Seminars in Cell and Developmental Biology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Seminars in Cell and Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

State of the art in (semi-)synthesis of Ubiquitin- and Ubiquitin-like tools

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ARTICLE INFO

Keywords:
Ubiquitin(l)
Activity-based probes
Chemical protein synthesis

ABSTRACT

Protein ubiquitination is a key post-translational modification in regulating many fundamental cellular processes and dysregulation of these processes can give rise to a vast array of diseases. Unravelling the molecular mechanisms of ubiquitination hence is an important area in current ubiquitin research with as aim to understand this enigmatic process. The complexity of ubiquitin (Ub) signaling arises from the large variety of Ub conjugates, where Ub is attached to other Ub proteins, Ub-like proteins, and protein substrates. The chemical preparation of such Ub conjugates in high homogeneity and in adequate amounts contributes greatly to the deciphering of Ub signaling. The strength of these chemically synthesized conjugates lies in the chemo-selectivity in which they can be created that are sometimes difficult to obtain using biochemical methodology. In this review, we will discuss the progress in the chemical protein synthesis of state-of-the-art Ub and Ub-like chemical probes, their unique concepts and related discoveries in the ubiquitin field.

1. Introduction

Protein post-translational modifications (PTMs) such as phosphorylation, glycosylation, and ubiquitination are key events regulating various biological processes in eukaryotic cells. Ubiquitination is the covalent attachment of ubiquitin (Ub) to substrate proteins catalyzed by a triad of enzymes (Fig. 1a), namely, E1-activating enzyme, E2-conjugating enzyme and E3 ligase enzyme, and is reversed by deubiquitinating enzymes (DUBs) [1–3]. The first step in the ubiquitination process is the ATP dependent activation of the Ub C-terminal carboxylate via formation of an E1~Ub thioester followed by a trans-thioesterification reaction with an E2 enzyme's active site cysteine to form an E2~Ub thioester. Finally, an E3 ligase mediates the transfer of Ub to the target, either directly by taking over the Ub cargo via an E3~Ub thioester and transferring it to a lysine ε-amine in the target protein or indirectly by acting as adaptor protein between Ub~E2 and target protein (Fig. 1a) [2]. The target protein can be decorated with a single or multiple Ub monomers or with homo- or heterotypic Ub chains, wherein Ubs are interconnected via the M1, K6, K11, K27, K29, K33, K48, or K63 residues of Ub (Fig. 1b) [4]. Yet, another layer of complexity is added to this spectrum of processes by the generation of hybrid chains containing ubiquitin and ubiquitin- like proteins (Ubls), such as interferon stimulated gene 15 (ISG15), small ubiquitin-like modifier (SUMO), and neuronal precursor cell expressed developmentally downregulated protein 8 (NEDD8) [5].

Ubiquitination is a key process in multiple cellular processes including proteasome mediated protein degradation, cell division, DNA repair and cell signaling and its dysfunction has been indicated in several diseases, including cancers, neurodegenerative diseases, and infectious diseases [6]. Research on the complex Ub system focuses on understanding the factors involved in writing, reading, and erasing the Ub code on a molecular level as well as applying this knowledge in combating deregulation of the Ub system. Small molecules specifically targeting the enzymes involved in the ubiquitination cascade are emerging as inhibitors for DUBs or E1-enzyme [7–9] or are used to direct E3 ligases to target proteins in the form of molecular glues or PROTACs that lead to ubiquitination and subsequent degradation of the selected protein [10,11].

Despite extensive research, the study of the ubiquitin proteasome system is far from finished and new and more advanced tools can offer important insights. The identification of specific E2 [12] and E3 ligases [13] allowed for the enzymatic preparation of homotypic polyUb chains and ubiquitinated substrates that have been of great importance in the classification of DUBs [14] and determination of specific interacting domains from associated proteins. However, this biochemical methodology also has limits, for instance the absence of ligases to prepare all polyUb chains selectively, the difficulty to control chain length and creation of homogenic chains in large quantities [15]. To overcome this

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https://doi.org/10.1016/j.semcdb.2021.11.025
Received 6 September 2021; Received in revised form 26 November 2021; Accepted 28 November 2021

obstacle several non-enzymatic methods have been developed which remedy these shortcomings. Such methods have resulted in various polyUb chains and ubiquitinated proteins which have demonstrated to be of great value in deciphering Ub signaling. Another advantage of chemical methodology is the possibility to add non-amino acid chemical groups to the Ub(l) proteins which can be used to generate non-hydrolysable Ub chains, activity-based probes (ABPs) or assay reagents (Fig. 2). Such chemically modified Ub chains and reagents can for instance be applied in studying active DUBs or Ub binding proteins in cell lysate, in studying the interaction between Ub and the involved enzymes using structural biology approaches or in high-throughput screens to identify enzyme specific inhibitors.

This review discusses some of the key methods and recent advances in the chemical preparation of Ub(l)-based probes and reagents and their application in the investigation of (de)ubiquitinating enzymes.

2. Chemical protein synthesis

2.1. Total linear chemical synthesis of Ub(l)

Chemical protein synthesis has given access to proteins that are difficult to prepare by molecular biology approaches, and proteins modified with chemical moieties such as fluorescent tags or warheads (chemical probes) [16]. The chemical synthesis of peptides, by coupling one amino acid to the next in a stepwise fashion in a process called solid phase peptide synthesis (SPPS) is however often limited to 50 amino acids, due to aggregation of the growing polypeptide chain on resin [17, 18]. The discovery of disaggregation building blocks has increased the diversity and accessibility of longer peptides including the linear synthesis of Ub (76 amino acids), Nedd8 (76 amino acids) and SUMO1–3 (92–96 amino acids) from 2010 onwards [19,20]. Commercially available amines, such as for instance propargylamine, can be coupled to the C-terminus of Ub using SPPS methodology, or carboxylic acids, such as for instance carboxyfluorescein, can be coupled to the N-terminus of Ub, creating modified Ub(l)s unattainable using biochemical methods.

2.2. Two segment condensation approaches

The linear synthesis of Ub(ls) requires specialistic equipment, protocol optimization and expensive disaggregation building blocks and hence alternative methods were applied to the synthesis of Ub(l)s and probes and reagents based on these proteins.

