

How to Target Viral and Bacterial Effector Proteins Interfering with Ubiquitin Signaling



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Abstract Ubiquitination is a frequently occurring and very diverse posttranslational modification influencing a wide scope of cellular processes. Ubiquitin (Ub) has the unique ability to form eight different lysine-linked polymeric chains, mixed chains and engages with ubiquitin-like (Ubl) molecules. The distinct signals evoked by specific enzymes play a crucial role in, for instance, proteasome-mediated protein degradation, cell cycle regulation, and DNA damage responses. Due to the large variety of cellular functions that this posttranslational modification influences, the enzymes that construct such Ub modifications, and subsequently control and degrade these signals, is enormous. In this chapter, we will discuss the current

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state-of-the-art of activity-based probes, reporter substrates, and other relevant tools based on Ub as recognition element, to study the enzymes involved in the complex system of ubiquitination.

1 Introduction

Protein activity is regulated by the attachment and removal of posttranslational modifications (PTMs). Nucleophilic side chain functionalities in the protein can be decorated with such PTMs aided by a large class of enzymes that can monitor the substrate and the need to activate or deactivate its activity. Some of those PTMs are intensively studied such as, for instance, phosphorylation, but other PTMs are less well understood due to their high complexity, such as glycosylation and ubiquitination. In the latter case, a 76 amino acid protein ubiquitin (Ub) is transferred mostly to the ϵ -amine of a lysine residue in a target protein. Ubiquitination is involved in almost all aspects of eukaryotic biology including the regulation of immune responses, cell cycle progression, and protein degradation. Ub is a stable protein with a denaturation temperature of over 80 °C that is highly conserved from yeast to man with only three respective changes. Another interesting feature of Ub is its ability to form polymeric Ub chains in which the ϵ -amine functionality of any of the seven internal lysine residues or the N-terminal amine of Ub can be linked to the C-terminal carboxylic acid of a subsequent Ub. Eight different topologies can thus be formed all exerting different signaling outcomes. Lys-48-linked poly-Ub, for instance, is involved in proteasome-mediated protein degradation, whereas the Lys-63-linked poly-Ub is implicated to play a role in DNA damage repair. Most linkage types have been connected to one or more specific functions but new discoveries keep expanding the repertoire of involvement of Ub in cell signaling. On top of this already complex system with eight different homotypic poly-Ub chains, the possibility to form mixed heterotypic chains and branched chains complicates the Ub system even further. Posttranslational modifications such as phosphorylation, acetylation and newly discovered ADP-ribosylation of Ub and hybrid chains with ubiquitin-like modifiers such as SUMO and NEDD8 are also found to play distinct roles. The numerical amount of combinations one could think of our vast, but are all biologically relevant?

The general mechanism of Ub attachment to a target protein or a predecessor Ub (forming a poly-Ub chain) depends on a series of so-called E1-, E2-, and E3-enzymes that work together to activate and ligate Ub to its target using ATP as energy source. This process, known as the canonical Ub cascade, can undergo repetitive cycles and lead to poly-ubiquitinated substrates. One of two E1 activating enzymes, known to date, adenylates the C-terminal carboxylic acid of Ub and subsequently forms a reactive thioester intermediate. One of ~ 40 E2 conjugating enzymes takes over the Ub cargo by means of a trans-thiolation reaction (Stewart et al. 2016). One of over 600 E3-enzymes encoded in the human genome either mediates the transfer of Ub from the E2 to the substrate or takes over the Ub cargo

itself prior to transferring it to the target, depending on whether the E3 belongs to the RING, HECT or RBR class of conjugating enzymes (Buetow and Huang 2016). The specific combination of E2/E3 not only ensures the correct target proteins are ubiquitinated but also dictates the type of poly-Ub linkage that is installed; actively controlling the signaling outcome of the ubiquitination event.

Counteracting the buildup of (poly-)ubiquitinated proteins is a group of deubiquitinating proteases (DUBs) that break down the ubiquitin modification, liberating the substrate protein, recycling Ub, and ending the Ub invoked signaling. Nearly a hundred of such deubiquitinating proteases have been identified in human cells that can be classified into seven distinct families. The subfamilies of ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumor domain proteases (OTUs), Machado–Joseph disease proteases (MJD), motif interacting with Ub-containing novel DUB family (MINDYs), and zinc finger with UFM1-specific peptidase domain protein (ZUFSPs) are cysteine proteases, whereas JAB1/MPN/MOV34 proteases (JAMMs) are zinc-dependent metallo-proteases.

To understand the complex signaling network brought about by the writers, readers, and erasers of the ubiquitin code, tools to study them in detail and on molecular level are of great interest. Below we describe the current state of the molecular toolbox available and three examples of their use in unraveling some of the secrets in viral and bacterial interference with ubiquitin biology.

2 The Current Toolbox

A diversity of tools has been developed to study the activities of enzymes involved in constructing and deconstructing ubiquitin chains and ubiquitinated proteins. The probes based on the Ub core as recognition element will be discussed below classified on their ability to target different enzymatic functions. Both chemical and semi-synthetic techniques are used to construct such probes, each having distinct advantages and drawbacks that, however, will not be discussed in great detail in this chapter.

