Synthetic tools to study ubiquitin biology

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Chapter 1

General Introduction: How chemical synthesis of ubiquitin conjugates helps to understand ubiquitin signal transduction

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Summary

Ubiquitin (Ub) is a small post-translational modifier protein involved in a myriad of biochemical processes including DNA damage repair, proteasomal proteolysis, and cell cycle control. Ubiquitin signalling pathways have not been completely deciphered due to the complex nature of the enzymes involved in ubiquitin conjugation and deconjugation. Hence, probes and assay reagents are important to get a better understanding of this pathway. Recently, improvements have been made in synthesis procedures of Ub derivatives. In this perspective, we explain various research reagents available and how chemical synthesis has made an important contribution to Ub research.

Introduction

Ubiquitin (Ub) is a small 76 amino acid protein that can be covalently attached to a target protein in a process called ubiquitination in order to regulate protein function or lifespan. Three different classes of enzymes E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) facilitate different types of ubiquitination of a target protein. [1, 2] E1 activates ubiquitin as a thioester of the active site cysteine residue at the expense of ATP. The activated ubiquitin molecule is then transferred to an E2 enzyme by the formation of a new thioester with the catalytic cysteine residue of an E2 enzyme. From the E2 enzyme, ubiquitin can either be transferred to the catalytic cysteine residue of HECT or RBR classes of E3 enzymes or the E2-Ub loaded complex binds to RING class E3 enzymes forming an Ub-E2-E3 complex (Figure 1A). Finally, an isopeptide bond is formed between the C-terminal glycine residue (Gly76) and ε-NH_2 of a lysine residue of a substrate protein. Target specificity is determined by precise E2/E3 combinations to regulate the fate of a substrate. Ubiquitination can be reversed by the removal of ubiquitin from target proteins by deubiquitinating enzymes (DUBs) (Figure 1B). More than 90 DUBs are encoded in the human genome. [3]

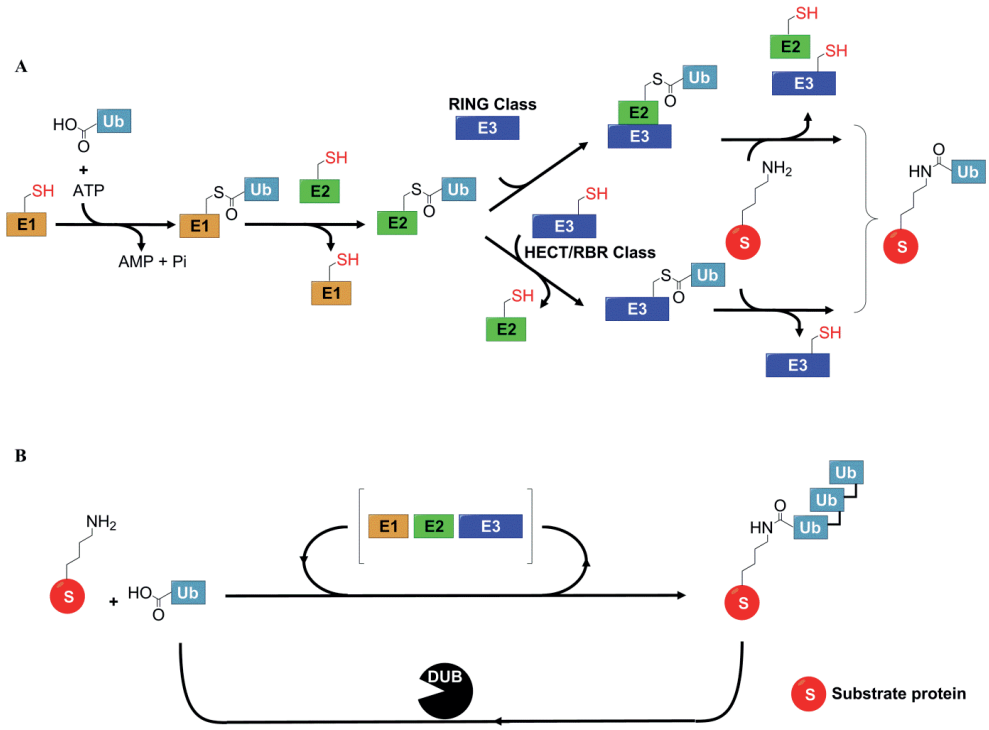

Figure 1: Schematic overview of the ubiquitin machinery. ***A***. *The ubiquitin conjugation pathway starts with the activation of ubiquitin as a thioester by a ubiquitin-activating enzyme E1, followed by transfer to a ubiquitin-conjugating enzyme (E2) and finally onto the target with the help of an E3 ligase.* ***B***. *Different types of ubiquitin chains are conjugated by the concerted action of different E1, E2 and E3 enzyme combinations. Ubiquitin removal is carried out by deubiquitinating enzymes (DUBs).*

Target proteins can be modified by monoubiquitination, multi-monoubiquitination or polyubiquitination. Ubiquitin contains an N-terminal methionine residue and seven internal lysine residues at positions 6, 11, 27, 29, 33, 48 and 63 which are all involved in the formation of different ubiquitin chains. [4, 5] Different types of ubiquitin chains are involved in many cellular events including proteasomal degradation, cell-cycle regulation, DNA repair and vesicle transport. The most well-studied Ub chains are the K48- and K63-linked polyUb chains. After the discovery that the degradation of proteins in the cell can be carried out by ubiquitin-mediated proteolysis, it was shown that cell cycle progression and protein degradation processes are abolished when the lysine 48 (K48) residue in Ub is changed to an arginine (R) residue suggesting that K48-linked polyubiquitin chains target proteins for proteasomal degradation. [6, 7] The K63-linked polyUb is important for other processes including DNA damage repair. [8] Furthermore, K11-linked polyUb has been associated with endoplasmic reticulum-associated degradation (ERAD)[9] and proteasomal degradation. [10]

DUBs are classified into USP, UCH, OTU and Josephin domain-containing proteases that belong to cysteine proteases class. A fifth DUB family, the JAMM/MPN+ domain-containing

