A new dawn beyond lysine ubiquitination

Squair, Daniel R.; Virdee, Satpal

Published in:
Nature Chemical Biology

DOI:
10.1038/s41589-022-01088-2

Publication date:
2022

A New Dawn Beyond Lysine Ubiquitination

Daniel R. Squair & Satpal Virdee*

MRC Protein Phosphorylation and Ubiquitylation Unit, University of Dundee, Scotland, UK

Email: s.s.virdee@dundee.ac.uk

Tel: +44 (0)1382 388738

Fax: +44 (0)1382 388500

*Corresponding author

Abstract

The ubiquitin system has become synonymous with the modification of lysine residues. However, the substrate scope and diversity of the conjugation machinery have been underappreciated bringing us to an epoch in ubiquitin system research. The striking discoveries of metazoan enzymes dedicated towards serine and threonine ubiquitination have revealed the significant role of non-lysine ubiquitination in endoplasmic reticulum-associated degradation, immune signalling and neuronal processes, whilst reports of non-proteinaceous substrates have extended ubiquitination beyond the proteome. Bacterial effectors which bypass the canonical ubiquitination machinery and form unprecedented linkage chemistry further redefine long-standing dogma. Whilst chemical biology approaches have advanced our understanding of the canonical ubiquitin system, further study of non-canonical ubiquitination has been hampered by a lack of suitable tools. This Perspective aims to consolidate and contextualise recent discoveries and propose potential applications of chemical biology, which will be instrumental in unravelling this new frontier of ubiquitin research.

(146 words)

Main

The ubiquitin (Ub) system is a vast eukaryotic regulatory mechanism underpinning almost all cellular processes[1]. By and large, research has focused on "canonical ubiquitination" which can be considered the attachment of the Ub carboxy-terminus to lysine residues within substrate proteins through an isopeptide bond[2]. Whilst the most common function of ubiquitination is to mark protein substrates for proteasomal degradation, it is also utilized by the cell to control processes such as immune system signalling, endocytosis and DNA repair[3]. Lysine residues within the Ub molecule itself can act as anchor points for further attachment and the resultant polyubiquitin (polyUb) chains and the different cellular signals they encode have been the subject of intensive research (for reviews see [4-6]). Remarkably, chain types with near indistinguishable topology have distinct cellular functions illustrating the cell's capacity to interpret even the most subtle differences in Ub signal[7]. Thus, the Ub system is highly versatile, enabling discrete cellular signalling through a combination of substrate selection and chain topology **(Figure 1)**.

Early studies on viral effectors hinted at non-lysine ubiquitination being an additional facet of the Ub system[8]. The viral protein kK3 from Kaposi's sarcoma-associated herpes virus was shown to trigger the lysosomal degradation of major histocompatibility class I (MHC-I) molecules by ubiquitinating them on cysteine residues[9,10]. Subsequent studies revealed that the related murine viral E3 ligase mK3 ubiquitinates MHC-I molecules on serine and threonine residues leading to their endoplasmic reticulum-associated degradation (ERAD) [11]. Strikingly, the source of non-lysine activity was a host Ub system enzyme that mK3 was simply co-opting[12]. However, as our understanding of the molecular principles of Ub conjugation has matured, in retrospect this early report revealed that non-lysine ubiquitination is a physiological and integral arm of the Ub system, rather than a viral peculiarity as perhaps first assumed. In recent years there have been a growing number of reports of non-lysine ubiquitination and unprecedented writer enzymes have been discovered, together with dedicated erasers[13-19].

Non-lysine ubiquitination expands the number of potential ubiquitination sites in the proteome and introduces an additional key variable - linkage chemistry between Ub and its substrates **(Figure 1)**. Ubiquitination of serine/threonine and cysteine residues generates ester and thioester bonds, respectively, which compared to the canonical lysine isopeptide, differ in intrinsic stability, proximity to substrate backbone, and rotational freedom. Non-lysine ubiquitination also affords additional Ub chain topologies containing ester-linkages[14,15],. and grants the potential for expanded crosstalk with other posttranslational modifications (PTMs). Thus, non-lysine ubiquitination has the potential to diversify Ub signals and afford additional regulatory paradigms. Pathogenic bacteria have also devised a remarkably distinct form of reversible non-canonical ubiquitination termed phosphoribosyl ubiquitination **(Figure 1)** [20-22], whilst ubiquitination of non-proteinaceous substrates including nucleotides, lipids and sugars is also an emerging area of investigation[16,23,24]. Taken together, these recent developments have revealed that the true scale of the Ub system has been underestimated bringing us to an epoch in Ub research and a growing field of non-canonical ubiquitination.

The Many Paths of a Ubiquitin Molecule

Ubiquitination occurs at the end of a multi-enzyme cascade which utilises a series of cysteine acylation events to transfer Ub to the substrate **(Figure 1)**. Ub is first activated by an adenosine 5'-triphosphate (ATP)-driven E1 enzyme, forming a reactive thioester bond between Ub and its own catalytic cysteine[2]. Subsequent steps are cofactor-independent and involve the transfer of Ub to the cysteine of an E2-conjugating enzyme forming a thioester-linked E2 conjugate (E2~Ub). E2~Ub is then engaged by E3 ligase enzymes (E3s) which control substrate specificity. Homologous to E6AP Carboxy-Terminus (HECT) and RING (Really Interesting New Gene)-in-between-RING (RBR) E3 classes contain a catalytic cysteine that undergoes a transthiolation step with E2~Ub to form a thioester-linked acyl intermediate. A further two classes of transthiolating E3 have now been discovered that both carry

out non-lysine ubiquitination. The sole member of the RING-Cys-Relay (RCR) class, MYCBP2, extends the cascade further by employing a second catalytic cysteine that facilitates an internal Ub relay mechanism **(Figure 1)** [13]. The Moyamoya disease-associated E3 RNF213 contains a novel ZF2 domain harbouring its active site cysteine[17,18]. In an autonomous manner, all transthiolating E3s control substrate recruitment and dictate the amino acid reactivity profile[25,26]. It should be noted that although thioester intermediates formed can be highly transient due to active site effects, the half-life of E2~Ub thioesters is on the time scale of hours[27], and would be expected to be even longer for cysteine ubiquitinated substrates .

Whilst transthiolating E3s have been implicated with a spectrum of physiological and pathophysiological processes, most E3 ligases are made up of the RING class[28]. RING E3s function like scaffolds by mediating direct Ub transfer from the E2 active site to substrate. When partnered with RING E3s, E2s influence the ubiquitination signal because they can direct ubiquitination to specific sequences and structural motifs[29-33]. A single E3 can also function with multiple E2s to shape the topology of the Ub signal on the substrate[34,35]. We now appreciate that for RING E3s, the amino acid reactivity profile is a hardwired feature of the E2[36], and the viral E3 mK3 that directed serine and threonine ubiquitination of MHC-I molecules was co-opting UBE2J2 - an E2 that favours esterification of hydroxy amino acids over lysine aminolysis[12,19]. The astounding number of potential RING E3-E2 interactions (~20,000), and the fact that the amino acid reactivity profile for many E2s is yet to be determined, points to a potential for gross underestimation of the scale of non-lysine ubiquitination.

Non-lysine Ubiquitination: Dedicated and Deliberate

As virally encoded E3s of the RING class were usurping the esterification activity of UBE2J2[8,9,12] this indicated it must have a physiological role. It is now understood to function with the RING E3 HRD1 to execute native ERAD and multiple ERAD substrates ubiquitinated on serine and threonine residues have now been identified[37-40]. In addition to serine and threonine ubiquitination, there are

growing accounts of cysteine ubiquitination having important cellular functions (for a comprehensive review on non-canonical ubiquitination see here[41]). In a recent example it has been shown that cysteine ubiquitination of TRAF3 by the HECT E3 NEDD4L primes the substrate for ubiquitination by other E3s and this positively regulates innate antiviral immunity[42].

It is now apparent that in addition to UBE2J2, several transthiolating E3s demonstrate esterification activity enabling them to attach the Ub carboxy-terminus to hydroxy groups within substrates[12-15]. Enabled by activity-based probes (ABPs) for E3 transthiolation activity[43], we showed that the E3 MYCBP2 belongs to an unprecedented RCR class and selectively ubiquitinates threonine residues preferentially over serine in model substrates[13]. MYCBP2 silencing in animals has revealed a conserved and essential role in synaptogenesis and positive regulation of programmed axon death (Wallerian degeneration) [44-47]. These phenotypes can be recapitulated with a knock-in mouse model that ablates MYCBP2 E3 activity, suggesting that its non-lysine esterification activity regulates neuronal processes[48]. Its apparent role in initiating programmed axon death is of particular interest and raises the possibility that inhibition of its RCR mechanism, or other aspects of its E3 activity, are promising strategies for treating neurological disorders[49]. MYCBP2 demonstrates broad activity towards model hydroxy substrates, including those that are non-proteinaceous. Thus, although several protein substrates have been assigned to MYCBP2[50-54], whether a threonine is the primary substrate in these contexts remains undetermined.

A second transthiolating E3 with non-lysine esterification activity has now come to light[14]. The linear ubiquitin chain assembly complex (LUBAC) regulates innate immunity and contains the protein HOIL-1, which possesses an RBR E3 module. There have been historical challenges in coaxing convincing E3 ligase activity from HOIL-1 in biochemical assays. It is now understood that HOIL-1 is an active E3 ligase that demonstrates non-lysine esterification activity. However, this required the selective enrichment of components from the Myddosome signalling pathway from wild type and HOIL-1 knock-in mice. These then needed to be specifically tested for non-canonical ubiquitination[14].