2.1 DUB Targeting Covalent Probes

The first activity-based probe based on Ub forming a covalent complex between probe and target protease was based on replacing Gly75-Gly76 with 4-aminobutyraldehyde (Pickart and Rose 1986). The ubiquitin aldehyde (Ubal) reagent was found to form a complex and inhibit the activity of the UCH protease studied. Modifications on this reactive C-terminal element with, for instance, a nitrile moiety (Lam et al. 1997) and glycine vinyl sulfone (VS) or glycine vinyl methyl ester amine (VME) (Borodovsky et al. 2001, 2002) led to the development

of a bigger panel of activity-based probes able to capture the active site cysteines of deubiquitinating enzymes. These electron poor vinyl motifs act as Michael acceptor element and allow the sulfur nucleophile of the active site cysteine to be trapped by forming a covalent intermediate in an irreversible reaction. Later on, the total chemical synthesis of Ub (mutants) using solid phase peptide chemistry (El Oualid et al. 2010; Kumar et al. 2009; Pasunooti et al. 2009) opened the way to prepare Ub probes carrying fluorescent labels or affinity handles on large scale (de Jong et al. 2012). Exploiting a similar mode of action as Ub-VME is Ub-propargyl (Prg), where the terminal alkyne of the propargyl moiety unexpectedly is able to react with the active site cysteine leading to a covalent adduct in the form of a vinyl thioether linkage (see Fig. 1a) (Ekkebus et al. 2013). Major advantage of this last variant, Ub-Prg, is its unreactiveness toward other cysteine proteases, making it really a DUB-specific probe. In general, specificity of such probes for DUBs is based on the Ub element of the probe to be recognized by the protease on a binding interface or so-called S1-pocket preceding the active site placing the reactive

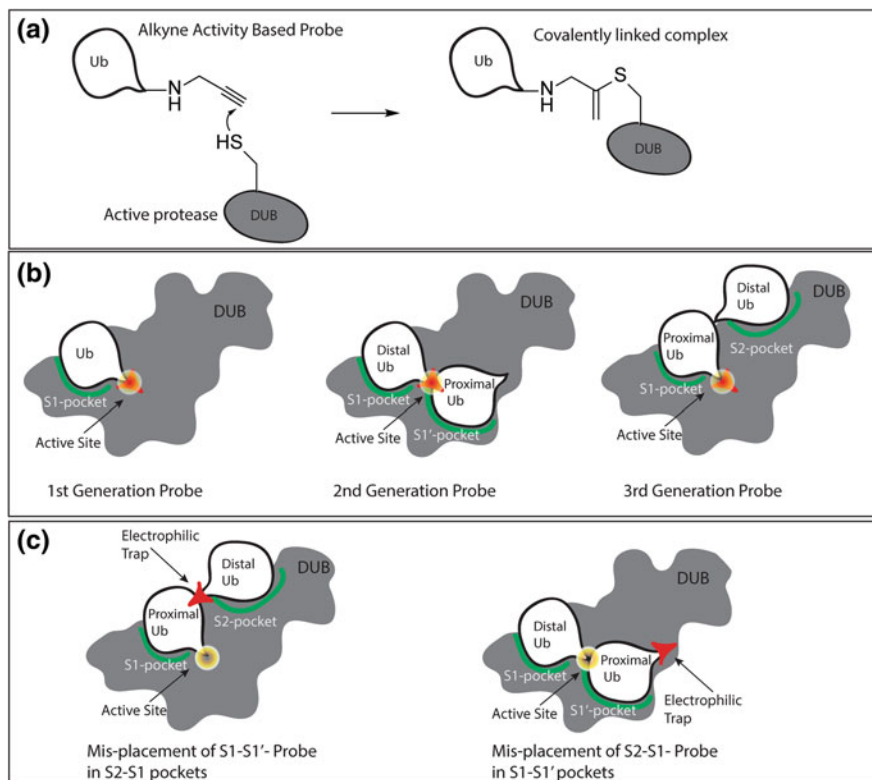


Fig. 1 Activity-based probes to target DUB activity. **a** Reaction details of cysteine protease reaction with Ub-Prg, **b** different probes targeting S1, S1-S1', or S1-S2 interactions, **c** misplacement of probes on DUBS allowing the study of different binding pockets

element directly over the active site cysteine. In this situation, the two reacting partners are optimally aligned and lead to the formation of a covalent adduct (see Fig. 1a).