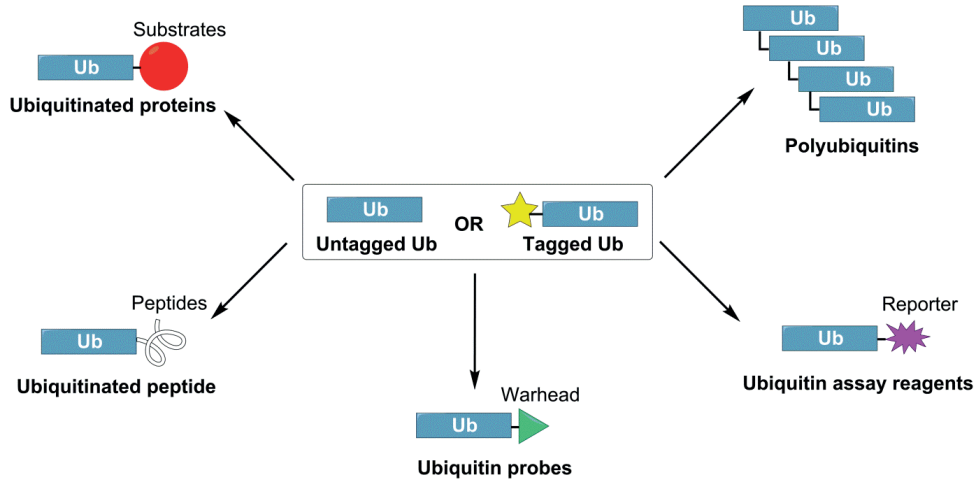

Figure 2: Overview of different DUB activity reporters*. Ub is chemically synthesized as tagged or untagged protein which can be further chemically modified into different Ub-conjugates, each used in different assays for DUB activity.*

proteins, is a family of metalloproteases. In the cysteine protease class of DUBs, the thiol group of the active site cysteine residue acts as a nucleophile that attacks the carbonyl group of the isopeptide bond between ubiquitin and the lysine residue of a target protein. [11] In the case of the JAMM/MPN+ metalloprotease class of DUBs, the active site contains a water molecule that is activated by a zinc atom coordinated by two histidine residues and a glutamate residue near the active site. This water molecule hydrolyzes the isopeptide bond thereby deconjugating ubiquitin from the target protein. [12, 13]

Defects in components of the ubiquitination process can contribute to disease pathogenesis, especially cancer and neurodegeneration. This makes them of interest as potential therapeutic targets. [14, 15] For example, the E3 ligase MDM2 ubiquitinates the tumour suppressor p53, regulating its levels in the cell by targeting them for proteasomal degradation. [16] The DUB USP7, in turn, regulates the stability of p53 by deubiquitination of both MDM2 and p53. [17]

Many ubiquitin-like modifiers (UBLs) have been identified as well. These UBLs include NEDD8, SUMO, ISG15, FAT10, Ufm1 and URM1. Although they are similar to Ub in sequence and structure, they have different functions. These UBL modifiers are attached to the target protein *via* their own dedicated E1, E2 and E3 enzymes and dedicated proteases recognize and deconjugate UBLs from the target although often crosstalk with enzymes from the ubiquitin machinery is observed. [18]

Ubiquitin-based probes and assay reagents are important as they help to provide insight into enzymatic activity and substrate recognition. Also, they have been important in the discovery of various new enzymes involved in the Ub machinery. Initially, reagents were generated by reversed proteolysis and later by intein chemistry. Various Ub conjugates have also been generated by chemical synthesis in order to study different components of the Ub signalling cascade (Figure 2). Such tools help in determining the kinetic properties and substrate specificity of the enzymes involved in the ubiquitination pathway, which in turn helps in the identification and characterization of interesting therapeutic targets.

Table 1A

Ub-based assay reagents	Applications	References
Ub-AMC	Fluorogenic assay reagent used to identify small molecule inhibitors for UCHL5 (see Figure 3A) (AMC: λ_{ex} 365nm; λ_{em} 440nm)	[19]
DiUb-AMC	Non-hydrolysable diUb molecule linked to AMC at the C-terminal end; Used to characterize special DUBs that have an additional Ub binding site (see Figure 3B) (AMC: λ_{ex} 365nm; λ_{em} 440nm)	[20]
Ub-Rho	Fluorogenic assay reagent with spectral properties not overlapping with most small molecule inhibitors (see Figure 3C) (Rho110: λ_{ex} 490nm; λ_{em} 550nm). An improved derivative based on morpholinecarbonyl-rhodamine 110 [21] is commercially available.	[22]
Ub-Lys-TAMRA-Gly	A fluorogenic reagent that is linked by an isopeptide bond; used in Fluorescence polarization assays (see Figure 3D) (TAMRA: λ_{ex} 550nm; λ_{em} 590nm)	[23]
Ub-(pep)TAMRA	Similar to Ub-Lys-TAMRA-Gly, but with substrate specificity for the DUBs determined by the peptide (see Figure 3E)	[23, 24]
Ub-Luc	Bioluminescence-based assay reagent with low background noise - suited for DUBs that require high substrate concentrations (see Figure 4)	[25]
diUb-based FRET pair	FRET-based assay reagents used to identify Ub linkage specificity of DUBs (see Figure 5A)	[26-29]
Tb-Ub/Fl-Ub	Ub with different fluorescently labelled dyes for FRET-based assays, used in characterizing UBC13, an E2 enzyme (see Figure 5B)	[30]

Table 1B

Activity-based probes with a Ub backbone	Applications	References
Ub-VS	An α,β unsaturated sulfone at the C-terminal end of Ub that can label DUBs. It was used to identify USP14 as a proteasome-associated DUB (see Figure 6A)	[31]

Ub-VME	An α,β unsaturated ester at the C-terminal end of Ub that can covalently react with many of the known cysteine protease DUBs (see Figure 6B)	[32, 33]
Ub-PRG	Relatively stable Ub-based probe for cysteine protease DUBs with an alkyne functionality as warhead at the C-terminal end of Ub (see Figure 6C)	[34]
DiUb-based DUB probes	DiUb molecules of different linkages equipped with a warhead positioned either between two Ub molecules or at the C-terminal end of the diUb to identify linkage-specific DUBs (see Figure 7)	[20, 35-39]
Ub-based E1/E2/E3 probe	Ub based probe containing a warhead at the C-terminal end of Ub that can target E1, E2 and E3 enzymes (see Figure 8)	[40-44]

***Table 1: Overview of currently available Ub-based assay reagents and active-site directed probes**. **A**. Representative Ub-based assay reagents to study DUB activity. **B**. Activity-based probes used to study different DUBs and E1, E2 and E3 enzymes.*

Various Ub-based probes and assay reagents are available especially for the cysteine protease class of DUBs and for conjugating enzymes. Examples are given in Table 1. In this review, we will discuss the development of Ub-based probes and activity reporters and how chemical synthesis has contributed to the current understanding of Ub signal transduction.