HOIL-1 also introduces ester-linked branch points within complex polyUb chains after cellular stimulation with tumor necrosis factor (TNF) [14,15]. Taken together, these observations indicate an important role of non-lysine ubiquitination in the innate immune system.

Non-lysine Deubiquitination

Posttranslational modifications with crucial regulatory functions invariably have dedicated eraser enzymes enabling reversible signalling. Ubiquitination is reversed by the action of deubiquitinating enzymes (DUBs) and ~100 DUBs belonging to 7 classes are encoded by mammals[55]. Whether DUBs can cleave non-lysine ubiquitination sites or if there are members that specifically carry out this task remained unknown until the recent application of chemical biological approaches. A small panel of DUBs tested for esterase activity towards a chemically synthesised threonine-ubiquitinated α-globin substrate revealed that some DUBs can cleave ester linkages, and this need not be mutually exclusive with lysine isopeptidase capacity[56].

Using model ester- and isopeptide-linked model substrates, prepared using semisynthetic and chemoenzymatic methods, we carried out a comprehensive screen of 53 DUBs representative of all 7 classes[19]. We found that 22 out of 24 USP DUBs analyzed could mediate esterase activity with deubiquitination kinetics comparable for both linkage types, suggesting reversible non-lysine ubiquitination is physiologic and occurs on a large scale. In contrast, the fourteen tested DUBs belonging to the OTU DUB class were generally devoid of esterase activity, consistent with their established role in editing isopeptide- and peptide-linked polyUb chains[57]. An interesting exception was a DUB encoded by Crimean Congo Haemorrhagic Fever Virus (vOTU) with high threonine esterase activity[19]. As non-lysine ubiquitination of substrates has been implicated with immune signalling, it will be interesting to see if their deubiquitination by vOTU plays a role in immune system evasion. Strikingly though, the Machado-Joseph Disease (MJD) class of DUBs demonstrates potent and highly selective Ub esterase activity toward both serine and threonine[19]. The MJD family member JOSD1

deubiquitinates a model threonine substrate with kinetics rivalling the most active DUBs with isopeptidase activity, and serine esterase activity is higher still[19]. JOSD1 is the most upregulated DUB in chemoresistant gynaecological cancers, which is associated with poor prognosis[58]. This has been linked to its ability to stabilise the anti-apoptotic BCL-2 family member MCL-1 via its DUB activity[58]. Intriguingly, apoptosis induction by the BCL-2 family member BID is dependent on the ubiquitination of a serine residue[59]. It is therefore tempting to speculate that non-lysine JOSD1 DUB activity is oncogenic and its inhibition might be of therapeutic value. A take-home message from these recent discoveries on E3s and DUBs is that when robust activity is not forthcoming in the context of isopeptide-linked ubiquitination, exploration of non-lysine sites is essential.

Phosphoribosyl Linkages

Deviating from Ub conjugation via its C-terminus, *Legionella pneumophila* effectors of the SidE family (SdeA, SdeB, SdeC and SidE) bypass the ubiquitination machinery entirely to conjugate an internal Ub residue to serine residues of host proteins through a phosphoribosyl linkage **(Figure 1 and 2)** [60]. Unexpectedly, the catalytic process requires nicotinamide adenine dinucleotide (NAD^+) and together with a mono ADP-ribosyltransferase (mART) domain, arginine 42 of Ub initially becomes ADP-ribosylated generating (ADPr-Ub) [20]. Next, a catalytic histidine within a distinct phosphodiesterase (PDE) domain cleaves the phosphodiester bond in ADPr-Ub releasing adenosine monophosphate (AMP) thereby forming a covalent phosphoramidate catalytic intermediate with phosphoribosyl Ub (PR-Ub) [21,61,62]. Subsequent steps result in phosphoribosyl ubiquitination of serine residues **(Figure 2)** [61,62]. Its requirement stems from *Legionella pneumophila* being an intracellular pathogen, which must carve its own microenvironment within host cells to survive and proliferate. Key to this is the formation of Legionella-Containing Vacuoles (LCVs), which protect the pathogen from the host defence system. The LCV is constructed through the fragmentation and recruitment of membranes of the host endoplasmic reticulum (ER) [63], a feat achieved through the targeted phosphoribosyl

ubiquitination of key ER remodelling enzymes such as Rab GTPases and RTN4[20,22]. Interestingly, tyrosine has also been shown to be a target of SidE effectors, raising the possibility that additional substrates of phosphoribosyl ubiquitination remain to be identified[64]. Illustrating the requirement for its sophisticated regulation, the *Legionella* enzymes DupA and DupB (Dups) are tasked with the removal of phosphoribosyl ubiquitination, which releases the substrate and PR-Ub[60]. These mediate their effect through a PDE domain similar to that found in SidE effectors but their opposing directionality is dictated by an enhanced affinity for Ub[22]. Why such an unconventional linkage chemistry is employed can only be speculated but ADPr-Ub and PR-Ub inhibit the native Ub conjugation machinery, which might also serve an important function[21].

Non-protein Substrates

Intriguingly, early studies showed that ADP-ribosylation of the Ub C-terminus occurs natively in mammalian cells and converts Ub to a latent form that cannot be conjugated to substrates. This is carried out by an E3 complex consisting of the RING E3 DTX3L and the mono ADP-ribosyltransferase PARP9 (DTX3L/PARP9) [24,65]. Consistent with this having a regulatory role, the ADP-ribose moiety can be removed by DUBs[65]. Further reports of alternative substrates of the Ub system have followed. RNF213 corresponds to a new mechanistic class of transthiolating E3[17,18]. RNF213 modifies invading bacteria with Ub marking them for bacterial autophagy[16]. Remarkably though, the ubiquitination target is the lipid A moiety of the lipopolysaccharide (LPS) bacterial coat – a non-proteinaceous substrate **(Figure 1)**[16]. This enables cytosolic bacteria to be recognized by receptors that facilitate their clearance by autophagy. The precise nucleophile that becomes acylated with Ub remains to be confirmed. This discovery demonstrates the important role non-proteinaceous ubiquitination can have in the immune system. An exciting possibility is that the innate immune response is bolstered by an ability to mark a range of non-proteinaceous species of pathogenic origin with Ub.

Strong links between non-proteinaceous ubiquitination and genetic disorders have also come to light[23]. Deficiency of the RBR E3 HOIL-1 leads to the accumulation of toxic polyglucosan deposits in the heart and other tissues resulting in death from heart failure in young adults[66,67]. E3 inactive HOIL-1 knock-in mice also present with aberrant polyglucosan deposits in the hind brain and heart. Further investigation into whether loss of Ub conjugation activity is associated with disease onset is called for. It has recently been shown that in addition to esterification activity towards serine and threonine residues, HOIL-1 is capable of ubiquitinating the carbohydrate glycogen **(Figure 1).** Using the oligosaccharide maltoheptaose as a model, the precise hydroxy moiety that becomes ester-linked to Ub has been mapped to the C6-hydroxy of maltoheptaose, which occurs in a mutually exclusive manner despite there being several C6 hydroxy units within maltoheptaose[23]. Furthermore, peptide and isopeptide-linked Ub chains stimulate this activity providing an example of crosstalk between the canonical Ub system and non-lysine ubiquitination.

These recently elaborated and remarkable substrates illustrate how the cell leverages non-lysine ubiquitination to degrade deleterious non-proteinaceous species. The true scale of Ub-mediated degradation of non-proteinaceous substrates is an open question and radically different tools will be required to define this comprehensively.

Emerging Commonalities

Approximately 30 Ub E2s exist, and many are poorly characterized. The catalytic mechanism underpinning how certain E2s facilitate acylation of side chains with the Ub carboxy terminus is reasonably well understood. However, studies have only been carried out in the context of aminolysis of a lysine acceptor. Key residues involved in pK_a suppression of the ε-amino group have been established and computational predictions suggest that through desolvation effects, they supress the lysine pK_a by 3 pH units[68,69]. Whilst sufficient for ε-amino group deprotonation (pK_a ~10), a requisite for lysine ubiquitination, an alternative mechanism must exist for E2s that esterify hydroxy amino

acids (pK_a ~16). Interestingly, the residues considered essential for lysine aminolysis are absent in the E2 UBE2J2 **(Figure 3a)** [12]. Furthermore, a highly conserved histidine-proline-asparagine (HPN) motif involved in activating the E2~Ub thioester bond is also missing in UBE2J2 **(Figure 3a)** [70]. Crystal structures for UBE2J2 exist but a region likely to be important for esterification activity is disordered. These observations point towards a distinct and uncharacterised mechanism for E2 esterification activity and to delineate its mechanism in detail, atomic resolution structures of an E2-hydroxy substrate complex will be instrumental.

Insights into how a transthiolating E3 carries out selective threonine esterification activity has been afforded from crystallographic studies on MYCBP2[13]. Here a fortuitous crystal packing interaction placed a threonine residue in proximity of the active site responsible for substrate modification where it formed several substrate-like interactions **(Figure 3b)** [13]. The active site engages the threonine side chain and optimally positions it for catalysis via docking of its side chain methyl into a hydrophobic pocket formed by a striking array of phenylalanine residues. In addition, an essential histidine proximal to the catalytic cysteine residue acts as a general base to deprotonate its hydroxyl group **(Figure 3b)**. Puzzlingly, the ~42 HECT/RBR E3s also utilize a proximal histidine to mediate lysine aminolysis activity so it is unclear if MYCBP2 esterification activity is brought about by idealized geometry or if additional factors are at play. It is also unknown if HECT/RBR E3s in addition to HOIL-1 can ubiquitinate hydroxy amino acids. Intriguingly, MJD DUBs also have hydrophobic phenylalanine or tryptophan residues at their catalytic sites which are important for threonine esterase activity and UBE2J2 has a hydrophobic leucine residue at position 117, rather than a hydrophilic residue typically displayed by lysine-specific E2s **(Figure 3a)** [19]. Thus, the aromatic and hydrophobic character of these active sites might provide a solvent environment conducive to both esterification and esterase chemistry that contributes to the non-canonical reactivity profile of these enzymes. Computational chemistry predictions should provide valuable insight into deciphering the catalytic mechanisms of these unusual components of the Ub system.