Some DUBs have been shown to have a preference to specifically cleave certain poly-Ub chains. In order to investigate such possible preferences, in a classical experiment all native diUb molecules are incubated with a purified recombinant DUB and cleavage of the diUb is monitored over time. Biggest drawback of this method is that it is not compatible with complex biological settings (such as cell lysate), and it is limited to isolated DUBs. To overcome such issues, a second generation of probes to investigate linkage-specific proteolysis of DUBs has emerged more recently. These probes generally consist of two ubiquitin moieties carrying a Michael acceptor element in the isopeptide linkage region in-between the two Ub moieties. Initial reports show the two Ub regions to be linked together using non-native connections such as a triazole (McGouran Joanna et al. 2013) and thiol ether linkage (Li et al. 2014). Two type of probes presenting either a dehydroalanine (Dha) (Haj-Yahya et al. 2014) or VME-like electrophilic trap (Mulder et al. 2014) mimic the native lysine–glycine linkage the closest using amide linkages. A panel of all seven isopeptide-linked diUb probes can be constructed and used to covalently capture the active site cysteine of the DUB showing its reactivity and preference toward certain linkage types. Using such probes the N-terminal or distal Ub molecule will be positioned before the active site in the so-called S1-pocket, and the C-terminal or proximal Ub molecule will be positioned after the active site cysteine in the so-called S1'-pocket. Due to the geometrical differences between al Lys-linked diUb probes, the DUB will only be able to position the probes mimicking its natural substrates in such a way that the active site cysteine is able to react with the reactive element (see Fig. 1b).

Some DUBs are able to recognize Ub chain topologies using other binding surfaces further away from the active site, such as, for instance, an S2-site preceding the S1-site. A third generation of probes targeting such S2 binding sites has been developed where a diUb molecule is equipped with a reactive element at the proximal C-terminus (Flierman et al. 2016). These probes are only able to react with DUBs that contain a S2-site that plays a determining role in positioning the diUb molecule in the S2- and S1-sites thereby placing the alkyne directly over the active site cysteine (see Fig. 1b). Noteworthy is that the isopeptide linkage between proximal and distal Ub has been replaced by a protease stable triazole linkage, prohibiting the protease of interest to degrade the probe during assays.

If a DUB recognizes such a third generation diUb probe using its S1- and S1'-sites, the alkyne will not be in the vicinity of the active site cysteine and no covalent adduct will be formed. Conversely, if a second generation probe will be reacted with a DUB recognizing the diUb moiety using its S2- and S1-sites, no reaction will occur since the reactive element will not be aligned with the reactive cysteine (see Fig. 1c). Having access to both second and third generation probes offers an exciting combination to investigate the binding interfaces that play a role in determining binding preferences of DUBs and cast a light on their molecular mechanism of action.

More recently a new probe able to reversibly capture cysteine DUBs followed by the release of the still active proteases has been published, making use of subtle chemistry to form, and disrupt disulfide bridges between the active site cysteine and Ub-based thiol-containing probe (de Jong et al. 2017). Although only the proof of principal studies has been performed, this novel technology holds great promise for the future capture, release, and follow-up investigations of native active cysteine DUBs.

Not all DUBs are cysteine proteases, however, and the metallo-DUB family currently is understudied due to the lack of probes. Selective probes targeting such proteases are needed and will no doubtably be developed in the future.

The covalent capture of active DUBs with Ub-based activity-based probes allows for purification of the formed complexes and subsequent crystallization efforts to study the interactions between protease and Ub in detail. A large number of crystal structures has been solved using such Ub-based ABPs as is reviewed by van Tilburg et al. (2016). Synthetic procedures to prepare above-mentioned probes and reagents on large scale are in place and are finding their way into the related fields of Ub-like proteins such as, for instance, SUMO biology (Mulder et al. 2018).

2.2 *E1–E2–E3 Targeting Covalent Probes*

Due to the diversity and complexity in the conjugating and ligation machinery, development of probes targeting the constructing of ubiquitinated substrates has only emerged more recently than probes targeting the deconstructing proteases, for the simple reason that targeting a sequential enzymatic cascade is more difficult than targeting a single proteolytic step.

The first step in the cascade is activation of the C-terminal carboxylate of Ub by and E1-activating enzyme. The E1 firstly adenylates the carboxylic acid group of glycine 76 at the expense of ATP in one region of the E1 and subsequently the catalytic cysteine of the E1 in another region of the enzyme takes over the Ub cargo by forming a thioester and expelling AMP. Initial probes based on Ub₇₁-CGG-vinyl sulfonamides show reactivity toward the cysteine of yeast E1 Uba1, although the C-terminal Arg-Leu-Arg-Gly-Gly region of Ub is replaced by the smaller Cys-Gly-Gly vinyl adenyl sulfonamide, rendering a shorter Ub mutant with a RL deletion (see Fig. 2a) (Lu et al. 2010). These Ub probes are based on small molecule adenyl sulfonamide analogues that can also be used to monitor intracellular E1 activity and specific inhibitory potential toward different E1's (An and Statsyuk 2013; Misra et al. 2017). Other probes based on Ub₇₇-dehydroalanine adenyl amides also show reactivity toward a. o. Uba1 although these probes are longer than the native Ub₇₆ substrate (An and Statsyuk 2016).

Combined this data implies that unlike the active sites of DUBs the E1's are more tolerant toward their substrates or the flexibility of the C-terminal tail of Ub corrects for possible misalignment during the Ub transfer from the ATP binding

cascade, Ub transfer from E2 to E3 can be interrogated. A ubiquitin-charged E2 carrying a TDAE element allows for trapping the trans-thiolation event toward an E3, forming a stable complex between Ub, E2, and E3 (see Fig. 2b) (Pao et al. 2016). Of note is that in this last TDAE derived probe, the C-terminal RGG motif of Ub is replaced by the reactive TDAE element, which might limit the generality of such probes as it is implicated that R74 and the diGly motif can play an important role of recognition of the appropriate Ubl.

