by site-specific ubiquitination to promote apoptosis
Immune Signaling and Host-pathogen interactions	1	Shin et al., 2020 [75]	1	Legionella possesses a suite of deubiquitinases that are selective for different ubiquitin chains and sites
	2	Schubert and Nguyen et al., 2020 [76]	2	Prediction and validation of deubiquitinases in pathogenic bacteria provides insight into ubiquitin chain site recognition
Neurodegeneration	1	Hakim-Eshed et al., 2020 [77]	1	Ubiquitination of Huntingtin leads to less toxic, larger aggregates and shapes aggregate morphology
	2	Munari et al., 2020 [78]	2	Aggregation propensities differ for tau monoubiquitinated with chemoselective disulfide approach vs. enzymatic/native isopeptide approach