2.2.1. Native chemical ligation

Native chemical ligation (NCL) is a chemo selective reaction used to prepare larger peptides/proteins forming a native amide bond between two unprotected peptide segments in solution by reacting a C-terminal fragment carrying a cysteine residue at the N-terminus, with a N-terminal fragment carrying a thioester at the C-terminus [21]. The need of an appropriately positioned cysteine residue in this approach at first hampered the widespread application of NCL and therefore efforts were put in the development of chemoselective desulfurization reactions of cysteine residues. A mild radical-based desulfurization reaction of cysteine was reported in 2007 by Wan and Danishesky to afford the corresponding alanine residue, expanding the scope of NCL significantly [22]. Using this NCL-desulphurization approach, full-length Ub was synthesized using two-fragment, i.e Ub(1−45)-thioester and Ub(46−76) carrying the A46C substitution (Fig. 3b) [23].

2.2.2. α-Ketoacid-Hydroxylamine (KAHA)-ligation

Another less frequently applied approach to prepare native amide bonds is the KAHA ligation, developed by the Bode group, in which an α-ketoacid fragment reacts with a 5-oxaproline fragment giving rise to a homo-serine at the site of conjugation. The Ubl proteins UFM1 [24] and SUMO2/3 [25] were prepared using KAHA ligation.

2.3. Strategies to form native isopeptide bonds in Ub-conjugates and Ub-chains

2.3.1. Auxiliary mediated ligation

The difference in Ub chain length and linkage type on ubiquitinated substrates establishes a specific and desired signaling code inside the cell which is counterbalanced by (linkage specific) DUBs. In order to probe the recognition of native ubiquitinated substrates by DUBs, their Ub-linkage specificity and molecular mechanism it is important to chemically construct the native isopeptide bonds in ubiquitinated proteins or peptides based thereon or Ub chains. Chatterjee et al. were the first to establish the chemical synthesis of ubiquitinated peptides utilizing an auxiliary group and a recombinant Ub thioester followed by photolytic or reductive removal of the auxiliary group resulting in ubiquitinated substrates (Fig. 3a) [26,27]. An alternative trifluoracetic acid labile auxiliary was used later for the synthesis of K48-linked diUb by introducing this auxiliary on recombinant proteins [28].

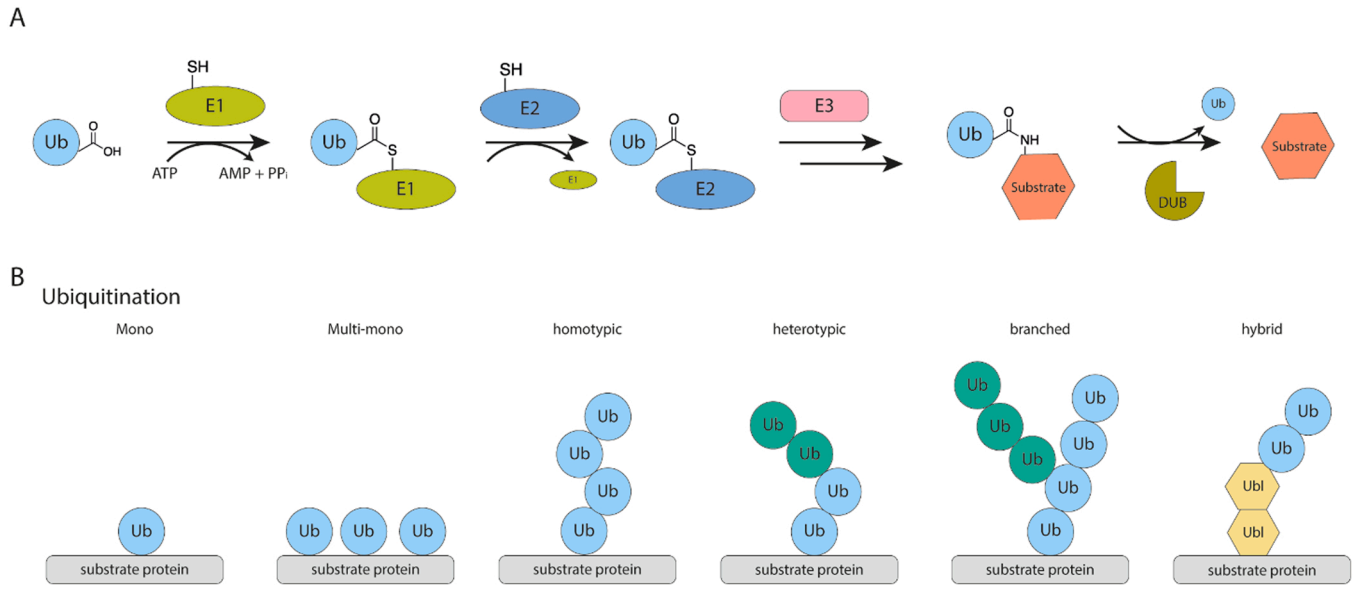

Fig. 1. A) Schematic representation of the Ub system. B) Ub chain topologies.