In-vitro generated ubiquitinated proteins and peptides

The fate of a ubiquitinated protein in a cell depends on the site and the type of ubiquitination involved. Polyubiquitination can lead to different responses based on the position of the isopeptide bond and the number of ubiquitin molecules attached to the target protein. K48- and K63-linked polyubiquitin chains are the best-studied chains. The functions of other polyubiquitin chains (lysine 6, 11, 27, 29, 33 and the methionine 1 linked chains) have been explored more recently. Not all enzymes that make specific polyubiquitin chains are currently known, but fortunately, several non-enzymatic methods have been recently developed to make well-defined ubiquitin conjugates.

Polyubiquitin chains bind to specific elements in "reader" proteins leading to different Ub signalling transduction events. To prevent premature chain cleavage, non-hydrolyzable ubiquitin conjugates that are resistant to DUB activity can be used. In 2000, Yin *et al.* reported the biochemical synthesis of non-hydrolyzable Ub dimers where Ub molecules containing cysteine residues at specific sites were cross-linked using dichloroacetone. [45] Recently, Lewis *et al.* used a similar approach to cross-link Ub with α-Synuclein and studied their mechanism of aggregation. [46] The triazole linkage is another example of a non-hydrolyzable link that can be formed by a click reaction between an alkyne and an azido group and serves as a good isostere of the naturally occurring peptide bond. In 2010, Eger *et al.* were able to incorporate an alkyne and an azide functionality in ubiquitin using orthogonal tRNA/tRNA synthetase pair in a bacterial expression system and made non-hydrolyzable ubiquitin dimers. *[47]* Similarly, Wiekart *et al.* were able to incorporate an alkyne functionality in SUMO and made non-hydrolyzable SUMO-peptide conjugates. *[48]* However, the use of a bacterial expression system limits the number of possible modifications. To overcome this problem, chemical synthesis of polypeptides provides an

attractive alternative. Jung *et al.* were able to incorporate a non-hydrolyzable thioether functionality in a lysine 63-linked diubiquitin molecule. *[49]* Shanmugham *et al.* incorporated an oxime functionality between the C-terminus of ubiquitin and specific lysine positions on polypeptides derived from PCNA, PTEN, H2A and H2B. *[50]* Such non-hydrolysable ubiquitin conjugates can be used for determining the substrate selectivity of certain DUBs.

Chemical synthesis of ubiquitin to make ubiquitin conjugates

The efficiency of the chemical synthesis of a polypeptide depends highly on the number of amino acid couplings carried out during synthesis. This makes the linear synthesis of proteins challenging. The first chemical synthesis of ubiquitin was reported in 1989 by Briand *et al.* and Ramage *et al.* [51] [52] Over the years, technologies for the synthesis of long polypeptides have improved allowing for an increase in the yield and purity of the produced polypeptides. Ubiquitin has been synthesized in a linear fashion using an optimized procedure[53] or in a step-wise modular fashion using native chemical ligation (NCL)[54] and subsequent desulfurization. [55] Chemical synthesis has the advantage that site-specific incorporation of unnatural amino acids, labels and pull-down handles is possible in virtually any position.

Using Fmoc-based solid-phase peptide synthesis (SPPS), the N-terminal end of Ub can be coupled to short peptide epitope tags like Human influenza hemagglutinin (HA), His_6 or Biotin. Such modified Ub molecules can be used in Western Blots (WB) or pull-down assays. Modifications of the internal residues of Ub, especially at one of the seven Lys position, are useful to chemically make Ub conjugates. This may involve a native chemical ligation handle or a 'click' handle instead of lysine. The C-terminal end of Ub can be modified by mild cleavage of the fully protected Ub from a trityl resin, followed by global deprotection and purification. In this manner, fluorogenic molecules or reactive moieties may be introduced that can selectively target enzymes involved in the ubiquitination pathway.

The site-specific incorporation of the unnatural amino acid thiolysine has allowed the synthesis of ubiquitinated reagents including ubiquitinated peptides, polyubiquitin chains and diubiquitin molecules. [53, 55] The synthesis and use of such conjugates have been reviewed extensively. [56-58] One development is the synthesis of diubiquitin-based reagents that have made it possible to monitor the chain specificity of DUBs including the USP enzymes[59] and the OTU proteases. [60, 61] Other diUb-based linkage specific reagents have been developed recently using chemical means which revealed the mechanism of action for specific DUBs. [20, 29] Also, these diubiquitin molecules have been used to determine the linkage specificity of specific DUBs and for the characterization of DUB inhibitors using mass spectrometry. [61] Polyubiquitin chains synthesized from recombinant proteins through click linkages have been used to study interactions with binding domains. [47, 62] Also, ubiquitinated peptides have also been generated to the study substrate-specificity of DUBs. [24]

Ubiquitin conjugates: activity-based probes and activity reporters based on ubiquitin

Ubiquitin conjugated to reporter molecules has been used to study the biochemical properties of many DUBs. Initially, such molecules were synthesized by purifying expressed Ub and introducing reporter molecules by reverse trypsinolysis. [63] Later, intein chemistry was used to generate Ub as an activated thioester. [31] More recently, chemical synthesis has provided an efficient route to such reagents. [24, 53, 55]

Ubiquitin assay reagents

Ub-based assay reagents consist of Ub attached to a reporter molecule at the C-terminal end. DUBs recognize Ub and cleave the reporter molecules that can be fluorogenic or bioluminescent. In case of DUBs that are specific for a particular Ub linkage, unlabeled chains can be used in a gel-based assay, or a labelled diUb-based reagent can be used for measuring enzyme kinetics. Sometimes, two different sets of fluorescent Ub molecules can be assembled using ubiquitinating enzymes and the resulting FRET readout can be used to characterize such enzymes. [30]

Fluorogenic ubiquitin reagents

Fluorogenic assay reagents based on Ub contain a fluorogenic molecule attached *via* an amide bond at the C-terminal end of Ub. DUBs recognize Ub and hydrolyze the amide linkage at the C-terminal glycine residue in Ub, releasing a fluorescent reporter molecule, which can be used to measure the DUB activity over time (Figure 3).