All approaches mentioned above are useful in specifically studying one step in the cascade of Ub-ligation. To amend the need to be able to pass down the probe through the complete cascade and target E1's, E2's, and E3's; simultaneously, a 'cascading'-probe based on a C-terminal dehydroalanine (Dha) moiety was developed (Mulder et al. 2016). This reactive element has been employed earlier in diUb probes targeting cysteine DUBs (Haj-Yahya et al. 2014), but was found in other studies to show low reactivity. This low reactivity can be harnessed in trapping the E1–E2–E3 enzymatic cascade consecutively (see Fig. 2c). The Ub₇₅Dha can be activated by an E1 to form a thioester intermediate and transferred to an E2 and subsequent RBR- or HECT-E3 via a trans-thiolation reaction. In each step, only a small portion of the active cysteines will be trapped by the Dha-moiety, while the rest of the cysteine will continue to function normal in the trans-thiolation step. By doing so, the probe will capture all components in the cascade, which has been shown to indeed be detectable using proteomic approaches in a proof of principle study.

The above-mentioned arsenal of probes has emerged very recently, and most likely the near future holds exciting insights in the ubiquitination cascade through implementation of these probes. Complicating factor in the investigation of E3-ligases is that of the three major classes of E3's only the HECT and RBR classes have an active cysteine residue that takes over the Ub cargo from an E2 and transfer it to the final substrate protein. RING E3s do not possess such an active site cysteine and merely serve as platforms to bring Ub charged E2's and substrates together, thereby making them unsuited for direct probing using ABPs.

2.3 Assay Reagents

Other type of probes or assay reagents based on a Ub scaffold have also been developed allowing the real-time monitoring of DUB or ligase activity. In contrast to the probes described above, these reagents do not have a Michael acceptor element and thus do not form a covalent complex with the targeted enzyme, but mostly rely on a fluorescent signal that is altered. Advantage of the probes described below is that one enzyme can perform multiple catalytic cycles, and hence, signal amplification occurs allowing a more accurate readout of the enzymes native activity and/or specificity.

An important class of Ub-based assay reagents is the fluorogenic Ub reagents, where a quenched fluorophore is conjugated at the C-terminus of Ub. DUB activity will cleave the amide bond at position 76, and the fluorophore will be released from the Ub and simultaneously start to fluoresce (see Fig. 3a). Hence, the increase in fluorescence is a direct measure of DUB activity. Fluorogenic reporters used in such type of reagents are aminomethylcoumarin (AMC) (Dang et al. 1998) or substituted rhodamine-110 (RHO) (Hassiepen et al. 2007) scaffolds that show favorable fluorescent properties. In a similar setup, DUB mediated aminoluciferin release can be assayed in a bioluminescence approach using a luciferase assay (Orcutt et al. 2012). All of these reporters are conjugated to a mono-Ub recognition element, and hence, the preference of a DUB to specific poly-Ub topologies cannot be assessed. To allow monitoring chain-specific proteolysis mediated by S1–S2 interactions on the DUB, diUb-AMC substrates were generated (Flierman et al. 2016). In analogy to the diUb-Prg covalent probes, these diUb-AMC substrates are linked via a non-hydrolysable triazole linker preventing proteolysis of the diUb entity during the assay.

One important note is that the fluorogenic substrates do not contain an isopeptide linkage at the side where the DUB would perform its proteolytic action, whereas the natural substrates for most DUBs would. To mimic this isopeptide link more closely, fluorescent polarization (FP) reagents were developed where Ub is conjugated via a native isopeptide linkage to a fluorophore carrying substrate derived peptide (Tirat et al. 2005; Geurink et al. 2012). Rationale behind these reagents is that once the fluorophore-containing peptide is linked to Ub, the large construct tumbles slowly in solution and light remains polarized, whereas after proteolysis the small fluorophore-containing peptide tumbles faster and hence the polarization of light decreases (see Fig. 3b). Such tools are not only reported for Ub but also for other Ubl's such as the three SUMO's, Nedd8, and ISG15.

Another type of reagent makes use of the fact that a fluorophore and quenching moiety can be placed in close proximity of each other on a diUb or Ub-substrate peptide and upon DUB action both entities are separated and hence fluorescence is restored (see Fig. 3c). Probes based on such a time-resolved fluorescence resonance energy transfer (FRET) principle consisting of Ub-peptide (Ohayon et al. 2012) and diUb moieties (Geurink et al. 2016; Ye et al. 2012) have been described. All seven lysine-linked diUb FRET pairs are available which form a platform to measure DUB preference and kinetic parameters in real time. Using similar technology, the E2 mediated constructing of poly-Ub chains equipped with a FRET acceptor onto a FRET donor-containing Ub results in a FRET signal (Madiraju et al. 2012). In this particular study, inhibitors for the E2 can be identified when a decrease in FRET signal is observed in the assay.