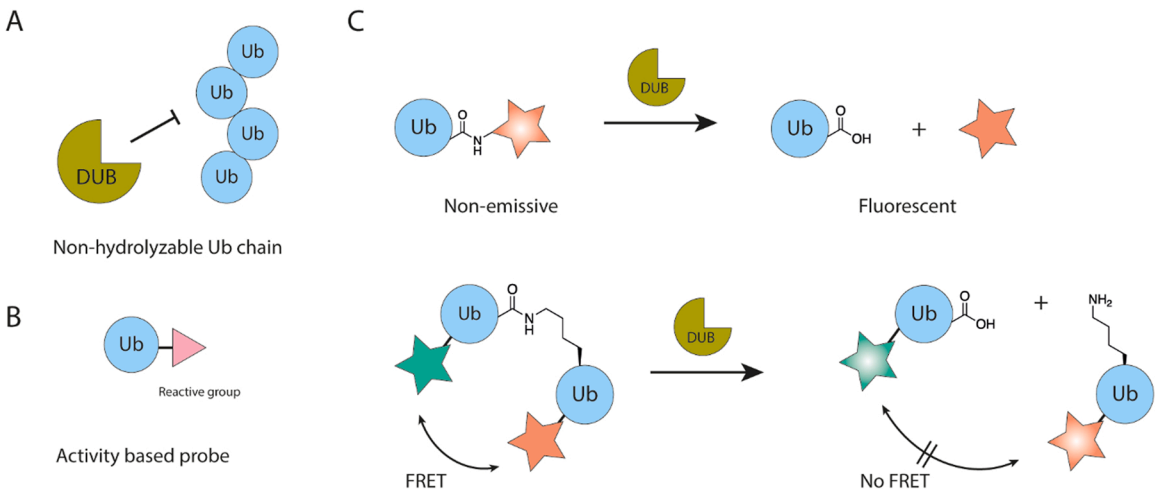

Fig. 2. A) Non-hydrolyzable Ub chain B) Representation of an ABP C) Fluorogenic assay reagent based on Ub and fluorescent energy transfer reagent.

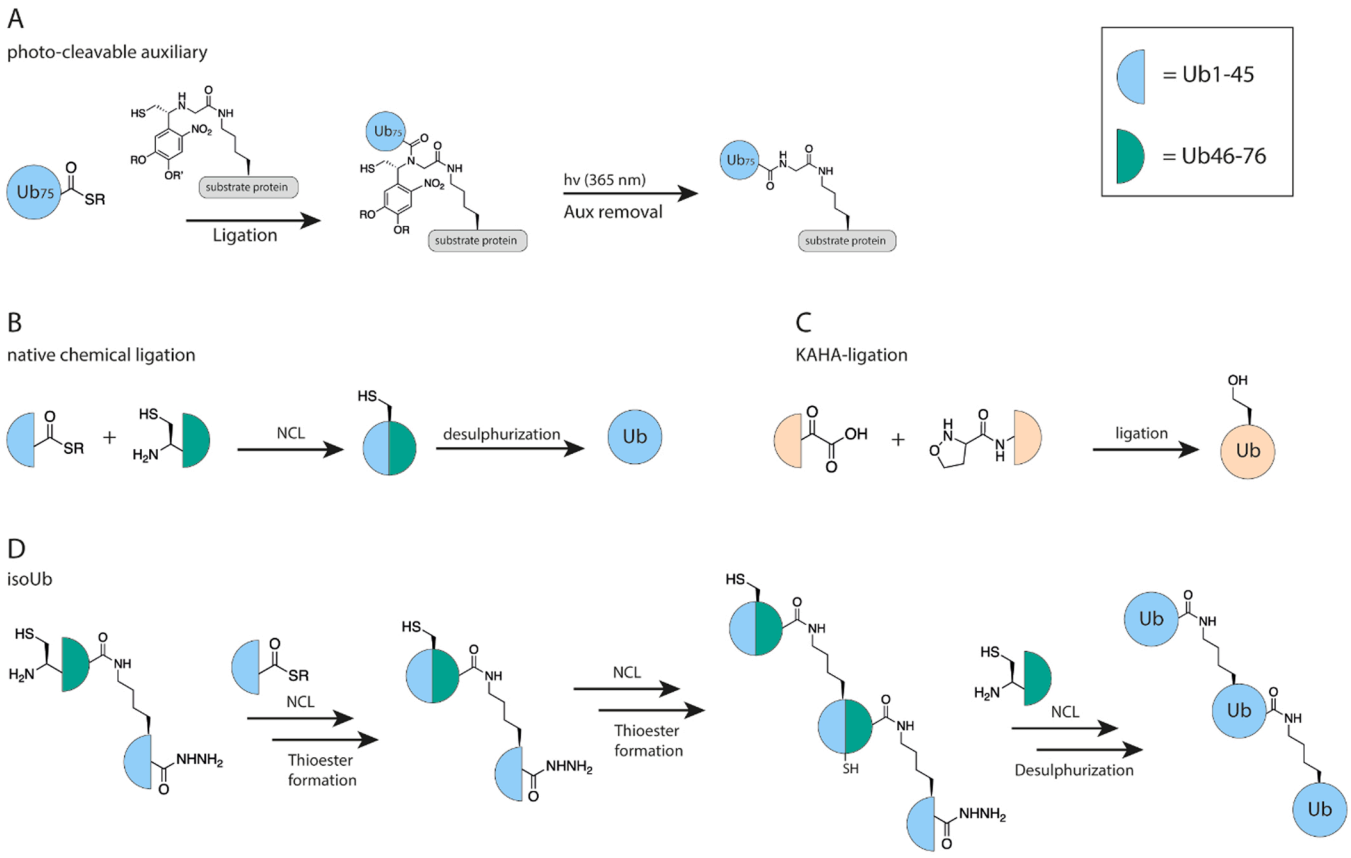

Fig. 3. A) Synthesis of ubiquitinated substrates using the auxiliary strategy B) Synthesis of full length Ub using NCL-desulphurization strategy C) Schematic representation of the KAHA-ligation D) Synthesis of Ub polymers using the isoUb strategy.

2.3.2. *Mercaptolysine mediated ligation*

The biggest limitation of the auxiliary strategies is the low reaction speed, therefore the more reactive δ- and γ-mercaptolysine building blocks designed by the Brik [29] and Liu [30] groups are more widely applied for chemo selective ubiquitination of peptides or Ub chain formation [31]. The thiol functionality in mercaptolysine building blocks serves as a temporary handle during NCL and the native lysine residue can be restored by selective desulphurization chemistry [29,30]. Several analogues of δ-mercaptolysine were prepared to extend its use in SPPS. The thiazolidine protected δ-mercaptolysine for instance was employed in the synthesis of various Ub chains and ubiquitinated substrates using sequential building up of polyUb chains [32]. δ-Mercaptolysine was used to synthesize di- and tetra-ubiquitinated α-synuclein to study the effect of α-synuclein ubiquitination and its association with Parkinson's disease (PD) [33]. Ub synthons carrying both a mercaptolysine and a thioester functionality were used in self-polymerisation approaches to gain access to higher order polyUb chains in a single reaction [34,35]. The successful genetic incorporation of protected mercaptolysine derivatives in recombinant expressed proteins furthermore allows non-chemistry labs to use this methodology, making such Ub materials

widely accessible [36].