One of the earliest reported fluorogenic substrates for DUBs is Ub amidomethyl coumarin (UbAMC) by Dang *et al.* (Figure 3A). [19] They were able to synthesize Ub-AMC using a transpeptidation reaction involving Ub, GlyGlyAMC and trypsin. This substrate was used to characterize UCH-L3 and Isopeptidase T enzyme kinetics. In addition, they were able to test the inhibition kinetics of Ubiquitin aldehyde, a DUB inhibitor. UbAMC can now be easily prepared by chemical synthesis. [53] Recently, Flierman *et al.* reported a fluorogenic assay reagent based on AMC where non-hydrolysable diUb molecules of different linkages were attached to AMC at their C-terminal end. [20] These reagents serve as a specific substrate for DUBs that have an additional Ub binding site (S2 binding site) (Figure 3B). Another fluorogenic substrate called Ub-Rho110 (Figure 3C) was first described by Hassiepen *et al*. [22] It was used in the screening of inhibitors for UCHL3 and USP2. Both Ub-AMC and Ub-Rho110 contains a peptide bond at the C-terminal end of Ub. However, the majority of naturally occurring Ub conjugates have an isopeptide bond instead. Although many DUBs cleave a normal peptide bond at the C-terminal end of Ub, an isopeptide linked Ub assay reagent would be a more relevant substrate for DUBs. The earliest report on an isopeptide-linked ubiquitin assay reagent was by Tirat *et al.* who used fluorescent polarization assays to study the biochemistry of DUBs. [23] They generated a short peptide sequence surrounding the K48 position in Ub by SPPS. A fluorescent molecule (TAMRA) was attached to the N-terminus of the peptide. Full-length ubiquitin, E1 and E2 enzymes were then added to this peptide to yield a fluorescent ubiquitin-peptide conjugate. Changes in the fluorescence polarization occur when a DUB cleaves the isopeptide bond between ubiquitin and a TAMRA-labelled peptide (Figure 3D). This reagent was used to validate the enzyme kinetics of UCHL3 and USP2. However, this technique requires the use of both E1 and E2 enzymes, which limits the nature of the peptide that can be conjugated to Ub.

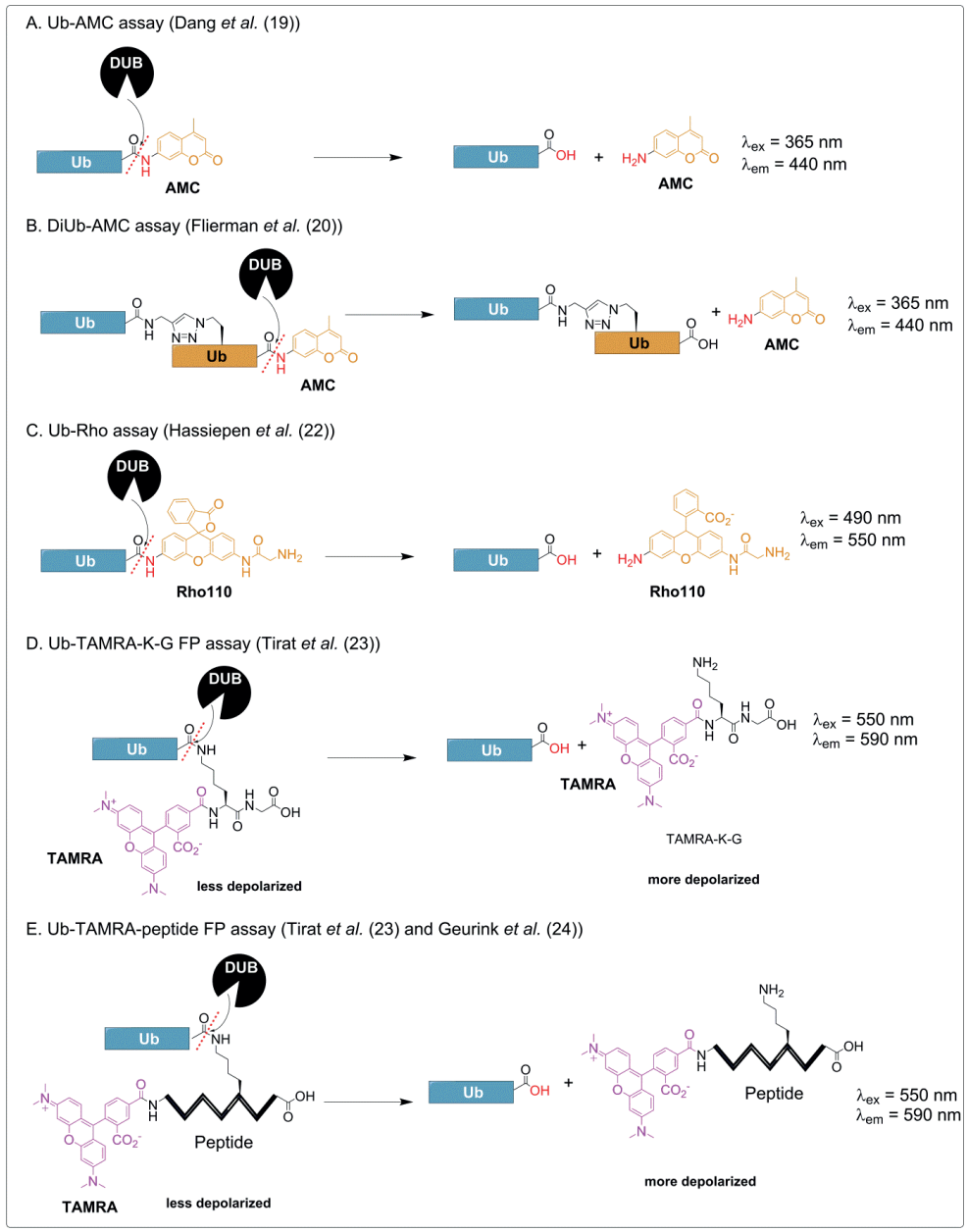

Figure 3: Ubiquitin-based assay reagents. A. *Ub-AMC,* ***B.*** *non-hydrolysable diUb-AMC and* ***C.*** *Ub-Rho assay reagents, all work on the principle of increase in fluorescence upon DUB cleavage.* ***D and E.*** *Fluorescent Polarization (FP) assay reagents. These reagents consist of a TAMRA-linked peptide attached to the C-terminal end of Ub by an isopeptide bond. Upon DUB-mediated cleavage, the TAMRA-containing peptide is released, and its fluorescence polarization is measured.*

In 2012, Geurink *et al.* synthesized a fluorescent Ub-peptide and UBL-peptide assay reagents using SPPS and subsequent NCL and desulfurization (Figure 3E). [24] Ub or UBL were activated as a thioester using E1 and MESNa (sodium 2-mercaptoethanesulfonate) in the presence of thiolysine containing peptides. The NCL products were then desulfurized and used in cleavage assays. Site-specific incorporation of the thiolysine handle by SPPS has expanded the choice of peptides that can be conjugated to Ub and also allows the generation of reagents based on UBLs like NEDD8 and SUMO-1, -2 and -3.