The relay mechanism demonstrated by MYCBP2, which is associated with its dedicated hydroxyl ubiquitination activity is intriguing[13,48]. It has been proposed that similarly to MYCBP2, HOIL-1 does not accept Ub from E2 but instead receives it from the catalytic intermediate consisting of the RBR E3 HOIP thioester-linked to Ub[15]. It is tempting to rationalise the association of Ub relay mechanisms and non-canonical ubiquitination activity **(Figure 1)**. For example, active sites endowed with esterification activity might have constraints rendering them incompatible with E2-E3 transthiolation. In this scenario, relay mechanisms would address this paradox whereby two active sites tailored for distinct catalytic steps cooperate in unison. Indeed, there is a precedent for distinct E3s cooperating to modify a single substrate[71,72].

Why Not Lysine?

With protein quality control processes, such as ERAD, where non-lysine ubiquitination has been implicated[37], dynamic cellular stresses might lead to aberrant proteins of arbitrary identity. It is conceivable that limiting ubiquitination to a lysine might be deleterious by not affording a general and timely mechanism for degradation. Expansion to non-lysine sites also provides the opportunity for cellular crosstalk with other PTMs and crosstalk between cysteine ubiquitination and redox regulation has been described[73]. Reactive oxygen species generated during myoblast differentiation oxidise two cysteine residues in INSIG-2, a negative regulator of lipid biosynthesis, to sulfenic acid which cannot be ubiquitinated. The ensuing INSIG-2 stabilization prevents lipogenesis by inhibiting the sterol regulatory element-binding proteins (SREBP) pathway. This enables a powerful regulatory paradigm where Ub-dependent substrate stability is linked to its local redox environment.

How the cell might exploit the alternative linkage chemistries may be tied to the respective cellular stabilities of ester, thioester, and amide bonds **(Box 1)**. Peptide and isopeptide bonds are extremely resistant to uncatalyzed hydrolysis with a half-life ~400 years[74], which allows protein turnover to be tightly regulated by proteases. Alternatively, the thioester bond is less

thermodynamically stable and intrinsically transient. Ubiquitinated glutathione as a model cysteine substrate is also readily cleaved by a small representative panel of DUBs[19]. Thus, cysteine ubiquitination is well-suited for processes requiring transient modification. For example, the Peroxisomal Targeting Signal Type 1 (PTS1) receptor Pex5p imports PTS1-proteins to the peroxisomal membrane, releases them into the lumen, and is ubiquitinated on a conserved cysteine[75,76]. As cytosolic export is dependent on deubiquitination kinetics[77], the intrinsic instability of the thioester might be utilized by the cell to enable rapid cycling of peroxisomal protein import, which is essential for the biogenesis of the organelle.

Esters exhibit less thermodynamic stability than amides, but greater stability than thioesters. In a cellular context, esters are generally considered to be highly unstable due to the action of promiscuous esterases - an activity exploited by prodrugs[78]. However, the cellular half-life of a model Ub ester is on the time scale of hours[19]. Therefore ester-linked ubiquitination could provide an enduring cellular signal, which could be reversed upon localised pH changes, or upon the action of a DUB with specific esterase activity. In this capacity, ester-linked Ub could function as a 'molecular switch'. For example, Ub chains primed with ester-linked Ub by HOIL-1 or MYCBP2 could be swiftly cleaved *en bloc* to abruptly attenuate the inflammatory or programmed axon death response, respectively. Whilst many compelling physiological roles for non-lysine ubiquitination can be speculated, new tools and technologies are urgently required to decipher this branch of the Ub code.

Text for Box:

Conventional amide-bonds formed during protein synthesis or canonical ubiquitination are stabilised as a resonance-hybrid as the nitrogen lone-pair electrons delocalize to the antibonding orbital of the carbonyl carbon. This gives the amide bond partial double-bond character whilst polarising the carbonyl group, limiting its susceptibility to nucleophilic attack and preventing the uncatalyzed hydrolysis of proteins.

Alternatively, the thioester bond is not stabilised by resonance due to poor overlap between the different valence electron shells of the carbonyl carbon and the sulphur atom. Coupled with the relatively low *pKa* of the sulfhydryl group that renders the thiolate a better leaving group, the thioester bond is more labile and less thermodynamically stable than the amide bond.

Esters are the 'Goldilocks' of the three linkages. Like the amide, the ester is stabilised through a resonance-hybrid due to the delocalisation of the oxygen lone-pair. However, the higher electronegativity of oxygen relative to the amide nitrogen results in reduced delocalization and a weaker bond so the ester carbonyl is more prone to nucleophilic substitution and hydrolysis in solution[79]. Thus, esters could enable context-dependent deubiquitination due to localised pH changes or the action of DUBs with esterase activity.

Box 1 figure inset.

New Tools for Non-canonical Ubiquitination

Probes for Thioester- and Ester-linked Ubiquitination

Although not a chemical probe per se, a proven strategy for cursory diagnosis of linkage chemistry between the Ub carboxy terminus and the protein substrate takes advantage of the different strengths of the thioester, ester and amide bonds **(Figure 4a)**. However, key to holistically understanding the function and true scale of non-lysine ubiquitination will be robust identification of ubiquitination sites using bottom-up proteomic technologies that analyse the peptides generated after protease treatment. Comprehensive coverage of lysine ubiquitination sites demands enrichment strategies, but it remains unclear how compatible established antibody-based approaches are with non-lysine attachment points[80-82]. Co-enrichment of higher abundance lysine peptides is also likely to mask non-lysine counterparts. Thus, tools that enable selective enrichment of ester-linked remnants will be of great value. However, it remains unknown if small ester containing peptides required for antibody generation would be sufficiently stable *in vivo* to raise an immune response. This might call for the design and synthesis of non-hydrolyzable analogues, which have been instrumental for other labile PTMs[83,84]. An alternative strategy in this instance might be the development of non-antibody based affinity reagents that can be developed using *in vitro* selection methods[85].

Enrichment might only be part of the problem though as the instability of the thioester and ester-linked peptides - due to intrinsic half-life, aforementioned promiscuous esterase activity, or inadvertent fragmentation within the mass spectrometer - might make detection challenging by mass spectrometry. The difficulties encountered with directly mapping ester-linked sites on immunoprecipitated proteins are evidence of this[39,86]. For answers on how this could be addressed for cysteine ubiquitination it is prudent to look beyond the Ub system. For example, palmitoylation is a PTM that involves cysteine acylation with lipid groups. In a bid to identify sites of cysteine palmitoylation in a proteome, Thinon *et al.* showed it is possible to exploit the reactivity of cysteine and thioesters with a "protect, expose and label" strategy **(Figure 4b)** [87]. Despite its limitations, this

technique could be applied immediately to thioester linked ubiquitination. Conceptually similar approaches might be applicable to ester-linked and phosphoribosyl ubiquitination where yet to be developed "enrichable" chemical probes selectively cleave ester- and phosphoribosyl-linked Ub with stable and concomitant attachment to the substrate. These would be particularly powerful in enabling proteome-wide mapping and imaging of unconventional ubiquitination sites and should address the pitfalls described with antibody-based enrichment strategies **(Figure 4c)**.

Another PTM that involves an ester linkage is ADP ribosylation of acidic Asp/Glu residues[88]. A "clickable" aminooxy probe has been developed that cleaves the ester and becomes covalently linked to the side chain acyl component, serving as a valuable tool for the detection and study of this PTM[89]. Similarly, it might be used to selectively cleave ester-linked Ub leaving a covalent probe on the C-terminus of Ub molecules that were once esterified, which could grant insights into the architecture of mixed polyUb chains containing both canonical and non-canonical linkages.

Probes for Phosphoribosyl Ubiquitination

Although catalytically inactive DupA variants can be used as substrate traps for identifying phosphoribosyl ubiquitinated substrates[22], site information is not reported and their modest affinity might compromise recovery of low abundance substrates. Complementary approaches might be learned from classic chemical biological strategies for mapping kinase phosphorylation sites[90]. Thio analogues of ATP are processed by many kinases leading to transfer of a thiophosphate group to substrates, the distinct chemistry of which enables a catch and release strategy[90]. As SidE proteins require a phosphate-containing NAD^+ cofactor, it might be possible to implement an analogous strategy for phosphoribosyl ubiquitination sites**.**

An important outstanding question is whether phosphoribosyl ubiquitination is a part of normal physiology or only a pathophysiological process in the context of Legionella infection.

Unbiased chemical probes are likely to be key to addressing this. Stable triazole-linked analogues of ADPr-Ub have been developed, which serve as affinity probes for enzymes that demonstrate phosphodiesterase activity and are effective at enriching DupA from lysates **(Figure 5a and b)** [91]. However, SidE-like ligases, likely to have lower affinity than Dups[22], might not be compatible with these probes. An alternative strategy might be to develop activity-based probes (ABPs) that form a covalent linkage with their enzyme targets[92]. ABPs contain an electrophilic warhead that typically modifies an active site nucleophile in the enzyme active site and a prime candidate in SidE and its potential homologues would be the anticipated histidine residue that forms the phosphoramidate intermediate (**Figure 2)**. ABPs that target histidine residues in a single step have not been reported. However, a lysine residue in the active site of most kinases can be targeted with ABPs containing an acyl phosphate warhead[93]. Judicious introduction of such an acyl phosphate moiety into a biotin carrying ADPr-Ub analogue might serve as an ABP for SidE-like ligases, potentially enabling the enrichment and discovery of mammalian homologues **(Figure 5b)**.