2.3.3. isoUb ligation

In 2017, Lei Liu and coworkers designed a new chemical strategy for the preparation of atypical Ub chains based on classical cysteine mediated NCL [37]. The innovative aspect of this approach is that rather than conjugating two full length Ubs together to forge the isopeptide bond at the designated lysine, a universal synthon $Ub_{Distal}(Cys^{46}\text{-}Gly^{76})\text{-}Ub_{Proximal}(Met^{1}\text{-}Phe^{45})$-hydrazide already containing the correct isopeptide linkage is first conjugated to $Ub_{Distal}(Met^{1}\text{-}Phe^{45})$ followed by repeatedly conjugation to itself and in a final stage completed with the C-terminal $Ub_{Proximal}(Cys^{46}\text{-}Gly^{76})$ fragment (Fig. 3c). Radical mediated desulfurization of the cysteine residues to alanines results in the fully native Ub chains and gives rise to homotypic and heterotypic Ub chains of unprecedented length.

2.3.4. GOPAL

The GOPAL approach (genetically encoded orthogonal protection and activated ligation) is a strategy in which genetic engineering is combined with chemical tools to produce diUb chains. It involves the genetic incorporation of a orthogonal protected lysine followed by selective protection of all other lysine residues and deprotection of the protected lysine of interest to allow site selective acylation of this desired lysine [38]. The less extensive researched K6- and K29 linked diUb, for instance, were obtained using GOPAL methodology and used to solve the crystal structure of K6-linked diUb as well as revealing the until then unknown K29 preference of OTU DUB TRABID [38].

2.4. Strategies to form non-native isopeptide bonds

Several research aspects of Ub signaling, for instance pull-down experiments, X-ray crystallography and DUB affinity measurements on active DUBs, cannot be performed with probes containing a native isopeptide bond due to the risk of proteolysis of the used probes by the studied DUBs. Therefore, many non-native isopeptide bond analogues have been developed, some more closely resembling the native isopeptide bond than others (Fig. 4). It has been shown that even slight modifications in the isopeptide region can have a large effect as introduction of a simple methyl group in the isopeptide region can completely prevent deubiquitination of synthetically prepared ubiquitinated substrates [39]. On the other hand, the recognition of triazole linked K48-linked diUb by the severe acute respiratory syndrome (SARS) Coronavirus Papain-like protease shows no interference of the unnatural linkage with the protease in X-ray crystallography [40]. Such unnatural linkages are produced using synthesized Ub analogues using inserted handles or rely on conjugation chemistry with an expressed cysteine mutant to form

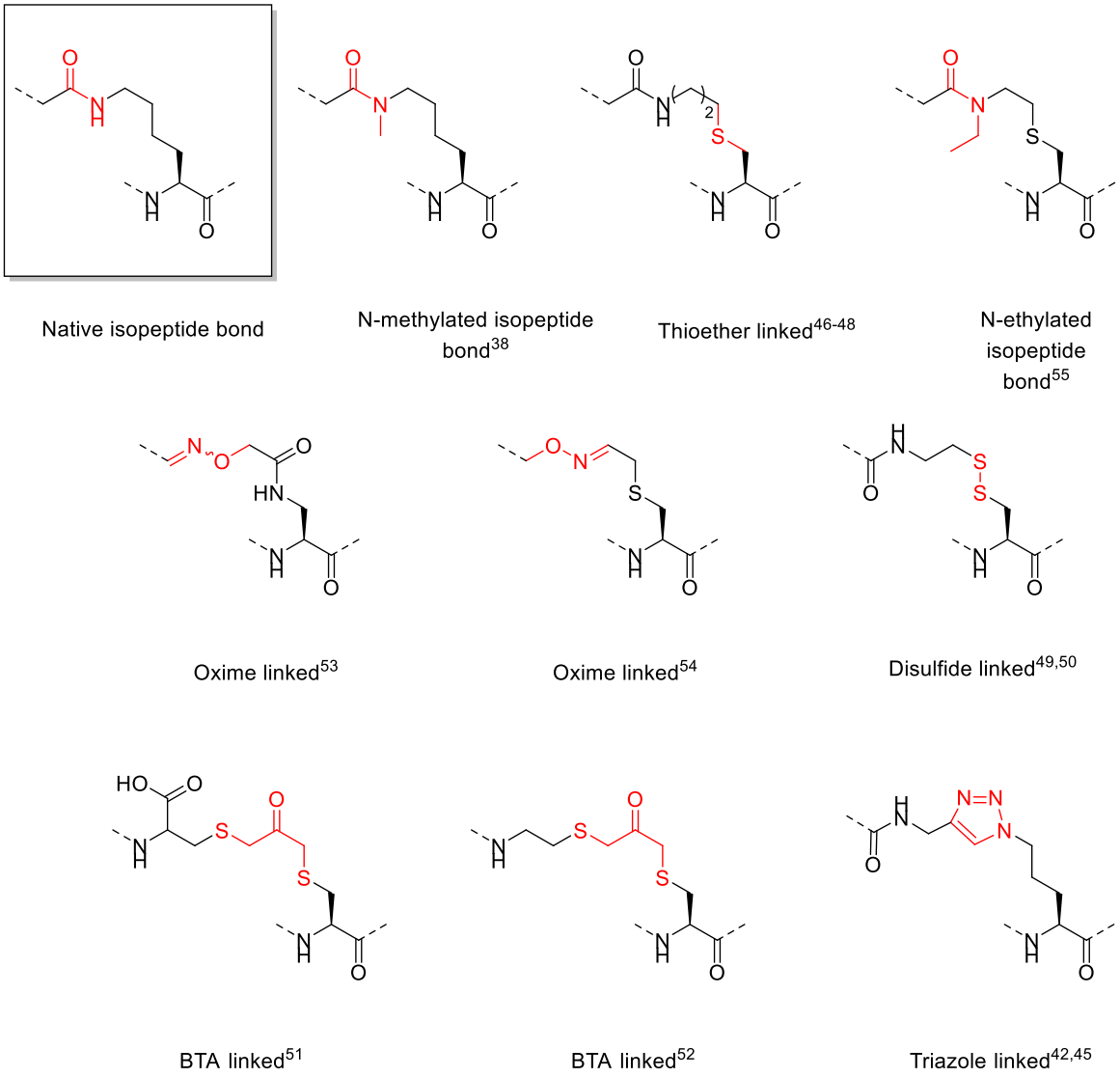

Fig. 4. Native and non-native isopeptide bond analogues.

thioether/disulfide linked bonds. The last technique is more accessible for labs with limited access to chemical methodology, however, often form bonds less stable to DUB proteolysis and are susceptible to reductive conditions in cellular environment.