Bioluminescent ubiquitin reagents

Some of the DUBs have lower affinity for their substrates due to which a higher substrate concentration is needed in a DUB assay. This often results in more background noise causing problems with the measurement. To avoid this problem, Orcutt *et al.* designed a bioluminescence-based assay to study DUBs at lower concentrations. [25]

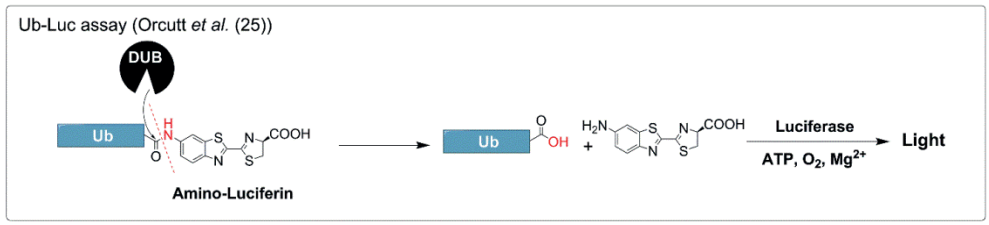

Figure 4: Ub-based bioluminescent assay reagent. *DUBs act on the Ub-Luc reagent which releases free amino-luciferin. Luciferase, that is already present in the reaction mix, acts on this amino-luciferin which leads to bioluminescence.*

The bioluminescent assay reagent consists of a Ub molecule linked to amino-luciferin at its C-terminal end (Ub-Luc). The DUB acts on the Ub-Luc releasing free amino-luciferin (Figure 4). Since amino-luciferin is not luminescent by itself, background noise is almost absent. Then, luciferase enzyme acts on the free luciferin in the presence of ATP and Mg^{2+}, leading to oxidation of aminoluciferin, thereby releasing photons. This technique was used to assay a panel of DUBs and in high throughput screening (HTS). However, care must be taken while using this reagent in an HTS assay because the small molecules may also inhibit luciferase.

FRET-based ubiquitin assay reagents

In order to study the Ub-linkage specificity of DUBs and their enzyme kinetics, labelled diUb-based assay reagents are used. In 2012, Ohayon *et al*. reported the chemical synthesis of a ubiquitinated peptide that was used in a FRET-based assay in HTS to identify small molecule inhibitors for UCHL3. [26] Later on, Ye *et al*. reported the enzymatic approach to make FRET-pair diUb reagents that were used to characterize ubiquitin-binding domains and DUBs. [27] Arnst *et al.* also used such a reagent in a Fluorescence Resonance Energy Transfer (FRET)-based assay in order to characterize the K63 linkage-specific metalloprotease-DUB AMSH (Figure 5A). *[28]* They used a diUb molecule in which two fluorescently labelled Ub modules were linked at position K63. Due to the proximity of the two fluorescent dyes, a FRET signal is observed. Upon adding AMSH, the FRET signal decreases over time. More recently, Geurink *et al.* have made a panel of all seven isopeptide-linked diUb-based FRET assay reagent using chemical synthesis. [29] The diUb-based FRET assay reagents were prepared by native chemical ligation using Rhodamine-Ub (FRET-

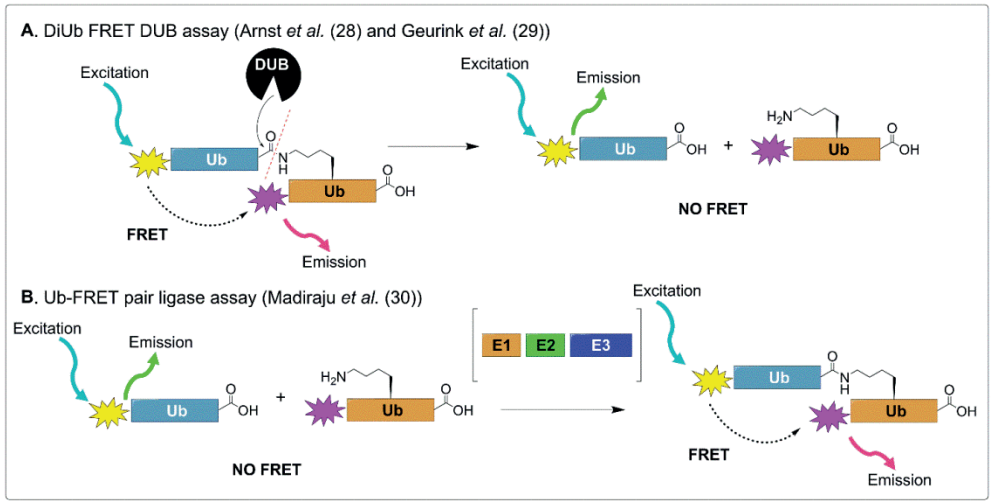

Figure 5: Ubiquitin-based assay FRET assay reagents A. *DiUb FRET assay reagent in which two different fluorescently labelled Ub molecules are linked by a native isopeptide bond. Upon DUB activity, the diUb-based reagent is cleaved, and the FRET signal decreases over time.* ***B.*** *FRET-based Ub ligase assays where two different fluorescently labelled Ub molecules form a diUb with the help of a specific E1, E2 and E3 enzyme combination. When the two fluorescent dyes are in close proximity, the FRET signal increases.*

donor) and TAMRA Ub (FRET-acceptor). The changes in the FRET signal upon DUB activity can be used to quantify the enzyme kinetics.The FRET-based assay can also be used to characterize ubiquitination enzymes. In 2012, Madiraju *et al.* used this technique to characterize the E2 enzyme UBC13-UEV1A (Figure 5B). [30] In this study, terbium-labelled Ub and fluorescein-Ub were used in a reaction containing E1 and UBC13-UEV1A (E2). Upon Ub chain formation and elongation due to the E1-E2 enzyme activity, the FRET signal between terbium and fluorescein is increased. This gives a real-time measurement of enzyme activity. The technique was used primarily in a high throughput screen for small molecules that can inhibit UBC13. In all these cases, the inherent nature of ubiquitin recognition by a DUB is used. However, DUBs often are specific to polyubiquitin chains, or ubiquitinated substrates. Hence the processing capacity of a DUB for these ubiquitin-small molecule conjugate assay reagents may not reflect the true activity of these enzymes. To measure the activity of a DUB more specific reagents are often needed.