Chemical Protein Synthesis and Semisynthesis

What has enabled breakthroughs in the field of canonical ubiquitination is the capacity to prepare defined Ub chains and ubiquitinated substrates using chemical rather than enzymatic methods. These have then been used to investigate the effects of ubiquitination on protein structure and function, and to determine the DUBs that process them **(Figure 6)** [94]. Access to homogenous Ub conjugates is a challenge because the enzymes that carry out the modification are often unknown, are not amenable to large scale reconstitution, or modify multiple sites *in vitro*. Fortuitously, the size and stability of the Ub molecule makes it the perfect subject for total chemical protein synthesis and semisynthesis. Thus, a suite of chemically produced Ub variants has been generated, serving as valuable tools for structural studies and biochemical studies into the canonical Ub code[95]. The sole report of chemical ester-linked ubiquitination is the total synthesis of the model substrate α-globin

ubiquitinated on a threonine residue[56]. To achieve site-specific attachment, esterification of a defined threonine side chain was achieved using orthogonal side chain protection. However, in the field of chemical lysine ubiquitination, amino acid derivatives that undergo chemoselective and traceless isopeptide bond formation have provided much-needed versatility, enabling convergent chemical synthesis of more complex conjugates and even chemoselective ubiquitination of recombinant proteins[94]. Application of these principles to non-lysine ubiquitination would enable access to defined non-lysine ubiquitinated substrates whose study will shed further light on the function of this modification. Another exciting strategy would be to program non-lysine ubiquitination in live cells by combining genetic code expansion with transpeptidation, as has been demonstrated with lysine ubiquitination [96].

As for protein ubiquitination, chemical biology methods enabling the production of homogenously ubiquitinated metabolites and analogues thereof will be instrumental to the study of non-protein substrates facilitating the delineation of non-protein ubiquitination, the identification of downstream effectors and processing enzymes. Conveniently for chemical biologists, the Ub molecule can be efficiently folded from a denatured state. Hence, an advantage with non-proteinaceous substrates is the freedom to explore diverse solvent systems, and in turn expand the repertoire of chemical reactions at their disposal.

Proteomics to Metabolomics

The study of non-proteinaceous ubiquitination presents novel challenges that will demand a venture into technologies not currently associated with Ub research. For example, the holistic analysis of non-proteinaceous substrates might be attainable by applying customized metabolomic technologies. Discovery and targeted mass spectrometry-based developments are available for analysing the metabolome and the signature diglycine remnant installed on ubiquitinated protein substrates would also remain on those that are non-proteinaceous[97]. As many metabolite spectra

remain unannotated[98,99], simply adding diglycine adducts to nucleophile-containing metabolites within existing metabolomic analysis might reveal further non-proteinaceous substrates. It is conceivable that these could be enriched using existing antibodies or chemical biology strategies discussed above.

Concluding Remarks

The advent of novel amino acid targets, linkage chemistries and non-proteinaceous substrates extends both the scope and regulatory mechanisms available to the Ub system. Of the amino acids that can be targeted by non-lysine ubiquitination only serine, threonine and cysteine have been reported. It remains to be seen if ubiquitination of tyrosine occurs in cells. Dedicated writers and erasers of ester-linked Ub demonstrate that these modifications are not a peculiarity, but a nuanced and significant branch of Ub signalling. Currently, a comprehensive understanding of the role and requirement of non-lysine ubiquitination can only be speculated and urgently needed tools such as those discussed herein will be instrumental for comprehensively mapping non-lysine ubiquitination sites on a systems level, thereby establishing its scale and enabling the testing of hypotheses relating to its cellular function. Exactly how many E2s have non-lysine activity is unknown. Excitingly, due to their modular nature where a single enzyme can support the activity of a multitude of E3s, new discoveries could associate non-lysine ubiquitination with a plethora of cellular functions. Key to gaining a deeper understanding of the underlying biology of non-lysine ubiquitination is understanding how it is dynamically regulated by the opposing action of E3s and DUBs. The realisation that one quarter of DUBs are loaded with the capacity to deubiquitinate threonine, together with DUBs that are highly selective, means non-lysine ubiquitination might be subject to extensive regulation.

Whilst the scale of non-lysine protein ubiquitination has caught the field by surprise, the discovery of non-proteinaceous ubiquitination has led to a seismic shift in how we should consider

ubiquitination, which should lead to a new dawn of Ub research where we encompass metabolomic expertise and methods. Thus, tools for identifying non-lysine substrates, whether they are proteinaceous or not, are urgently needed. Chemical biology has provided a plethora of techniques and technologies, the principles of which could be applied to non-canonical ubiquitination. It is hoped that this article will inspire interest and innovation in the field and precipitate the development of new tools to accelerate further study.

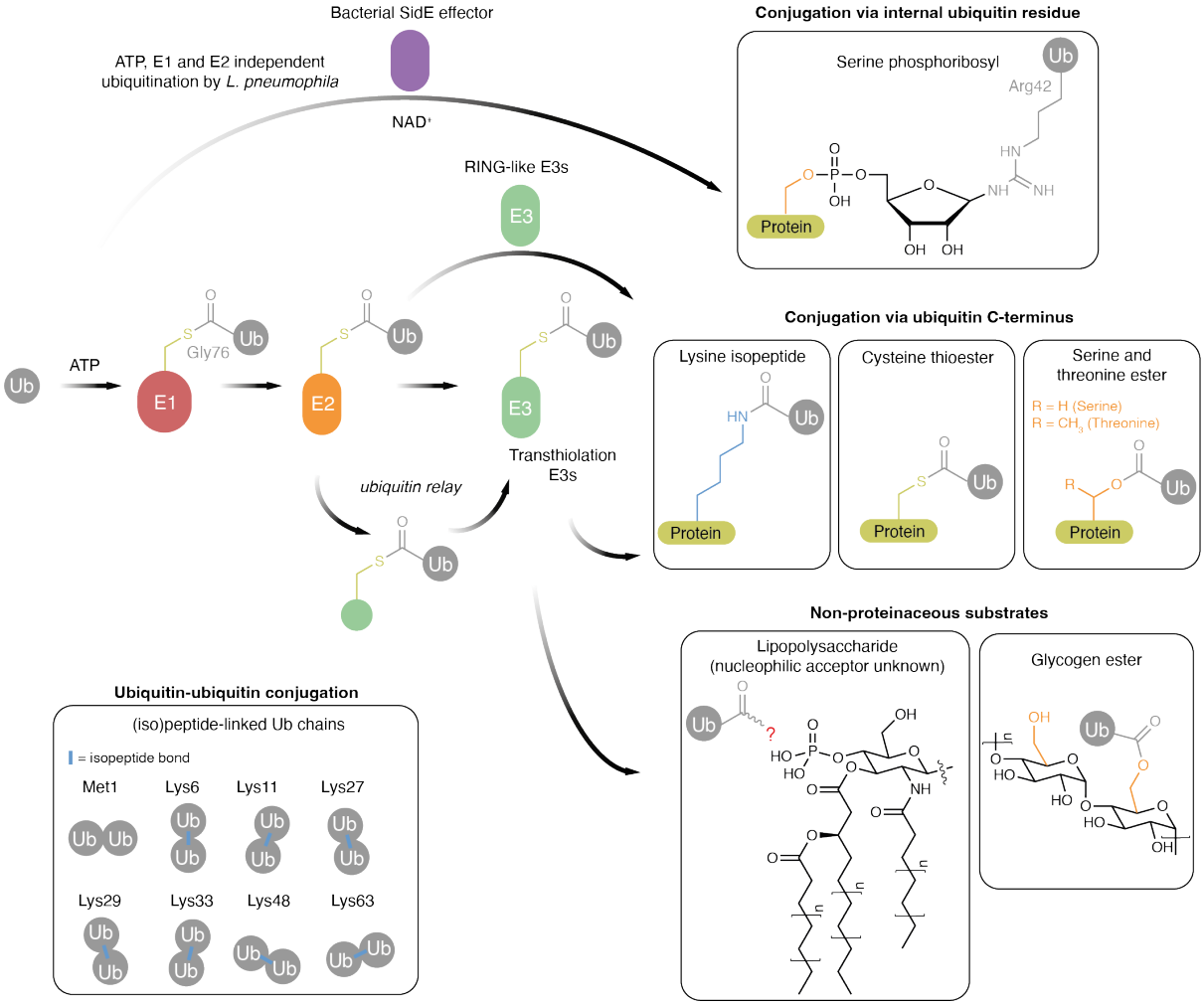

Figure 1. Substrate classes and linkage chemistries of the ubiquitin system. Ubiquitin transfer is an ATP-initiated process carried out by E1, E2 and E3 enzymes. Ubiquitin relay, where an intermediary catalytic cysteine operates in between E2 and the conventional E3 cysteine, is a newly discovered paradigm that might be associated with E3s with unconventional substrates. E3 enzymes recruit substrates and eukaryotic enzymes conjugate the C-terminal glycine 76 residue of ubiquitin to nucleophilic acceptors within protein substrates. Acceptor chemistry can be determined by E2 or E3. Ubiquitin itself can also be a substrate giving rise to elaborate ubiquitin chains of multiple linkage types. Non-proteinaceous substrates of the eukaryotic ubiquitin system are beginning to emerge. The bacterium *Legionella pneumophila* encodes effectors of the SidE family and uses NAD^+ as a cofactor to conjugate arginine 42 of ubiquitin to serine residues in protein substrates via a phosphoribosyl linkage.