Although it was long assumed that in order to ubiquitinate the target substrate the complete cascade, so E1, E2, and E3 functions are needed, recent reports show that Ub-thioesters can be used in *in vitro* studies to monitor HECT- or RBR-E3 ligase activity. In these assays, the E1- and E2-functions are bypassed and

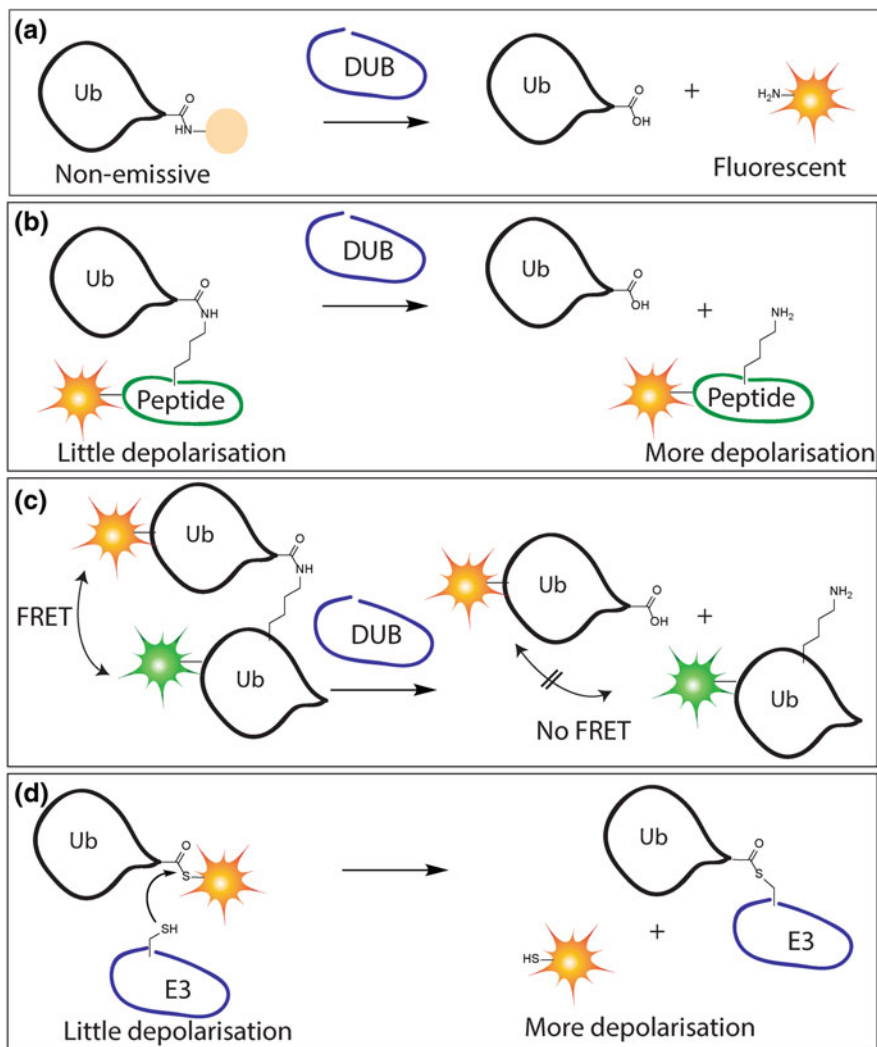


Fig. 3 Assay reagents based on Ub. **a** Fluorogenic substrates, **b** fluorescent polarization reagents to monitor DUB activity, **c** fluorescent energy transfer reagents, **d** fluorescent polarization reagents to monitor E3-ligase activity

Ub-thioesters [Ub-MES (Park et al. 2015) or fluorescent analogue Ub-FLUOR (Krist et al. 2016)] are directly trans-thiolated by the active cysteines in several E3's. The fluorescent thioester allows real-time quantitative monitoring of E3-ligase activity using a fluorescence polarization set up (Park et al. 2017) (see Fig. 3d).

2.4 (Poly)Ub Chains

All probes mentioned above are based on mono-ubiquitinated peptides, mono-Ub or diUb carrying a reactive or fluorescent entity. Moving forward in our understanding of the Ub system, it would be of great value to have access to well defined larger Ub chains and probes made thereof. Synthetic, semi-synthetic and biochemical methods are in place to produce poly-Ub chains, carrying native isopeptide linkages or artificial (non-hydrolysable) linkages.

Biochemical methodology to obtain all except K27-poly-Ub chains, are reported and rely on the use of linkage-specific E2/E3 enzyme combinations as has been reviewed elsewhere (Faggiano et al. 2016) (see Fig. 4a). Although the majority of poly-Ub chains can be produced, several drawbacks come with such methods. Crucial in biochemical production of Ub chains is that in order to have a defined polymer length, the mixture of simultaneously produced poly-Ub's needs to be separated, most frequently done using cation exchange chromatography. Inherent in producing polymers is that the isolated yield of one specific poly-Ub species is low and scalability of biochemical methodology hence can be an issue. Some of the E2/E3 combinations are not absolute topology specific and additional treatment using DUBs might be necessary to guarantee a homogenous preparation, further complicating procedures. All in all six out of seven lysine-linked poly-Ub chains can be prepared biochemically although preparation of high quantities of pure material still is a laborious task.