2.4.1. Triazole linked Ub

The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) is the reaction of an azide with an alkyne forming a triazole linkage. The triazole linkage has proven to be a good isostere of the native glycine-ε-lysine isopeptide bond and is stable to DUB hydrolysis, providing a good isostere to use in the study of DUBs (Fig. 4) [41–43]. Several sets of diUb probes were prepared using CuAAC, including diUb probes with an electrophilic trap at the position of the native isopeptide bond [44] and homogenous polyUb chains [45]. Both sets of probes were used in proteomics studies to define DUB specificity for distinct polyUb linkages in cellular context [44] or identification of interaction partners [45]. Moreover, non-hydrolyzable diUb probes, containing a C-terminal warhead, were prepared, targeting the S1-S2 pocket on DUBs. These probes were used to study the contribution of additional binding interfaces on the reactivity of DUBs and their impact on linkage specificity [40,46].

2.4.2. Other linkages

Introduction of a lysine to cysteine mutation opens the possibility to introduce several different non-native isopeptide bonds such as, a thioether bond [47–49], a disulfide bond [50,51], or a bis-thio-acetone (BTA) linkage [52,53](Fig. 4). Oxime linked Ub has also been used for DUB profiling due to its non-hydrolyzable nature (Fig. 4) [54,55]. More recent, an E1-catalyzed chemoenzymatic strategy was developed to create DUB stable N-ethylated isopeptide bonds (Fig. 4). This new generation probe was able to capture interactors in pull-down experiments that were not detected before by the triazole diUb or polyUb probes [56].

3. Ubiquitin toolbox

The great advantage of synthetic and semi-synthetic methods is the possibility to add specific handles to Ub(l) proteins, which can be used to generate for example activity-based probes (ABPs) and assay reagents (Fig. 2). ABPs are powerful chemical tools that mimic the natural substrates of enzymes involved in the ubiquitination cascade, such as DUBs and E1-, E2-, E3-enzymes, however they contain a warhead that reacts with the active site residues to form a covalent bond with the involved enzymes [57]. Since ABPs only react with active enzymes they can be used as a direct measure of enzyme activity rather than just report on their expression levels. Furthermore, by exchanging the warhead by a fluorogenic group the appearance of fluorescence can be used as real-time read-out for DUB activity [58].

3.1. Probes targeting DUBs

In 1987 the first Ub probe, an Ub containing a C-terminal 4-aminobutyraldehyde (Ubal) was prepared and proved to be an important tool in the early studies of DUBs [59]. Afterwards, many Ub probes with different warheads were synthesized (reviewed in [60]) based on semi-synthesis of Ub and were key in identifying and studying DUBs and ligases [61,62]. Several of these warheads have been incorporated between two Ub moieties, facilitating the capture of linkage specific DUBs [61,63–65]. Furthermore, by placing a warhead in between Ub and its substrate, DUBs involved in deubiquitinating specific substrate proteins can be identified. The dehydroalanine (DHA) warhead, for instance, was introduced in an α-globin-Ub-DHA probe and identified ubiquitin specific peptidase 15 (USP15) as a DUB reversing α-globin ubiquitination [66].

In 2012, Ovaa and coworkers published the total chemical synthesis of Ub-propargylamide (Ub-PA), a novel ABP [67]. Although alkynes were considered to be bio-orthogonal and hence should not react with proteins, Ub-PA was found to react with the active site cysteine of DUBs forming a stable vinyl thioether. Due to its high stability in cellular environment and selectivity towards DUBs, Ub-PA has become the golden standard in Ub ABPs [67]. The reactivity of several substituted alkyne Ub-PA probes was further investigated by Mons et al. [68] on recombinantly expressed DUBs in isolation and in cell lysate. Interestingly, covalent adducts can be formed with internal alkynes and terminal alkynes with substituents (on the internal C3 carbon), in contrast to what was observed previously [67]. Interestingly Ub-PA was initially also shown to react with only one HECT E3 enzyme (HUWE1) and recently, the crystal structure of HUWE1 in complex with Ub-PA was solved [67,69], showing that the coordination of the C-terminal tail of the ligase to the N-lobe and the C-terminal region of Ub is critical for the transfer of Ub to the substrate.

Not all DUBs, however, contain an active site cysteine, as the metalloprotease family of DUBs contain a Zn^{2+}-ion in the active site and therefore does not react with any of the active site cysteine targeting ABPs mentioned above. The first probe targeting this class of DUBs was designed by Shahul Hameed et al. and contains a 8-mercaptoquinoline (8-MQ) Zn^{2+} chelating group at the C-terminus of Ub [70]. The probe shows improved inhibition for Rpn11/Rpn8 compared to full-length Ub and can pull-down metalloDUBs from HeLa cell lysate. Taken all together, this probe shows potential to gain a better understanding of the biochemical processes involved in metalloDUB mediated Ub regulation.

3.1.1. Ubiquitin like probes

In addition to Ub ABPs, several Ubl ABPs have been developed to study the Ubl enzymatic cascades. For example, Rhodamine-SUMO1, 2 and 3-propargylamide were synthesized using SPPS and introduced into HeLa cells where they show colocalization with overexpressed SUMO-specific proteases (SENPs) [20]. In addition, a diSUMO probe containing a reactive vinylamide moiety between the proximal and distal SUMO was synthesized, showing specific labelling of endogenous and ectopically expressed SENPs [20].