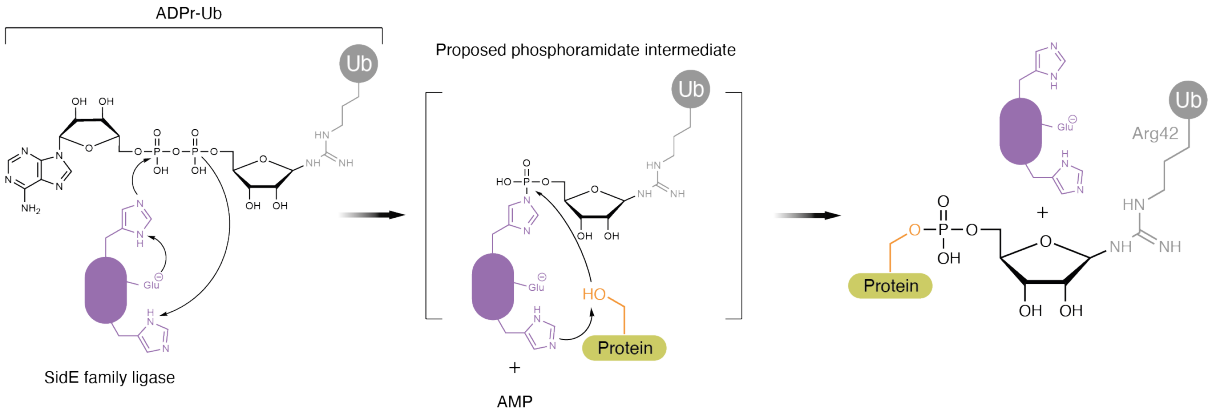

Figure 2. Mechanism of phosphodiesterase domain (PDE) catalyzed phosphoribosyl ubiquitination of serine residues by SidE effector ligases. Initial ADP-ribosylation of Ub Arg42 by the mono ADP-ribosyltransferase (mART) domain generates ADPr-Ub with release of nicotinamide (not shown). Next (shown), two catalytic histidine residues in a distinct PDE domain catalyze phosphoribosyl ubiquitination of a serine residue in a protein substrate. Facilitated by Glu340 and His407, His277 undergoes nucleophilic attack of the β-phosphate in ADPr-Ub to form a phosphoramidate intermediate. His407 also serves as general base to deprotonate the serine hydroxy group of the substrate.

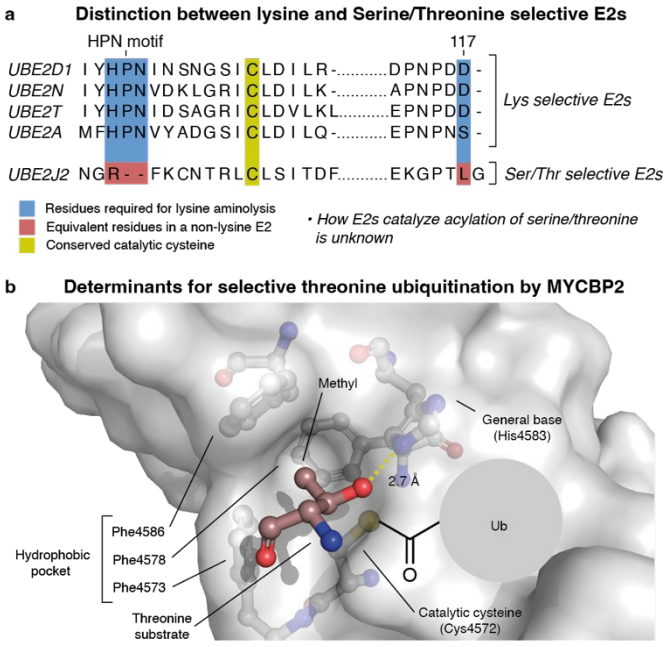

Figure 3. Current understanding of the determinants of non-lysine ubiquitination. (A) E2s with demonstrated lysine reactivity contain an HPN motif and a D/S residue (blue) at position 117 (UBE2D1 residue numbering). Strikingly, UBE2J2 which preferentially ubiquitinates Ser/Thr residues over Lys lacks the HPN motif and unusually contains a leucine at position 117. **(B)** The RCR E3 MYCBP2 has two catalytic cysteines that participate in a ubiquitin relay mechanism. In the crystal structure of the

downstream site (shown in gray ball and stick and surface representation) a proximal threonine residue (mauve ball and stick) forms several substrate-like interactions. For clarity, a catalytic thioester-linked Ub intermediate has been added in cartoon format. PDB ID: 5O6C.

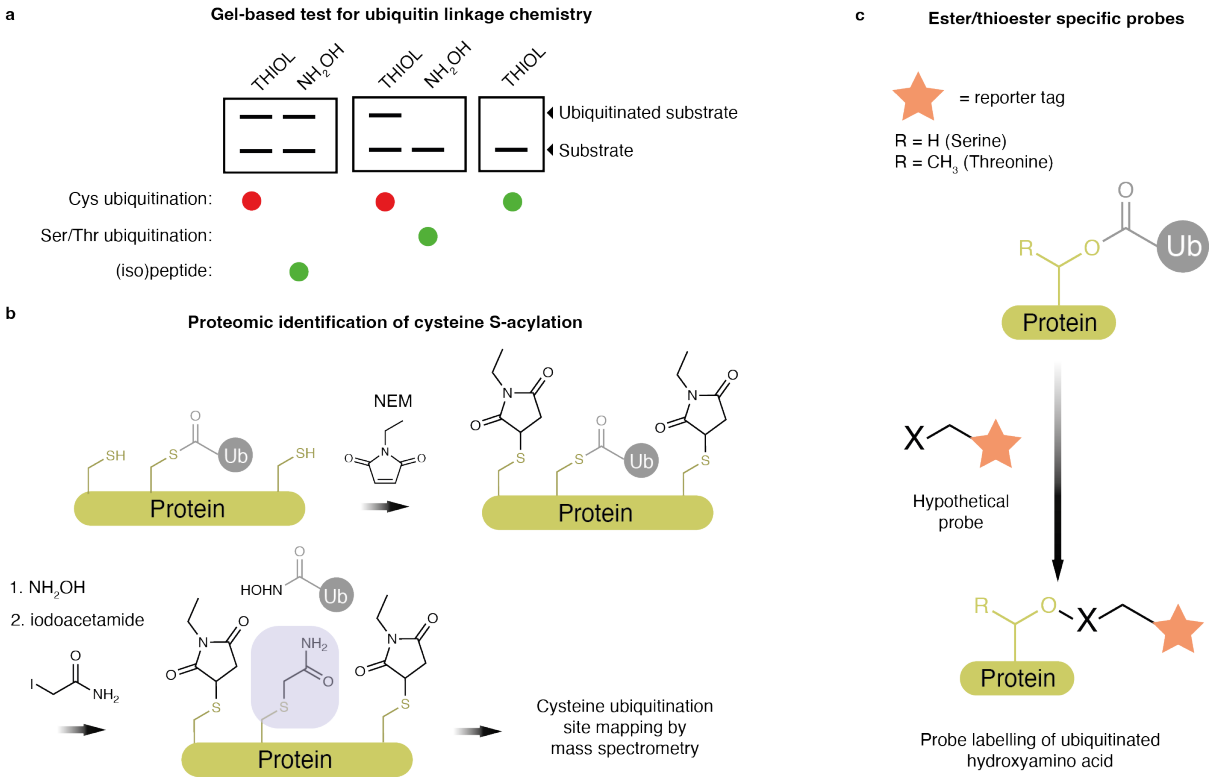

Figure 4. Established, transferrable and hypothetical approaches for studying non-lysine ubiquitination. A) Except for the very largest of proteins, ubiquitinated and non-ubiquitinated proteoforms can be resolved by SDS-PAGE. Therefore, prior treatment of the ubiquitinated protein sample with reagents that selectively cleave the different ubiquitin linkages can be used to diagnose their chemistry. This is because thioesters undergo thiolysis in the presence of thiols (e.g. 2-mercpatoethanol) whereas esters and amides are resistant. On the other hand, thioesters and esters can be cleaved with hydroxylamine (NH_2OH) and moderate hydroxide concentrations, whereas amides are resistant to both treatments. **B**) As thioester-linked cysteine residues are chemically protected from alkylation, unmodified cysteines can be capped with N-ethylmaleimide (NEM). Thioesters formed through S-acylation can then be cleaved with hydroxylamine followed by orthogonal cysteine capping with iodoacetamide. Cysteine carbamidomethylation (+57 Da) can be detected by mass spectrometry and used as a proxy for S-acylation sites, which would include cysteine ubiquitination. **C)** Probes that selectively cleave ester/thioester-linked Ub and become covalently attached to the acylated residues within protein substrates would be powerful tools enabling enrichment, proteomic mapping, and cellular imaging of non-lysine ubiquitination sites.