On the other hand, the total chemical synthesis of poly-Ub chains has been undertaken. Several methods using auxiliaries, different thiol modified amino acids and ligation/desulfurization strategies are in place as reviewed elsewhere (Spasser and Brik 2012), allowing for the sequential construction of Ub chains with the impressive highlight of K48-linked tetra-Ub (Kumar et al. 2011). Although for such time-consuming approaches a high level of expertise is required, it does allow the introduction of fluorophores, affinity handles or conjugation to substrate peptides or proteins. Of note is that using an isoUb native chemical ligation approach also K27 tetra-Ub chains and mixed chains have been prepared and employed in structural studies (Tang et al. 2017). In contrast to sequential strategies, chemical polymerization approaches using a bifunctional thiolysine/thioester-Ub mutants (van der Heden van Noort et al. 2017; Moyal et al. 2012) (see Fig. 4b) leading to native isopeptide-linked Ub chains, bifunctional thiol/ene Ub mutants leading to thioether-linked Ub chains (Trang et al. 2012) (see Fig. 4c) or azide/alkyne-Ub mutants leading to triazole-linked Ub chains (Schneider et al. 2014) (see Fig. 4d) give rise to larger poly-Ub analogues including the biochemical unavailable K27-linked Ub chains. Such stable poly-Ub chains can be employed to study the interacting proteins or 'readers' of the Ub code by performing pull-down experiments and subsequent proteomic analysis using mass spectrometry approaches (Zhao et al. 2017; Zhang et al. 2017).

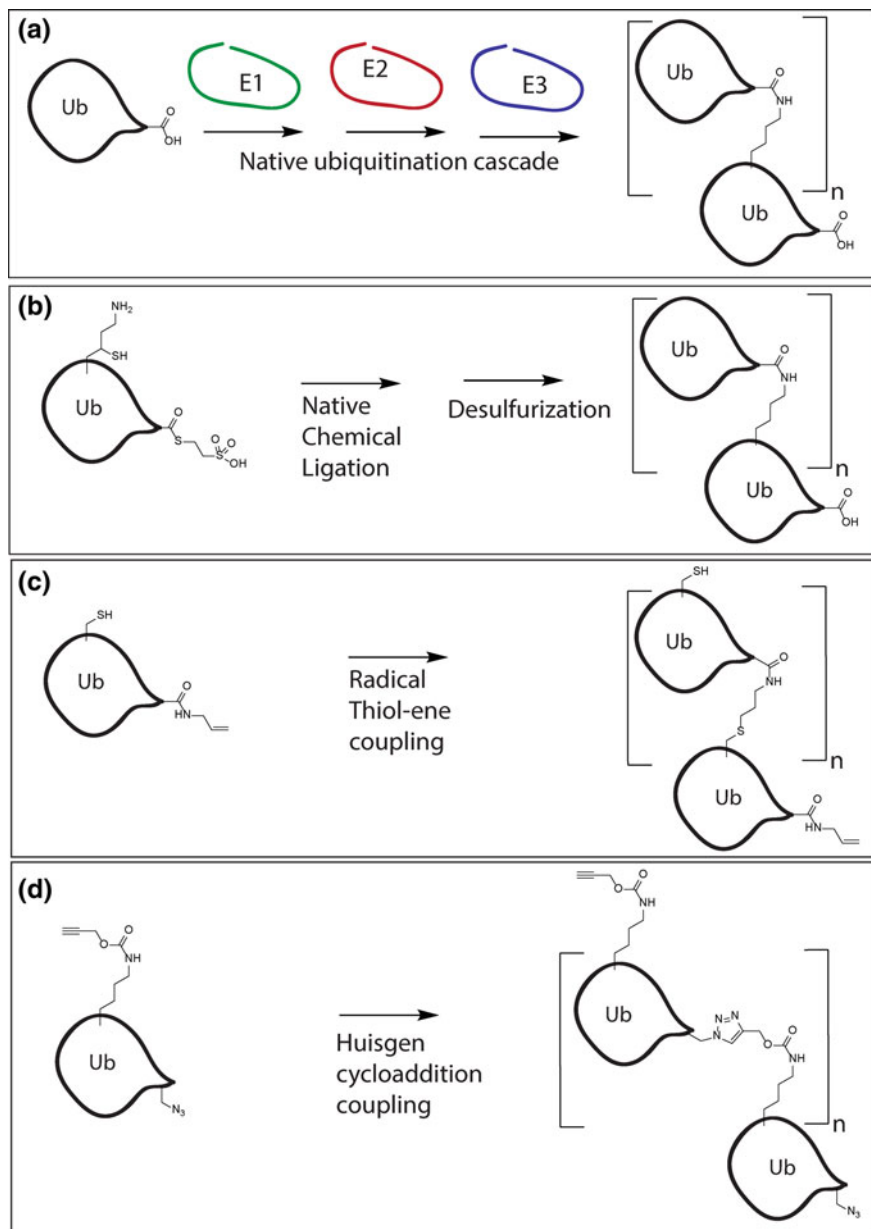


Fig. 4 Poly-Ub generation using **a** the native E1–E2–E3 cascade, **b** a native chemical ligation approach, **c** a thiol–ene radical coupling approach, and **d** a copper-catalyzed Huisgen cycloaddition approach

3 Applications

The above-described arsenal of ubiquitin-based probes, substrates, chains, and analogues are each designed to target-specific enzymatic functions or give insight into unanswered question regarding the ubiquitin–proteasome system. Apart from the proof of principle studies conducted during the development of these probes, it is important to note that these tools have made the translational step from the drawing board to actual biochemical and structural studies on viral and bacterial enzymes playing a role in the manipulation of (human) host pathways.