This trend was continued for UFM1, however due to inefficient linear synthesis the protein was prepared using NCL. Both a propargylamide and dehydroalanine probe were synthesized and validated in vitro, showing potential applicability in the interrogation of the enzymatic activities of the UFMylation cascade [71]. Besides KAHA ligation and NCL based total synthesis of UFM1, a recent report describes the semi-synthesis of UFM1-PA and UFM1-AMC using an intein based strategy followed by aminolysis with propargylamine or glycinylaminomethylcoumarine respectively, giving access to such probes for virtually all biochemistry laboratories [72]. Similar as for UFM1, ISG15 proved too challenging to synthesize using a linear approach and was therefore synthesized using two fragments and NCL. The mouse version of Rhodamine-ISG15-propargylamide was prepared and validated in vitro, leading to an expansion of the ISG15 toolbox and thereby allows the interrogation of ISG15 biology [73]. Expanding on the two fragment strategy to prepare ISG15 is the three fragment condensation described by Brik and coworkers who prepare the ISGylated Ubiquitin hybrid chain [74].

3.2. Assay reagents for DUB screening

DUBs are key components in the ubiquitination pathway and DUB deregulation is associated with various diseases providing attractive therapeutic targets [8]. Hence, the development of assays and the therein needed assay reagents, to monitor DUB activity in real-time are highly important in the search for novel and selective DUB inhibitors.

Several fluorogenic Ub-probes such as Ub-aminomethylcoumarine (Ub-AMC) [58] and Ub-Rhodamine (Ub-Rhod) [75] have been developed which contain a fluorogenic moiety at the C-terminus. This moiety is non-emissive when conjugated to the protein, but upon the action of a

DUB, proteolysis of the amide bond release the compound as amine and in this process restores fluorescence (Fig. 2). For this reason, these assay reagents are ideal to study DUB kinetics and DUB inhibition by small molecule compounds and have been key in the identification of several potent DUB inhibitors [8,76,77]. Komander et al. identified a covalent (FT827) and non-covalent (FT671) selective USP7 inhibitor using an Ub-Rhod assay [76]. Crystal structures with these selective inhibitors gave insights in the molecular basis for compound specificity. In a similar screen setup Kooij et al. used the Ub-Rhod assay to identify a small-molecule ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1) inhibitor. Further development led to the first selective cell-permeable activity-based UCHL1 probe in which the cyanimide moiety of the inhibitor reacts with the active site cysteine to form a covalent adduct. By introducing an azide functionality on the inhibitor a reporter group could be installed using CuAAC and several fluorescent and biotinylated probe derivatives were synthesized. These probes were used to label active UCHL1 in cells and to monitor UCHL1 activity during the development of zebrafish [7].

3.3. Ubiquitin variants and selective tools

Recently, the development of DUB probes targeting only one specific DUB has gained attention, in the interest of studying the function of individual DUBs in their native context and in the development of therapeutics. Following the pioneering work by Ernst et all. [78] on combinatorial Ub-variant (Ubv) libraries, Gjonaj et al. combined structural analysis, modelling and mutational predictions to introduce mutations in Ub to develop a selective USP7 Ub-variant (M6) and USP16 variant (M20) [79,80]. Charged with a fluorescent label and a propargyl warhead both probes could selectively pull-down USP7 or USP16 from cell lysate. In addition, a selective fluorogenic substrate for USP16 (M20-AMC) was developed capable of following USP16 activity in cell lysate and is therefore a useful tool to study USP16 biology. The M6 Ubv has been further equipped with a cell penetrating peptide in order to deliver TAMRA labelled Ubv-PA into live cells to investigate USP7 activity in vivo [81].

Additionally, DUB selectivity can be achieved by modifying the conserved C-terminal LRGG motif of Ub [82]. Rut et al. performed a high throughput screen (HTS) of fluorescent tetrapeptides derived from the LRGG motif to find a selective sequence for UCHL3 and MERS-PLpro. The mutations of the LRGG motif were introduced in fluorogenic and activity based Ub probes to verify the selectivity of the selected sequences [83]. As already shown by Ernst et all. Ubvs can also selectively interact with E2 enzymes and Middleton et al. used phage display to isolate Ubvs as inhibitors of the E2 enzyme Ube2k. Two potent and specific Ube2k inhibitors were identified with nM affinity. The Ubvs inhibit Ube2k function by two mechanism, firstly by impeding charging of the E2 enzymes by blocking the Ub-fold domain (UFD) and secondly, by disrupting the E3 ligase catalysed Ub discharge [84].

3.4. Fluorescent probes

3.4.1. Fluorescence polarisation probes

Geurink et al. developed isopeptide-linked Ub(l)-based fluorescent polarization (FP) reagents (Fig. 2c) [85] that contain an fluorescently labelled peptide linked via an isopeptide bond to the Ub C-terminus. DUB mediated cleavage of this bond leads to a change in light polarisation, serving as read out for DUB activity. FP-reagents were a.o. applied to study the proteolytic preference of SARS-CoV2 protease PLPro for ISG15 over K48 diUb or monoUb [86]. Another emerging niche is the study of non-lysine ubiquitination, where the ubiquitination sites are serine, threonine, or cysteine residues. It is still unclear which DUBs are involved in the deubiquitination of these unusual sites. An initial high-throughput matrix- assisted laser desorption/ionization time-of-flight (MALDI-TOF) DUB assay on 53 DUBs, showed that most USP- and UCH-class DUBs have both isopeptidase and esterase activity. One big exception is the Machado-Josephin (MJD) family of DUBs, which showed high preference for the Ub-Thr substrate. To validate these results ester linked Ub-Thr-TAMRA FP substrates were synthesized to determine kinetics of the enzymes and might prove valuable in a screening platform for MJD DUB inhibitors [87].

3.4.2. FRET probes

The above mentioned fluorogenic assay reagents do not allow the identification of Ub linkage specific DUB activity. In order to study such preferences FRET probes based on diUb have been prepared chemically and were instrumental in determing kinetic parameters of a variety of DUBs (Fig. 2c) [88]. Kost et al. designed a diSUMO FRET pair using recombinantly expressed diSUMO-2 containing a single azide and cysteine residue for modification with the fluorophores. This FRET sensor proved a useful tool to study the importance of biochemical and structural properties of SUMO chains and multi-SIM proteins [89].