Ubiquitin-based probes for the covalent capture of DUBs

Most of the DUBs are cysteine proteases, containing a catalytically active cysteine residue. Ub-based DUB probes can be used to visualize such DUBs in biochemical assays using SDS PAGE-based readouts. A thiol-reactive functionality is positioned in the probe in such a way that a covalent adduct with the active site cysteine residue of the DUB is formed. Such probes have been reviewed extensively by Ekkebus *et al.* [64] In this review, we give only a brief account of the different probes reported so far.

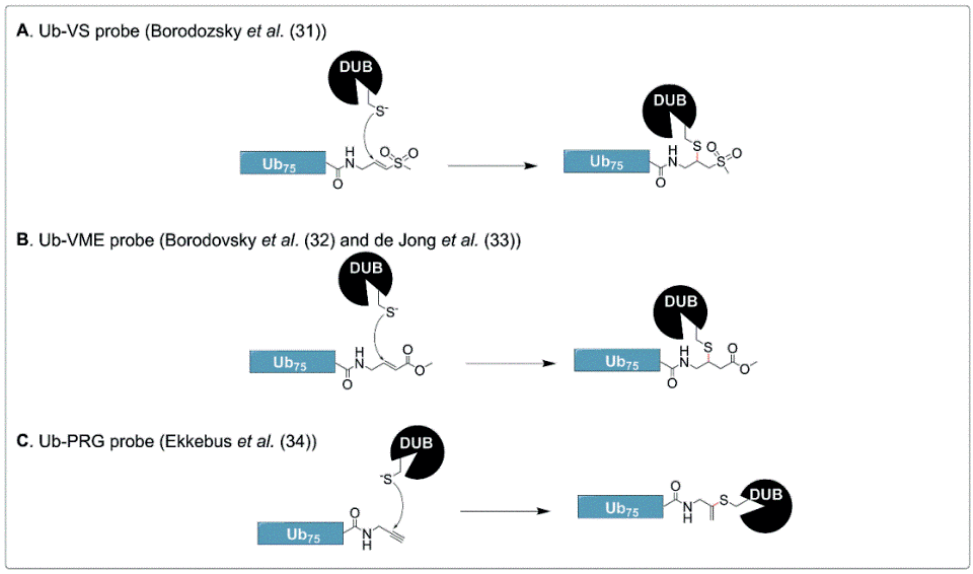

Figure 6: Monoubiquitin-based probes for cysteine protease class of DUB. A. *Ub-vinyl sulfone (UbVS) probe. In this monoUb-based probe, a vinyl sulfone Michael acceptor is positioned at the C-terminal end of Ub and used to capture DUBs at their active site.* ***B.*** *Ub-vinyl methyl ester (UbVME) probe. In this reagent, a vinyl methyl ester is used to covalently trap the active site of DUBs.* ***C.*** *Ub-propargyl (Ub-PRG) probe. The Michael acceptor is a terminal alkyne that can form a covalent adduct with the active site of DUBs.*

Many types of Ub-based DUB probes (Figure 6) have been developed using intein-fusions, including ubiquitin vinyl sulfone (Ub-VS)[31] and several derivatives including ubiquitin vinyl methyl ester (Ub-VME). [32] Using intein chemistry ubiquitin-like probes can also be prepared. [65] One of the main advantages of such covalent inhibitors is the use of epitope-tagging at the N-terminal end of ubiquitin allowing retrieval and detection or identification of DUBs. This includes tags that can be used in western blot and pull-down assays or fluorescent molecules that can be used to visualize DUBs in an SDS-PAGE protein gel. In 2012, de Jong *et al.* reported the total chemical synthesis of Ub-based probes. [33] In this method, a reactive vinyl methyl ester (VME) moiety was coupled to the C-terminus of ubiquitin by chemical synthesis. The free N-terminus was then labelled with a fluorescent dye like TAMRA, or peptide epitope tag such as HA. In 2013, Ekkebus *et al.* reported another activity-based DUB probe based on the unexpected reactivity of a propargyl group incorporated at the C-terminus of ubiquitin. [34] Sommer *et al.* independently reported the development of a SUMO-based propargyl probe for SUMO-specific proteases. [66] In this case, the SUMO protein was expressed as an intein-fusion and then modified with a terminal alkyne. To characterize DUBs that specifically hydrolyze different lysine-linked ubiquitin chains,[59, 61, 67] diUb-based probes have been used. In such probes, both the Ub moieties can bind to linkage-specific DUBs. Generally, in a diUb molecule, the Ub that is linked at its C-terminal end is called distal Ub while the lysine-linked Ub is called proximal Ub. In a diubiquitin-based probe, a reactive group is positioned at the site of action of the DUB, which can be either between the two ubiquitin moieties or at the C-terminal end of the diubiquitin molecule (Figure 7).

Iphofer *et al.* reported the first linkage-specific DUB probe where the C-terminus of a distal ubiquitin is linked to short peptides surrounding K48 or K63 of another ubiquitin and a reactive Michael acceptor is positioned instead of the isopeptide bond between the two Ub modules (Figure 7B). [35] This reacts specifically with DUBs that recognize this linkage by forming a covalent adduct at the active site of the DUB. This technique can be extended to other peptides derived from different substrates of DUBs. Later on, McGouran *et al.* developed a diubiquitin based probe containing the reactive trap at the site of isopeptide bond between two full-length Ub molecules that are linked by a non-hydrolyzable triazole moiety (Figure 7C). [36] Later on, different versions of a diUb-based probe were developed independently by Li *et al.*[37] (Figure 7D) who used a vinyl amide warhead positioned between two ubiquitin molecules and Haj-Yahya *et al.*[38] (Figure 7E) who used a dehydroalanine group. In 2014, Mulder *et al.* reported another diUb-based DUB probe that can be prepared in large quantities (Figure 7F). [39] Here, native chemical ligation was used to synthesize a diubiquitin molecule in which the subsequent elimination of a thiol residue resulted in the formation of a Michael acceptor warhead positioned at the appropriate site for reaction with a cysteine residue in the DUB catalytic site. These probes form a stable covalent interaction with the DUBs, which can be further used for structural studies. More recently, Flierman *et al.* reported another diUb-based probe with a propargyl warhead positioned at the C-terminal end of the proximal Ub (Figure 7G). [20] With this reagent, DUBs that have an additional Ub-binding site (S2) in poly-Ub chains can be characterized.