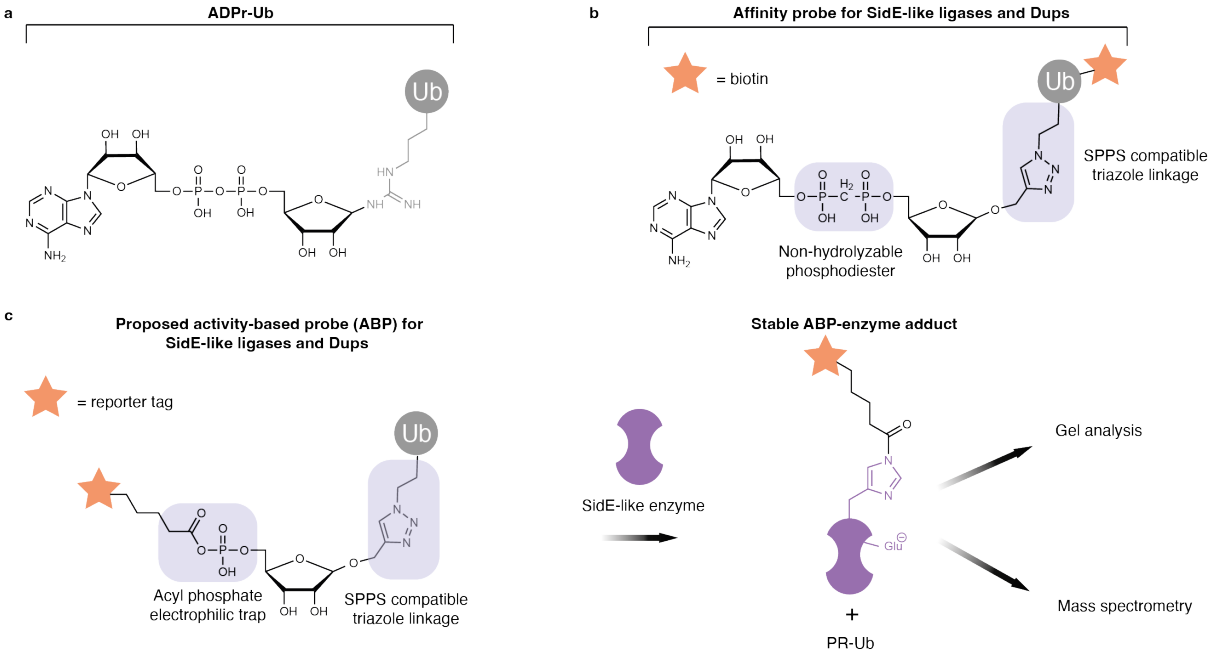

Figure 5. Chemical probes for studying writers and erasers of phosphoribosyl ubiquitination. (A) ADP-ribosyl-ubiquitin (ADPr-Ub) consists of a catalytically labile phosphodiester and a heat and base labile Arg-ribose linkage. **(B)** An ADPr-Ub analogue that serves as an affinity probe has been developed that contains a non-hydrolyzable phosphodiester[91]. To facilitate solid phase peptide synthesis (SPPS), a triazole mimic of the labile Arg-ribose linkage was introduced. **(C)** Hypothetical activity-based probe for ADPr-Ub phosphodiesterase activity. Electrophilic acyl phosphate traps have been successfully used to covalently label lysine residues and might be suitable for activity-based covalent labelling of the catalytic histidine in SidE effectors and potential mammalian SidE-like ligases.

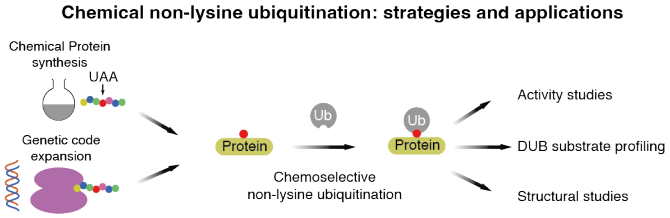

Figure 6. Chemoselective non-lysine ubiquitination. The design and incorporation of an unnatural amino acid (UAA) that undergoes chemoselective non-lysine ubiquitination would provide a necessary and general strategy for the preparation of ubiquitinated substrates. Study of these (semi)synthetic conjugates would enable insights into the function of non-lysine ubiquitination.

Acknowledgements

We thank members of the Virdee lab and Dr Ian Kelsall for critical feedback on our manuscript. S.V. and D.S. are funded by the UK Medical Research Council (MC_UU_12016/8). We also acknowledge pharmaceutical companies supporting the Division of Signal Transduction Therapy (Boehringer-Ingelheim, GlaxoSmithKline and Merck KGaA).

Competing Interests

S.V. is a founder and shareholder of Outrun Therapeutics, a biotech company investigating the ubiquitin system. D.R.S. has no competing interests.

References

1 Oh, E., Akopian, D. & Rape, M. Principles of Ubiquitin-Dependent Signaling. *Annu Rev Cell Dev Biol* **34**, 137-162, doi:10.1146/annurev-cellbio-100617-062802 (2018).

2 Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu Rev Biochem* **67**, 425-479, doi:10.1146/annurev.biochem.67.1.425 (1998).

3 Liao, Y., Sumara, I. & Pangou, E. Non-proteolytic ubiquitylation in cellular signaling and human disease. *Commun Biol* **5**, 114, doi:10.1038/s42003-022-03060-1 (2022).

4 Komander, D. & Rape, M. The ubiquitin code. *Annu Rev Biochem* **81**, 203-229, doi:10.1146/annurev-biochem-060310-170328 (2012).

5 Akutsu, M., Dikic, I. & Bremm, A. Ubiquitin chain diversity at a glance. *J Cell Sci* **129**, 875-880, doi:10.1242/jcs.183954 (2016).

6 French, M. E., Koehler, C. F. & Hunter, T. Emerging functions of branched ubiquitin chains. *Cell Discov* **7**, 6, doi:10.1038/s41421-020-00237-y (2021).

7 Komander, D. *et al.* Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. *EMBO Rep* **10**, 466-473, doi:10.1038/embor.2009.55 (2009).

8 Wang, X., Connors, R., Harris, M. R., Hansen, T. H. & Lybarger, L. Requirements for the selective degradation of endoplasmic reticulum-resident major histocompatibility complex class I proteins by the viral immune evasion molecule mK3. *J Virol* **79**, 4099-4108, doi:10.1128/JVI.79.7.4099-4108.2005 (2005).

9 Cadwell, K. & Coscoy, L. Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase. *Science* **309**, 127-130, doi:10.1126/science.1110340 (2005).
This manuscript describes cysteine ubiquitination carried out by a viral E3 ligase and represents the first report of non-lysine ubiquitination.

10 Cadwell, K. & Coscoy, L. The specificities of Kaposi's sarcoma-associated herpesvirus-encoded E3 ubiquitin ligases are determined by the positions of lysine or cysteine residues within the intracytoplasmic domains of their targets. *J Virol* **82**, 4184-4189, doi:10.1128/JVI.02264-07 (2008).

11 Wang, X. *et al.* Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. *J Cell Biol* **177**, 613-624, doi:10.1083/jcb.200611063 (2007).

12 Wang, X. *et al.* Ube2j2 ubiquitinates hydroxylated amino acids on ER-associated degradation substrates. *J Cell Biol* **187**, 655-668, doi:10.1083/jcb.200908036 (2009).
This paper reveals that the endoplasmic reticulum-associated E2 conjugating enzyme UBE2J2 preferentially ubiquitinates serine and threonine residues thereby establishing a physiological role for non-lysine ubiquitination.

13 Pao, K. C. *et al.* Activity-based E3 ligase profiling uncovers an E3 ligase with esterification activity. *Nature* **556**, 381-385, doi:10.1038/s41586-018-0026-1 (2018).
Here, activity-based probes (ABPs) are used to discover that the neuron-associated E3 ligase MYCBP2 belongs to a novel RING-Cys-Relay (RCR) class of transthiolating E3 with non-lysine ubiquitination activity.

14 Kelsall, I. R., Zhang, J., Knebel, A., Arthur, J. S. C. & Cohen, P. The E3 ligase HOIL-1 catalyses ester bond formation between ubiquitin and components of the Myddosome in mammalian cells. *Proc Natl Acad Sci U S A* **116**, 13293-13298, doi:10.1073/pnas.1905873116 (2019).
This report reveals that the RBR E3 ligase HOIL-1 ubiquitinates components of the Myddosome signalling complex establishing a role for non-lysine ubiquitination in immune system signalling.

15 Rodriguez Carvajal, A. *et al.* The linear ubiquitin chain assembly complex (LUBAC) generates heterotypic ubiquitin chains. *Elife* **10**, doi:10.7554/eLife.60660 (2021).

This paper demonstrates that ester linkages within complex polyubiquitin chains are formed in cells by the E3 HOIL-1 in response to tumor necrosis factor (TNF) stimulation.

16 Otten, E. G. *et al.* Ubiquitylation of lipopolysaccharide by RNF213 during bacterial infection. *Nature* **594**, 111-116, doi:10.1038/s41586-021-03566-4 (2021).
This paper shows that the Moyamoya disease-associated E3 ligase RNF213 ubiquitinates the lipopolysaccharide bacterial coat of infecting *Salmonella* thereby restricting its proliferation.

17 Ahel, J. *et al.* E3 ubiquitin ligase RNF213 employs a non-canonical zinc finger active site and is allosterically regulated by ATP. *bioRxiv*, 2021.2005.2010.443411, doi:10.1101/2021.05.10.443411 (2021).

18 Ahel, J. *et al.* Moyamoya disease factor RNF213 is a giant E3 ligase with a dynein-like core and a distinct ubiquitin-transfer mechanism. *Elife* **9**, doi:10.7554/eLife.56185 (2020).

19 De Cesare, V. *et al.* Deubiquitinating enzyme amino acid profiling reveals a class of ubiquitin esterases. *Proc Natl Acad Sci U S A* **118**, doi:10.1073/pnas.2006947118 (2021).
The first comprehensive assessment of whether DUBs can remove ubiquitin from non-lysine amino acids and reveals that the Machado-Joseph Disease (MJD) DUB class have highly selective esterase activity towards model substrates.

20 Qiu, J. *et al.* Ubiquitination independent of E1 and E2 enzymes by bacterial effectors. *Nature* **533**, 120-124, doi:10.1038/nature17657 (2016).
A striking paper reporting that SidE-effectors from *Legionella* ubiquitinate ER proteins independent of E1 and E2 enzymes but require NAD^+.