3.1 Applications in Virology

Viruses have defense mechanism in place to either utilize the host's ubiquitin system in their own advantage or to combat the host's immune response by suppressing pro-inflammatory Ub signaling. Human coronaviruses (hCoV), responsible for pandemic outbreaks of severe acute respiratory syndrome (SARS) in 2003 and Middle East respiratory syndrome (MERS) in 2012, are two of such viruses that are interfering with the host's Ub signaling. One way these coronaviruses act is by dampening the immune response through action of viral proteases that possess deubiquitinase and deISGylase functions. Interferon stimulated Gene 15 (ISG15) is a ubiquitin-like (Ubl) modification that has two Ub domains in tandem and is upregulated and attached to substrates during an antiviral response. The papain-like proteases (PLpro's) in both MERS and SARS hCoV are identified to cleave ISG15 and Ub from cellular proteins. In a study comparing MERS and SARS PLpro, it was found that fluorogenic substrates Ub-AMC and ISG15-AMC were both cleaved to a similar extent by MERS PLpro, where SARS PLpro preferred ISG15-AMC (Békés et al. 2015). Covalent capture of these proteases by ABP Ub-propargyl again showed a higher reactivity for the MERS then for the SARS protease. Cleavage assays using native K48-linked tetra-Ub also show a distinct pattern. SARS PLpro cleaves the Ub₄ into Ub₂ and is hardly able to process Ub₂ any further, whereas MERS PLPro cleaves Ub₄ into a mix of Ub₃, Ub₂, and Ub. SARS PLpro seems to have a di-distributive mechanism only making a cut after a Ub₂ moiety, whereas MERS is mono-distributive cleaving after one Ub residue. DiUb ABP's having the reactive element either at the proximal side of the Ub₂ or in-between the Ub moieties help to explain how these two different mechanism function, as MERS PLpro is able to react with the in-between probe and not the proximal probe, and SARS PLpro shows opposite reactivity toward these two probes (Bekes et al. 2016). Hence, activity of SARS PLPro seems to be largely influenced by an S2 site, whereas MERS PLpro does not seem to contain such a site. A crystal structure of the complex between the S2–S1 targeting diUb ABP and SARS PLpro allows identification of the crucial contacts in the S2-site of the DUB and the diUb ABP. All in all these hCoV proteases, although homologous,

are shown to have a different mode of action using the three generations of activity-based probes (discussed in Sect. 2.1) and accompanying kinetic parameters could be deduced using fluorogenic mono- and diUb-AMC substrates (discussed in Sect. 2.3).

3.2 Applications in Bacteriology

Similar to viral interference with host immune response, bacteria also have sophisticated mechanisms in place to counteract host immune responses and create optimal conditions for the bacterium to survive and promote replication. Bacterial effector proteins typically are directly secreted into the host cells to interfere with host kinase activities or Ub(l) signaling pathways. Interestingly, those bacteria do not have Ub or Ubl systems themselves and such effector proteins are only in place to promote survival within the host organism. Much of these effector proteases fall in the class of CE clan effector enzymes, which in humans consists of SUMO- and NEDD8-specific proteases. Bacterial CE clan proteases do not only show activity toward Ubl's but some members also show acetyltransferase activity. In a comparative study on such CE clan effectors from intracellular pathogens *Salmonella typhimurium*, *Chlamydia trachomatis*, *Escherichia coli*, *Yersinia pestis*, *Rickettsia bellii*, *Shigella flexneri*, and *Legionella pneumophila*, activity, specificity, and structure were investigated (Pruneda Jonathan et al. 2016). Although all share a common fold most but not all of them were able to react with ABP Ub-Prg. Using Ub-, SUMO1-, NEDD8- and ISG15-FP substrates (discussed in Sect. 2.3), the three effectors unreactive toward Ub-Prg were also shown to be unreactive toward any of the FP-reagents whereas the others showed reactivity toward both Ub and NEDD8 reagents. In contrast to their unreactivity toward Ubl's, the three CE effectors did show acyl transferase activity. Using diUb substrates, the panel of bacterial DUBs was all shown to have a strong preference for K63-linked chains, followed by K48 and K11 at later time points or higher enzyme concentrations. This specificity mostly was shown to be regulated by the S1'-site of these DUBs. Taken together, this study uses both mutational analyses, crystal structure information, and data obtained by Ub(l)-based probes and assay reagents to classify and study the molecular details of a bacterial class of effector proteins in detail.