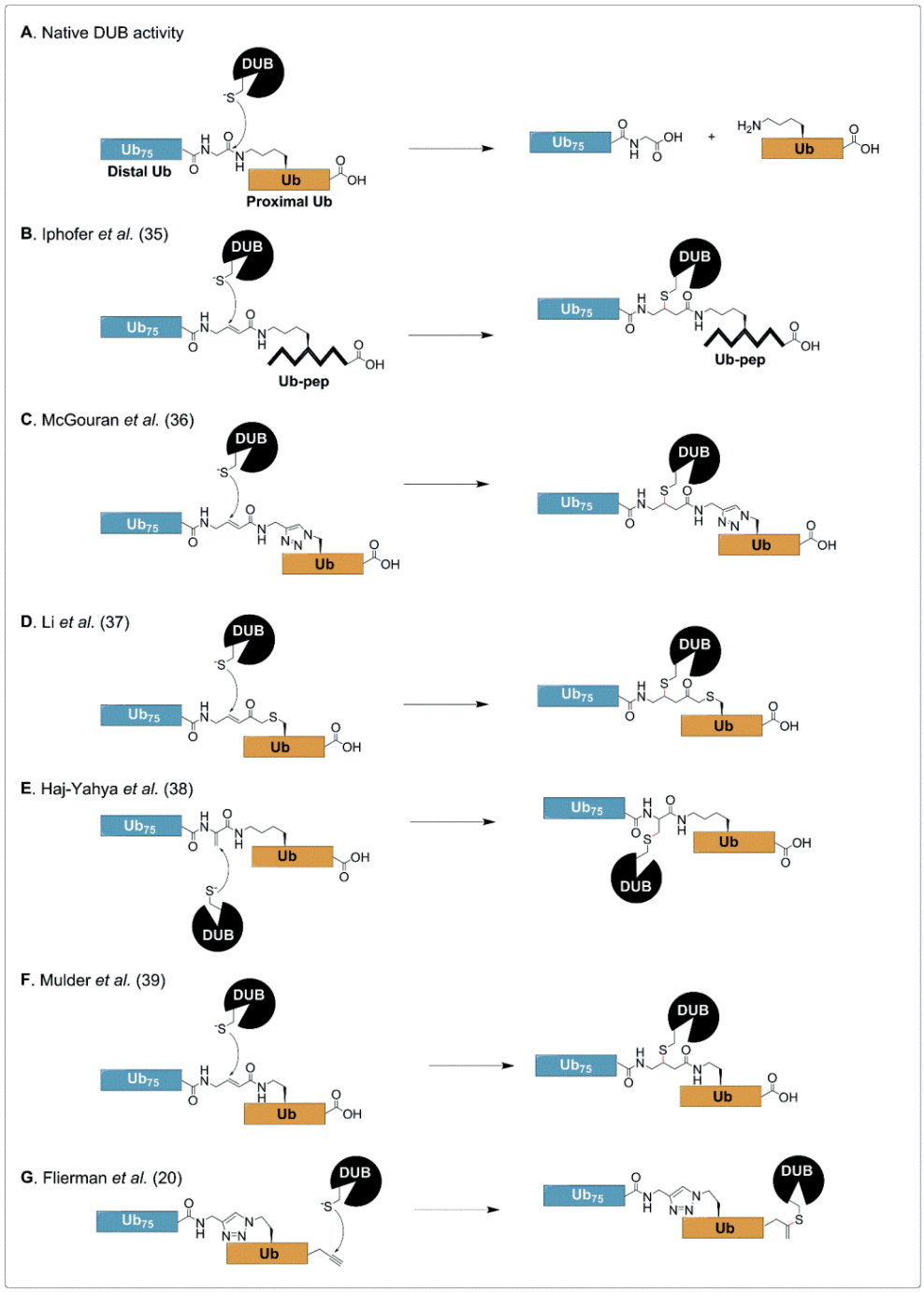
A. Native DUB activity
DUB
Ub75
Distal Ub
Ub
Proximal Ub
B. Iphofer et al. (35)
Ub-pep
C. McGouran et al. (36)
D. Li et al. (37)
E. Haj-Yahya et al. (38)
F. Mulder et al. (39)
G. Flierman et al. (20)

Figure 7: Diubiquitin-based probes. ***A.*** *Mechanism of diUb cleavage. The active site cysteine of a DUB acts on the carbonyl group of the C-terminal glycine residue in the distal Ub module. This makes the bond scissile and the resulting thioester intermediate is hydrolysed by a water molecule.* ***B.*** *Substrate-specific DUB probe using Ub and a short peptide derived from a substrate protein with an electrophilic centre positioned in between.* ***C–F.*** *Various diUb-probes using different types of Michael acceptors positioned between the distal and the proximal Ub.* ***G.*** *DiUb-based DUB probe where an alkyne group is positioned at the C-terminal end of the proximal Ub module.*

Ubiquitin-based probes for E1, E2 and E3 enzymes

Although several Ub-based probes have been developed for DUBs, probes that can target the ubiquitination enzymes (E1, E2 and E3) are challenging to make, due to the sequential trans-thioesterification reactions in the ligation cascade (Figure 8A). In particular, UbE1 activates Ub as a Ub-adenylate intermediate at the expense of ATP. This is then exchanged for the thiol in the active site of E1 to form an E1-Ub thioester complex. Upon subsequent trans-thioesterification by E2 and E3 (HECT/BRB class) or with the help of a RING E3 ligase, Ub is transferred onto a substrate protein forming a peptide or an isopeptide bond between them. However, few Ub-based probes for such enzyme cascade have been reported very recently. Among the earliest Ub-based probes for E1 are the Ub-AMP mimics. In 2010, Lu *et al.* have shown that a non-hydrolyzable Ub/UBL-adenylate mimic could be used to covalently trap the E1 enzyme (Figure 8B). [40] Later on, An and Statsyuk reported a mechanism-based AMP-derived compound that mimics the Ub/UBL-adenylate upon reacting with the E1-Ub/UBL thioester, which was used as an activity-based probe for E1 enzymes for Ub and different UBLs and as selective inhibitors (Figure 8C). [41] More recently, the same authors reported a dehydroalanine analogue of the adenylate mimic in order to study the structural dynamics of the E1 during the activation of Ub (Figure 8D). [42]

In 2016, Pao *et al.* reported a specific E2-loaded Ub probe that can target nucleophiles in E3 ligases. [43] In this technique, Ub containing thioacrylate and thioacrylamide electrophiles are loaded onto E2 and then incubated with their corresponding E3. The resulting bisthioether of Ub with both E2 and E3 can be used to characterize the enzyme kinetics of an E3 ligase (Figure 8E). Recently, Mulder *et al.* reported the synthesis and use of a transferable Ub-based probe where the C-terminal glycine is replaced with a dehydroalanine moiety which can either form a covalently trapped enzyme intermediate (Figure 8F, a', c', e') or a transferable thioester intermediate with the enzymes that are then transferred to subsequent enzymes in the cascade (Figure 8F, b', d', f'). [44]