3.5. Tools to interrupt Ub chain recognition

Ubiquitination is a key process in proteasome mediated protein degradation and several drugs that inhibit the proteasome have been approved for anti-cancer treatment, underscoring the importance of the protein degradation process [90]. K48-linked polyUb chains target proteins for proteasomal degradation, hence molecules that can bind selectively to K48-linked chains could potential interfere with the recognition and subsequent degradation of the ubiquitinated target. Biotin-K48-linked tetraUb was prepared chemically and used in the random non-standard peptides integrated discovery (RaPID) system for the screening of novel cyclic peptide inhibitors. A first and second generation of cyclic peptides were identified as highly selective K48-linked tetraUb interactors and show in vitro and cellular activity comparable to known proteasome inhibitors [91,92]. To simplify the process of finding macrocyclic peptide inhibitors for K48-linked tetraUb a fluorescent-based competitive high-throughput screening (HTS) assay was developed based on a peptide library. Highly potent next generation dimeric peptide inhibitors were developed, highlighting the potential of this technique to find inhibitors for other complex targets, such as hybrid chains [93].

3.6. Probes targeting the conjugation machinery

The Ub ligase cascade is more complex to target due to the collaborative actions of E1, E2 and E3. Many probes have been developed over the recent years targeting this complex machinery, which have been reviewed recently by Schulman et al. [94]. Of interest for this review is that some of the chemical tools and methods used to prepare DUB probes are also applied to target components of the ligase machinery.

One of these probes targeting the conjugation machinery is a cascading probe synthesized by Mulder et al. The probe contains an electrophilic dehydroalanine moiety at the G76 position of Ub designed to target the Ub conjugation machinery. The probe either undergoes sequential trans-thioesterification reactions as it cascades from the E1 to the E2 and then to the E3 or is irreversible trapped by the active site cysteine of one of the enzymes [95] and hence is capable of capturing the entire E1, E2 and E3 pathway. More recently an effective DHA formation strategy was applied to form a variety of Ub(l)-DHA probes [96].

Activity based E2-probes have recently emerged as effective tools for studying the molecular mechanism of E3 ligases. Virdee et al. developed an Ub charged E2 conjugating enzyme prepared by CuAAC chemistry, which carries a warhead that can selectively react with the activity site cysteine of a RBR-type E3 to produce a stable E2-E3-Ub intermediate (Fig. 5b) [97]. In addition, Schulman et al. developed a thioether-linked E2-Ub probe which more closely resembles the native structure (Fig. 5b). This probe enabled the visualization by cryo-electron microscopy of key steps in the ubiquitination actions of two different E3 ligases; Neddylated SCF and ARIH1 [98]. More recent, activity-based

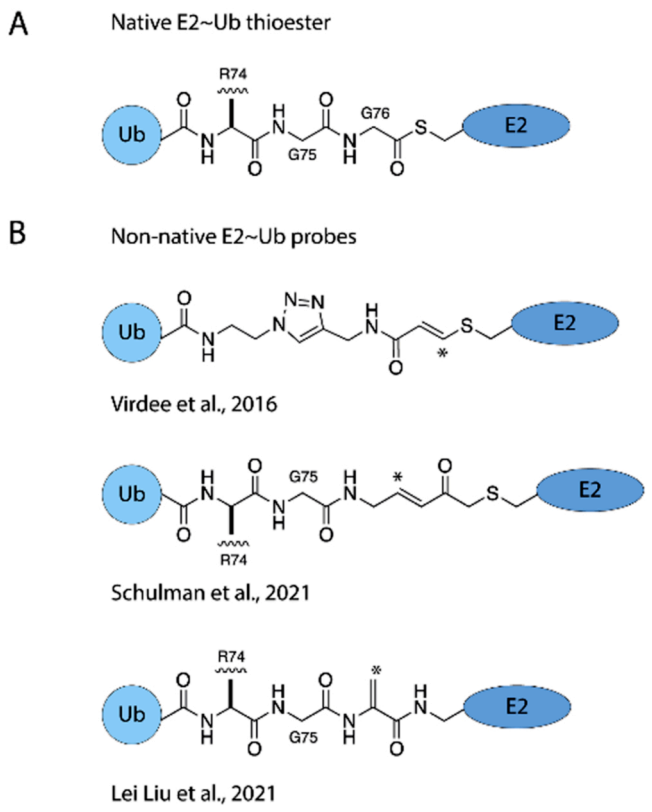

Fig. 5. Chemical structures of A: the native E2~Ub thioester and B: activity-based E2-Ub probes. * Indicates the reactive site upon which the active site cysteine residue reacts.

E2-Ub probes were prepared where a dehydroalanine mutation is inserted on or near the catalytic cysteine of UBED2D2 or UBE2L3 by Jing Shi and coworkers and Lei Liu and co-workers, respectively (Fig. 5b) [99,100]. This ubiquitinated E2 probe is a close structural mimic of the native E2-Ub complex. The probe was found to be active against HECT family member NEDD4 and RBR family member Parkin in crosslinking experiments [100]. Mathur et all. report on the development of a photocrosslinking tool based on a stabilized Ub charged E2 that allows the covalent capture of RING ligases [101].

In order to investigate the chemistry of Ub chain formation by E2/E3 enzymes Liwocha et al. chemically prepared Ub acceptor molecules in which the Lysine residue was systematically replaced by longer and shorter amine acceptors [102]. Many of the studied Ub chain forming enzymes were found to be reliant on the optimal geometry of a lysine residue as accepting amino acid.

3.7. Probes to find ubiquitin interactors

As mentioned before non-hydrolyzable diUb or polyUb chains are essential tools in investigating interaction partners [43], due to their stability against DUBs and other native conditions.