21 Bhogaraju, S. *et al.* Phosphoribosylation of Ubiquitin Promotes Serine Ubiquitination and Impairs Conventional Ubiquitination. *Cell* **167**, 1636-1649 e1613, doi:10.1016/j.cell.2016.11.019 (2016).
This paper demonstrates that SidE effectors carry out phosphoribosyl ubiquitination by linking Arg42 of ubiquitin to serine residues within protein substrates. It is revealed that a cryptic phosphodiesterase domain is required for conjugation and the ADPr-Ub intermediate inhibits native ubiquitin conjugation.

22 Shin, D. *et al.* Regulation of Phosphoribosyl-Linked Serine Ubiquitination by Deubiquitinases DupA and DupB. *Mol Cell* **77**, 164-179 e166, doi:10.1016/j.molcel.2019.10.019 (2020).

23 Kelsall, I. R. *et al.* HOIL-1 ubiquitin ligase activity targets unbranched glucosaccharides and is required to prevent polyglucosan accumulation. *EMBO J*, e109700, doi:10.15252/embj.2021109700 (2022).

24 Yang, C. S. *et al.* Ubiquitin Modification by the E3 Ligase/ADP-Ribosyltransferase Dtx3L/Parp9. *Mol Cell* **66**, 503-516 e505, doi:10.1016/j.molcel.2017.04.028 (2017).

25 Scheffner, M., Nuber, U. & Huibregtse, J. M. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* **373**, 81-83, doi:10.1038/373081a0 (1995).

26 Wenzel, D. M., Lissounov, A., Brzovic, P. S. & Klevit, R. E. UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. *Nature* **474**, 105-108, doi:10.1038/nature09966 (2011).
This paper discovers the RING-in-Between-RING (RBR) class of E3s, seeding the notion that E3 diversity has been under appreciated. Also, provides important insights into how the amino acid reactivity profile an E2 is determined.

27 Song, J. *et al.* Stability of thioester intermediates in ubiquitin-like modifications. *Protein Sci* **18**, 2492-2499, doi:10.1002/pro.254 (2009).

28 Cruz Walma, D. A., Chen, Z., Bullock, A. N. & Yamada, K. M. Ubiquitin ligases: guardians of mammalian development. *Nat Rev Mol Cell Biol*, doi:10.1038/s41580-021-00448-5 (2022).

29 Chen, Z. & Pickart, C. M. A 25-kilodalton ubiquitin carrier protein (E2) catalyzes multi-ubiquitin chain synthesis via lysine 48 of ubiquitin. *J Biol Chem* **265**, 21835-21842 (1990).

30 Hofmann, R. M. & Pickart, C. M. Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell* **96**, 645-653, doi:10.1016/s0092-8674(00)80575-9 (1999).
31 Rodriguez, M. S., Dargemont, C. & Hay, R. T. SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J Biol Chem* **276**, 12654-12659, doi:10.1074/jbc.M009476200 (2001).
32 Sampson, D. A., Wang, M. & Matunis, M. J. The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for SUMO-1 modification. *J Biol Chem* **276**, 21664-21669, doi:10.1074/jbc.M100006200 (2001).
33 Jin, L., Williamson, A., Banerjee, S., Philipp, I. & Rape, M. Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. *Cell* **133**, 653-665, doi:10.1016/j.cell.2008.04.012 (2008).
34 Christensen, D. E., Brzovic, P. S. & Klevit, R. E. E2-BRCA1 RING interactions dictate synthesis of mono- or specific polyubiquitin chain linkages. *Nat Struct Mol Biol* **14**, 941-948, doi:10.1038/nsmb1295 (2007).
35 van de Weijer, M. L. *et al.* A high-coverage shRNA screen identifies TMEM129 as an E3 ligase involved in ER-associated protein degradation. *Nat Commun* **5**, 3832, doi:10.1038/ncomms4832 (2014).
36 Stewart, M. D., Ritterhoff, T., Klevit, R. E. & Brzovic, P. S. E2 enzymes: more than just middle men. *Cell Res* **26**, 423-440, doi:10.1038/cr.2016.35 (2016).
37 Shimizu, Y., Okuda-Shimizu, Y. & Hendershot, L. M. Ubiquitylation of an ERAD substrate occurs on multiple types of amino acids. *Mol Cell* **40**, 917-926, doi:10.1016/j.molcel.2010.11.033 (2010).
38 Ishikura, S., Weissman, A. M. & Bonifacino, J. S. Serine residues in the cytosolic tail of the T-cell antigen receptor alpha-chain mediate ubiquitination and endoplasmic reticulum-associated degradation of the unassembled protein. *J Biol Chem* **285**, 23916-23924, doi:10.1074/jbc.M110.127936 (2010).
39 Anania, V. G., Bustos, D. J., Lill, J. R., Kirkpatrick, D. S. & Coscoy, L. A Novel Peptide-Based SILAC Method to Identify the Posttranslational Modifications Provides Evidence for Unconventional Ubiquitination in the ER-Associated Degradation Pathway. *Int J Proteomics* **2013**, 857918, doi:10.1155/2013/857918 (2013).
40 Burr, M. L. *et al.* MHC class I molecules are preferentially ubiquitinated on endoplasmic reticulum luminal residues during HRD1 ubiquitin E3 ligase-mediated dislocation. *Proc Natl Acad Sci U S A* **110**, 14290-14295, doi:10.1073/pnas.1303380110 (2013).
41 McClellan, A. J., Laugesen, S. H. & Ellgaard, L. Cellular functions and molecular mechanisms of non-lysine ubiquitination. *Open Biol* **9**, 190147, doi:10.1098/rsob.190147 (2019).
42 Gao, P. *et al.* E3 ligase Nedd4l promotes antiviral innate immunity by catalyzing K29-linked cysteine ubiquitination of TRAF3. *Nat Commun* **12**, 1194, doi:10.1038/s41467-021-21456-1 (2021).
43 Pao, K. C. *et al.* Probes of ubiquitin E3 ligases enable systematic dissection of parkin activation. *Nat Chem Biol* **12**, 324-331, doi:10.1038/nchembio.2045 (2016).
44 Schaefer, A. M., Hadwiger, G. D. & Nonet, M. L. rpm-1, a conserved neuronal gene that regulates targeting and synaptogenesis in C. elegans. *Neuron* **26**, 345-356, doi:10.1016/s0896-6273(00)81168-x (2000).
45 Wan, H. I. *et al.* Highwire regulates synaptic growth in Drosophila. *Neuron* **26**, 313-329, doi:10.1016/s0896-6273(00)81166-6 (2000).
46 Zhen, M., Huang, X., Bamber, B. & Jin, Y. Regulation of presynaptic terminal organization by C. elegans RPM-1, a putative guanine nucleotide exchanger with a RING-H2 finger domain. *Neuron* **26**, 331-343, doi:10.1016/s0896-6273(00)81167-8 (2000).

47 Burgess, R. W. *et al.* Evidence for a conserved function in synapse formation reveals Phr1 as a candidate gene for respiratory failure in newborn mice. *Mol Cell Biol* **24**, 1096-1105, doi:10.1128/MCB.24.3.1096-1105.2004 (2004).
48 Mabbitt, P. D. *et al.* Structural basis for RING-Cys-Relay E3 ligase activity and its role in axon integrity. *Nat Chem Biol* **16**, 1227-1236, doi:10.1038/s41589-020-0598-6 (2020).
49 Virdee, S. An atypical ubiquitin ligase at the heart of neural development and programmed axon degeneration. *Neural Regen Res* **17**, 2347-2350, doi:10.4103/1673-5374.338992 (2022).
50 Han, S. *et al.* Pam (Protein associated with Myc) functions as an E3 ubiquitin ligase and regulates TSC/mTOR signaling. *Cell Signal* **20**, 1084-1091, doi:10.1016/j.cellsig.2008.01.020 (2008).
51 Xiong, X. *et al.* The Highwire ubiquitin ligase promotes axonal degeneration by tuning levels of Nmnat protein. *PLoS Biol* **10**, e1001440, doi:10.1371/journal.pbio.1001440 (2012).
52 Richter, K. T., Kschonsak, Y. T., Vodicska, B. & Hoffmann, I. FBXO45-MYCBP2 regulates mitotic cell fate by targeting FBXW7 for degradation. *Cell Death Differ* **27**, 758-772, doi:10.1038/s41418-019-0385-7 (2020).
53 Desbois, M. *et al.* Ubiquitin ligase activity inhibits Cdk5 to control axon termination. *PLoS Genet* **18**, e1010152, doi:10.1371/journal.pgen.1010152 (2022).
54 Crawley, O. *et al.* Autophagy is inhibited by ubiquitin ligase activity in the nervous system. *Nat Commun* **10**, 5017, doi:10.1038/s41467-019-12804-3 (2019).
55 Lange, S. M., Armstrong, L. A. & Kulathu, Y. Deubiquitinases: From mechanisms to their inhibition by small molecules. *Mol Cell* **82**, 15-29, doi:10.1016/j.molcel.2021.10.027 (2022).
56 Sun, H., Meledin, R., Mali, S. M. & Brik, A. Total chemical synthesis of ester-linked ubiquitinated proteins unravels their behavior with deubiquitinases. *Chem Sci* **9**, 1661-1665, doi:10.1039/c7sc04518b (2018).
57 Clague, M. J., Urbe, S. & Komander, D. Breaking the chains: deubiquitylating enzyme specificity begets function. *Nat Rev Mol Cell Biol* **20**, 338-352, doi:10.1038/s41580-019-0099-1 (2019).
58 Wu, X. *et al.* JOSD1 inhibits mitochondrial apoptotic signalling to drive acquired chemoresistance in gynaecological cancer by stabilizing MCL1. *Cell Death Differ* **27**, 55-70, doi:10.1038/s41418-019-0339-0 (2020).
59 Tait, S. W. *et al.* Apoptosis induction by Bid requires unconventional ubiquitination and degradation of its N-terminal fragment. *J Cell Biol* **179**, 1453-1466, doi:10.1083/jcb.200707063 (2007).
60 Tomaskovic, I., Gonzalez, A. & Dikic, I. Ubiquitin and Legionella: From bench to bedside. *Semin Cell Dev Biol*, doi:10.1016/j.semcdb.2022.02.008 (2022).
61 Kalayil, S. *et al.* Insights into catalysis and function of phosphoribosyl-linked serine ubiquitination. *Nature* **557**, 734-738, doi:10.1038/s41586-018-0145-8 (2018).
62 Akturk, A. *et al.* Mechanism of phosphoribosyl-ubiquitination mediated by a single Legionella effector. *Nature* **557**, 729-733, doi:10.1038/s41586-018-0147-6 (2018).
63 Robinson, C. G. & Roy, C. R. Attachment and fusion of endoplasmic reticulum with vacuoles containing Legionella pneumophila. *Cell Microbiol* **8**, 793-805, doi:10.1111/j.1462-5822.2005.00666.x (2006).
64 Zhang, M. *et al.* Members of the Legionella pneumophila Sde family target tyrosine residues for phosphoribosyl-linked ubiquitination. *RSC Chem Biol* **2**, 1509-1519, doi:10.1039/d1cb00088h (2021).
65 Chatrin, C. *et al.* Structural insights into ADP-ribosylation of ubiquitin by Deltex family E3 ubiquitin ligases. *Sci Adv* **6**, doi:10.1126/sciadv.abc0418 (2020).
66 Nilsson, J. *et al.* Polyglucosan body myopathy caused by defective ubiquitin ligase RBCK1. *Ann Neurol* **74**, 914-919, doi:10.1002/ana.23963 (2013).