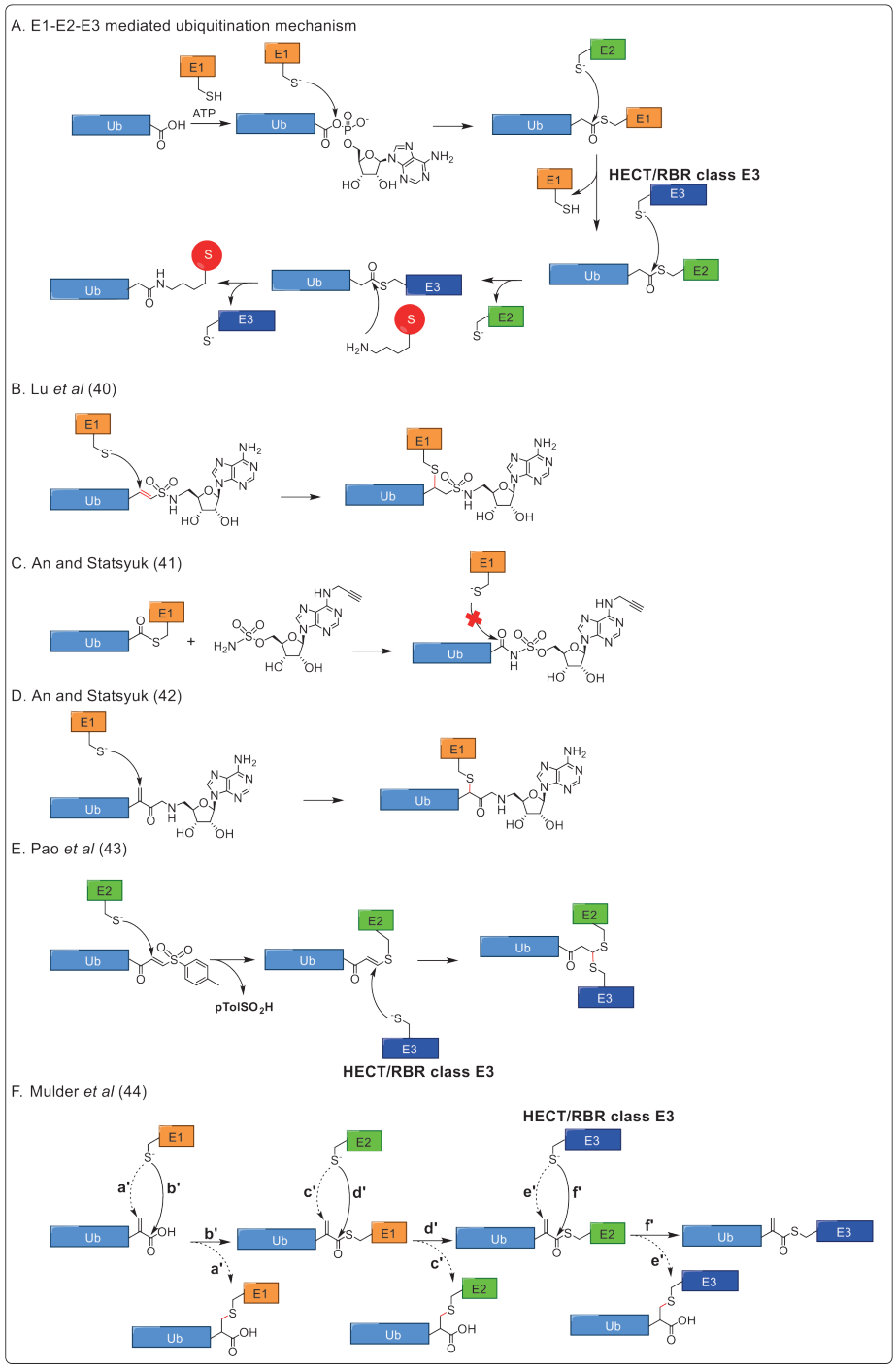
A. E1-E2-E3 mediated ubiquitination mechanism
E1
SH
ATP
Ub
OH
E2
HECT/RBR class E3
E3
S
B. Lu et al (40)
C. An and Statsyuk (41)
D. An and Statsyuk (42)
E. Pao et al (43)
pTolSO2H
HECT/RBR class E3
F. Mulder et al (44)
HECT/RBR class E3
a
b'
c'
d'
e'
f'

Figure 8: Ub-based probes for E1, E2 and E3 enzymes A. *General mechanism of ubiquitin activation by E1 and subsequent action leading to the formation of a ubiquitinated protein.* ***B-D.*** *Ub-based E1 probes that mimic the Ub-adenylate intermediate thereby inhibiting UbE1 enzyme.* ***E.*** *E2-Ub conjugate probe that can react covalently with the corresponding E3 using a Michael acceptor between the C-terminal end of Ub and the E2 enzyme. This probe can capture both E2-E3 in complex with Ub.* ***F.*** *Ub containing a dehydroalanine moiety instead of the terminal glycine is used to either trap the ubiquitinating enzymes or form a transferable Ub-based probe that can be transferred to subsequent enzymes in the cascade pathway.*

Conclusions

The chemical synthesis of ubiquitin has accelerated research in the ubiquitin field. Due to the availability of different assay reagents and probes, many DUBs have been characterized and studied. The next step in the chemical synthesis of reagents is to synthesize an assay reagent or a probe specific for a single DUB. This facilitates enzyme-specific characterization in a cellular environment. This can rely on the identification of ubiquitin mutants that are very specific to a particular DUB. Ernst *et al.* elegantly reported the use of such a Ub mutant library to specifically modulate DUBs, E2/ E3 ligase activity and to develop high-affinity Ub binders for DUBs and Ubiquitin Binding Domains (UBDs). [68] Zhang *et al.* also used a Ub mutant library to develop high-affinity Ub variants for USP7. [69]

Ub-based probes for the cysteine protease class of DUBs have revealed much information on mechanisms of their identity and enzymatic action. However, a different class of probes is needed to study the DUB metalloprotease family because, unlike the cysteine proteases, they contain a Zn^{2+} ion coordinated in their active site. Due to this, the development of a probe is challenging.

Recently, ubiquitin has shown to be phosphorylated at specific sites. Such post-translational modifications on Ub plays regulatory roles in ubiquitin chain processing and chain recognition. [70] At this point, many reagents can be quickly designed and tailored for specific applications.

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