3.3 Legionella Effector Enzymes: The Odd-Ones Out

We thought we understood the basis of the Ub system, but do we actually? Several enzymes have been recently identified to control the Ub system by attaching adenosine diphosphate ribose (ADPr) onto specific positions in Ub, exerting a new layer of control. The intracellular pathogenic bacterium *L. pneumophila*, the cause of Legionnaires disease, releases effector proteins into its host to hijack the host Ub

pool (Bhogaraju et al. 2016). These multi-domain SidE family proteins catalyze the ubiquitination of substrates proteins *independent* of the E1-E2-E3 cascade and without the use of energy source ATP. Instead they use NAD⁺ (see Fig. 5—structure II) to attach ADPr on the nucleophilic side chain of arginine 42 of Ub using their mono ADPr transferase (mART)-domain (see Fig. 5—structure I (Qiu et al. 2016). Subsequently, the phosphodiesterase (PDE) domain within SidE catalyzes the reaction between a serine-containing substrate (see Fig. 5—structure IV) and the ADP-ribosylated Ub (see Fig. 5—structure III), affording a ribosyl phosphate linkage between Ub and the target protein (see Fig. 5—structure V) (Kotewicz et al. 2017). Besides a mART and PDE domain SidE family members also contain a N-terminal canonical DUB domain that is not necessary for bacterial replication, but is crucial in regulating the extend of ubiquitination at the bacterial vacuolar surface. Cleavage assays using diUb substrates show a preference of K63 over K48 and K11, and crystallization efforts of the formed complex with ABP Ub-VME reveal a different binding modus, not involving the I44 patch on Ub, of the cysteine DUB and Ub (Sheedlo et al. 2015). Besides the multi-domain multi-function SidE's, Legionella also expresses a deubiquitinase SidJ that is able to cleave the formed phosphodiester linkage to Ub, liberating the substrate protein and phosphoribose modified ubiquitin (see Fig. 5—structure VI) (Qiu et al. 2017). This phosphoribose-Ub also interferes with the host Ub machinery as it prevents the canonical E1 to activate and further process the Ub molecule (Puvar et al. 2017). By controlling the construction and deconstruction of modified Ub, without competing with the canonical system, the bacterium tries to hijack the host Ub system.

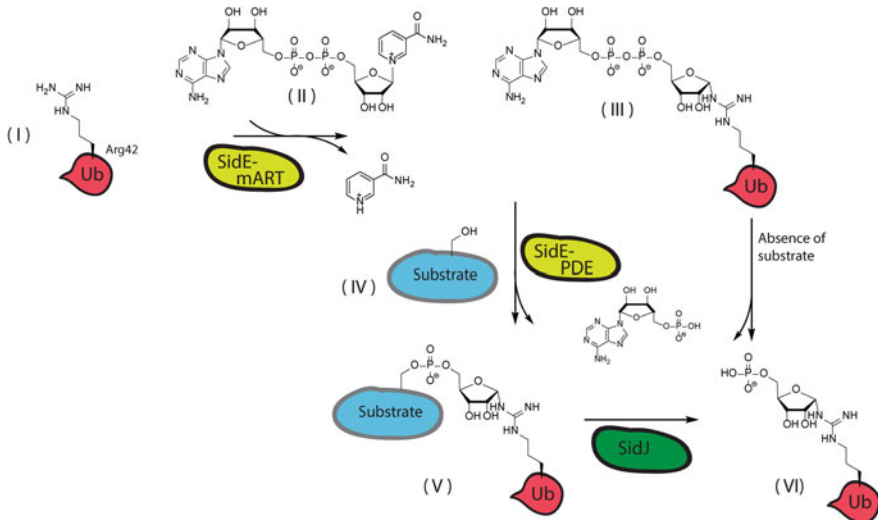


Fig. 5 Actions of SidE effector enzymes ADP-ribosylating Ub(Arg42) and subsequent coupling to substrates forming a phosphoribose linkage between substrate and Ub

Lots of questions on the mechanism of action, substrate recognition, and scope of this E1–E2–E3 independent ubiquitination and attached deubiquitination process call for the development of novel probes specifically targeting such pathways. So far the development of ADP-ribosylated Ub carrying a protease stable triazole linkage between the ribose and Arg42 of ubiquitin has been shown to reflect the native auto-ubiquitination activity of SidE family member SdeA (Liu et al. 2018). No doubtably probes targeting this interesting new way of Ub conjugation to substrates will appear in the near future.

4 Conclusions

The complexity of the ubiquitin system and its involvement in a wide variety of important biological processes makes it a widely and intensively studied field. The roles Ub play in neurodegenerative disease and cancers, for instance, make it a potential target for therapeutic intervention. Unraveling the complexity of this, highly sophisticated system is aided greatly by the development of (activity-based) probes reporting on the dynamics and structural mechanisms used by activating enzymes, ligases, and proteases counteracting the buildup of (poly)-ubiquitinated substrates. Ranging from probes that covalently trap the active cysteines involved in catalysis to probes that have altered fluorescent properties upon enzyme activity and stabilized substrates or poly-Ub chains allowing preference studies and profiling of the Ub interactome have been developed over the past decade or so and already have shed their light on some fundamental question in Ub biology. The more knowledge is gathered on the functioning of those (de)ubiquitinating proteins the more tailored solutions to interrogate their biology, and hence, specific probes involved in such approaches become. Without a doubt will the next generation of Ub-based tools help to increase our knowledge on the system and perhaps ultimately lead to diagnostic tools or therapeutics making it to the clinic.

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