67 Wang, K. *et al.* Whole-genome DNA/RNA sequencing identifies truncating mutations in RBCK1 in a novel Mendelian disease with neuromuscular and cardiac involvement. *Genome Med* **5**, 67, doi:10.1186/gm471 (2013).
68 Yunus, A. A. & Lima, C. D. Lysine activation and functional analysis of E2-mediated conjugation in the SUMO pathway. *Nat Struct Mol Biol* **13**, 491-499, doi:10.1038/nsmb1104 (2006).
69 Eddins, M. J., Carlile, C. M., Gomez, K. M., Pickart, C. M. & Wolberger, C. Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation. *Nat Struct Mol Biol* **13**, 915-920, doi:10.1038/nsmb1148 (2006).
70 Wu, P. Y. *et al.* A conserved catalytic residue in the ubiquitin-conjugating enzyme family. *EMBO J* **22**, 5241-5250, doi:10.1093/emboj/cdg501 (2003).
71 Horn-Ghetko, D. *et al.* Ubiquitin ligation to F-box protein targets by SCF-RBR E3-E3 super-assembly. *Nature* **590**, 671-676, doi:10.1038/s41586-021-03197-9 (2021).
72 Scott, D. C. *et al.* Two Distinct Types of E3 Ligases Work in Unison to Regulate Substrate Ubiquitylation. *Cell* **166**, 1198-1214 e1124, doi:10.1016/j.cell.2016.07.027 (2016).
73 Zhou, Z. S. *et al.* Competitive oxidation and ubiquitylation on the evolutionarily conserved cysteine confer tissue-specific stabilization of Insig-2. *Nat Commun* **11**, 379, doi:10.1038/s41467-019-14231-w (2020).
74 Radzicka, A. & Wolfenden, R. Rates of uncatalyzed peptide bond hydrolysis in neutral solution and the transition state affinities of proteases. *Journal of the American Chemical Society* **118**, 6105-6109, doi:DOI 10.1021/ja954077c (1996).
75 Carvalho, A. F. *et al.* Ubiquitination of mammalian Pex5p, the peroxisomal import receptor. *J Biol Chem* **282**, 31267-31272, doi:10.1074/jbc.M706325200 (2007).
76 Williams, C., van den Berg, M., Sprenger, R. R. & Distel, B. A conserved cysteine is essential for Pex4p-dependent ubiquitination of the peroxisomal import receptor Pex5p. *J Biol Chem* **282**, 22534-22543, doi:10.1074/jbc.M702038200 (2007).
77 El Magraoui, F. *et al.* The deubiquitination of the PTS1-import receptor Pex5p is required for peroxisomal matrix protein import. *Biochim Biophys Acta Mol Cell Res* **1866**, 199-213, doi:10.1016/j.bbamcr.2018.11.002 (2019).
78 Rautio, J., Meanwell, N. A., Di, L. & Hageman, M. J. The expanding role of prodrugs in contemporary drug design and development. *Nat Rev Drug Discov* **17**, 559-587, doi:10.1038/nrd.2018.46 (2018).
79 Choudhary, A. & Raines, R. T. An evaluation of peptide-bond isosteres. *Chembiochem* **12**, 1801-1807, doi:10.1002/cbic.201100272 (2011).
80 Kim, W. *et al.* Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell* **44**, 325-340, doi:10.1016/j.molcel.2011.08.025 (2011).
81 Akimov, V. *et al.* UbiSite approach for comprehensive mapping of lysine and N-terminal ubiquitination sites. *Nat Struct Mol Biol* **25**, 631-640, doi:10.1038/s41594-018-0084-y (2018).
82 Xu, G., Paige, J. S. & Jaffrey, S. R. Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling. *Nat Biotechnol* **28**, 868-873, doi:10.1038/nbt.1654 (2010).
83 Fuhs, S. R. *et al.* Monoclonal 1- and 3-Phosphohistidine Antibodies: New Tools to Study Histidine Phosphorylation. *Cell* **162**, 198-210, doi:10.1016/j.cell.2015.05.046 (2015).
84 Kee, J. M., Villani, B., Carpenter, L. R. & Muir, T. W. Development of stable phosphohistidine analogues. *J Am Chem Soc* **132**, 14327-14329, doi:10.1021/ja104393t (2010).
85 Tans, R. *et al.* Affimers as an alternative to antibodies for protein biomarker enrichment. *Protein Expr Purif* **174**, 105677, doi:10.1016/j.pep.2020.105677 (2020).
86 Lei, L., Bandola-Simon, J. & Roche, P. A. Ubiquitin-conjugating enzyme E2 D1 (Ube2D1) mediates lysine-independent ubiquitination of the E3 ubiquitin ligase March-I. *J Biol Chem* **293**, 3904-3912, doi:10.1074/jbc.RA117.001322 (2018).

87 Thinon, E., Fernandez, J. P., Molina, H. & Hang, H. C. Selective Enrichment and Direct Analysis of Protein S-Palmitoylation Sites. *J Proteome Res* **17**, 1907-1922, doi:10.1021/acs.jproteome.8b00002 (2018).
88 Kraus, W. L. PARPs and ADP-Ribosylation: 50 Years ... and Counting. *Mol Cell* **58**, 902-910, doi:10.1016/j.molcel.2015.06.006 (2015).
89 Morgan, R. K. & Cohen, M. S. A Clickable Aminooxy Probe for Monitoring Cellular ADP-Ribosylation. *ACS Chem Biol* **10**, 1778-1784, doi:10.1021/acschembio.5b00213 (2015).
90 Blethrow, J. D., Glavy, J. S., Morgan, D. O. & Shokat, K. M. Covalent capture of kinase-specific phosphopeptides reveals Cdk1-cyclin B substrates. *Proc Natl Acad Sci U S A* **105**, 1442-1447, doi:10.1073/pnas.0708966105 (2008).
91 Kim, R. Q. *et al.* Development of ADPribosyl Ubiquitin Analogues to Study Enzymes Involved in Legionella Infection. *Chemistry* **27**, 2506-2512, doi:10.1002/chem.202004590 (2021).
92 Niphakis, M. J. & Cravatt, B. F. Enzyme inhibitor discovery by activity-based protein profiling. *Annu Rev Biochem* **83**, 341-377, doi:10.1146/annurev-biochem-060713-035708 (2014).
93 Patricelli, M. P. *et al.* Functional interrogation of the kinome using nucleotide acyl phosphates. *Biochemistry* **46**, 350-358, doi:10.1021/bi062142x (2007).
94 Huppelschoten, Y. & van der Heden van Noort, G. J. State of the art in (semi-)synthesis of Ubiquitin- and Ubiquitin-like tools. *Semin Cell Dev Biol*, doi:10.1016/j.semcdb.2021.11.025 (2021).
95 Gui, W., Davidson, G. A. & Zhuang, Z. Chemical methods for protein site-specific ubiquitination. *RSC Chem Biol* **2**, 450-467, doi:10.1039/d0cb00215a (2021).
96 Fottner, M. *et al.* Site-specific ubiquitylation and SUMOylation using genetic-code expansion and sortase. *Nat Chem Biol* **15**, 276-284, doi:10.1038/s41589-019-0227-4 (2019).
97 Giera, M., Yanes, O. & Siuzdak, G. Metabolite discovery: Biochemistry's scientific driver. *Cell Metab* **34**, 21-34, doi:10.1016/j.cmet.2021.11.005 (2022).
98 Thiele, I. *et al.* A community-driven global reconstruction of human metabolism. *Nat Biotechnol* **31**, 419-425, doi:10.1038/nbt.2488 (2013).
99 Zamboni, N., Saghatelian, A. & Patti, G. J. Defining the metabolome: size, flux, and regulation. *Mol Cell* **58**, 699-706, doi:10.1016/j.molcel.2015.04.021 (2015).