Zhang et al. developed a label-free ubiquitin interactor affinity enrichment mass spectrometry (UbIA-MS) method to identify interactors in an unbiased manner. All seven linked triazole linked diUbs were used in pull-down studies and confirmed or identified linkage-selective Ub interactors in several cell types [103]. Zhao et al. used a similar method and CuAAC to synthesize all seven Ub polymers and performed an affinity enrichment experiment with K27-, K29-, and K33-linked polyUb chains [45]. Zhao et al. identified protein interactors that were not found by Zhang et al., suggesting that linkage specific interactions can be affected by the length of the Ub chain. Further studies on the interaction between UCHL3 and K27 diUb by Pan et al. revealed an unique inhibition mechanism where the crossover loop of UCHL3 binds between the two Ub moieties putting UCHL3 in a constraint conformation [104]. Van Tilburg et al. further showed that K27-linked Ub chains can form a thioester bond with the catalytic cysteine of the DUB [105]. The substrate and UCHL3 work together as a signal response pair where increasing concentrations of substrate give higher chances of inhibiting UCHL3 in a so-called ''kinetic trap''.

Variations in Ub chain length give rise to signalling differences and DUBs may have preference for longer chains like USP5 [106], or shorter chains like UCHL3 [107]. To investigate differences in chain length preference Lutz et al. generated K27-, K29-, and K33-linked Ub chains of defined length and linkage type using click chemistry and gel-eluted liquid fraction entrapment electrophoresis. Lengths up to tetramers could be obtained in high purity, whereas bigger chains were obtained as a mixture. Clear length-dependent and linkage-selective binding of proteins was detected in affinity enrichments assays in cell lysate. More interaction partners were found using longer chains compared to the dimers. Interestingly, it was found that K29- and K33-linked hexamers and longer chains bind metabolite interconversion enzymes and that K29- and K33-linked shorter chains have a preference to bind protein modifying enzymes [108].

3.7.1. Photo cross linking reagents

Delivering proteins to the 26 S proteasome for degradation is one of the best documented Ub signalling pathways, however difficult to study due to the transient nature of these interactions. To investigate such transient interactions photo cross-linking reagents have proven to be useful [109,110]. Photo cross-linking reagents contain chemical moieties that are transformed into highly reactive species upon irradiation, after which they form a covalent bond with proteins in close proximity. This approach enables unbiased identification of binding partners. Ub containing a photoleucine (pLeu) at position 8 or 73 was synthesized and incorporated into homogenous K48- and K63-linked polyUb chains (polyUb^{PT}). PolyUb^{PT} were able to trap Rpn10 and Rpn13 from an intact 26 S proteasome complex and identified Rpn1 as a third proteasome Ub-associating subunit. Its ability to irreversibly trap protein-protein interactions of a hydrophobic nature makes polyUb^{PT} an unique reagent for studying Ub-associating proteins in complex or isolation [109].

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To study the transient interactions between SUMO and other proteins Taupitz et al. incorporated photo-inducible crosslinkers into SUMO1 probes. Benzoyl-phenylalanine (BPA) was genetically encoded in proximity to the SUMO-SIM binding interface of SUMO1 and successfully pulled-down SUMO1 interacting protein RanBP2_SIM1 from cell lysate [110]. This concept was expanded to diSUMO-2(BPA) and tetraSUMO-2 (BPA) containing a BPA at position R50 of the proximal SUMO subunit. A novel protein list with potential SUMO binders was identified and also included proteins that do not contain a SIM motif [111].

3.8. ABPs in studying viral and bacterial ubiquitination pathways

3.8.1. Coronaviruses

Ubl signalling plays a key role in the immune response, including involvement in pro-inflammatory pathways that antagonize viruses such as the coronavirus severe acute respiratory syndrome coronavirus (SARS-CoV) responsible for the worldwide outbreak of the coronavirus disease outbreak in 2003 and global pandemic in 2019 (COVID-19). SARS papain-like protease (PLpro) is a viral DUB produced by SARS-CoV and specifically cleaves K48-linked polyUb chains into K48-linked diUb units. This specificity was determined using several K48-linked non-hydrolyzable diUb-ABPs and is caused by the interaction of K48-linked polyUb with the S2-S1 pockets of SARS-CoV-PLpro. In contrast to SARS CoV-PLpro, SARS CoV2-PLpro preferentially cleaves ISG15 from substrates [86,112]. Several ABPs including ISG15-PA, ISG15-AMC, K48-linked diUb-PA and K48-linked diUb-AMC probes were used to determine the protease activity and change in specificity of SARS

CoV2-PLpro compared to SARS CoV-PLpro. Ub- and ISG15-FP probes were used to determine catalytic efficiency of SARS CoV2-PLpro, showing the ISG15-TAMRA FP reagent is hydrolysed 350-fold more efficient then the Ub-TAMRA FP reagent (Fig. 6) [86].

3.8.2. *Mycobacterium tuberculosis*

Tuberculosis (TB) is caused by infection with the *Mycobacterium tuberculosis* (Mtb) bacteria, which belongs to one of the few bacterial orders that has a prokaryotic Ub-like protein (Pup) protease system. This is an attractive target to develop new drugs against as it is Mtb specific. A truncated Pup (Pup33–63)-AMC assay was developed by Merck et al. and applied in a HTS in pursue of new inhibitors for the Pup proteasome system that could be further developed into selective drugs against TB (Fig. 6) [113,114]. 1280 pharmaceutically active compounds were screened using this assay and two hits were identified. A SAR-study revealed important structural features of the inhibitor that were found to inhibit both the pupylating and depupylating enzymes of Mtb.

3.8.3. *Legionella pneumophila*

Damaged organelles and proteins are cleared by the cell using a conserved ''self-eating'' process called autophagy. Autophagosomes are key components in the autophagy process by engulfing and sequestering cytoplasmic components. The Ubl proteins microtubule-associated protein light chain 3 (LC3) C-terminus is conjugated to phosphatidyl-ethanolamine (PE) which is crucial for autophagosome membrane dynamics, substrate recruitment and localization. In 2013, Yang et al. synthesized LC3-PE using expressed protein ligation (EPL) and used the protein to investigate the function of LC3-PE in membrane tethering and fusion [115]. Simultaneously, Lei Liu and coworkers synthesized LC3-PE containing a fluorophore using a photo cleavable solubilizing tag needed to combat the hydrophobic nature of the PE [116]. The semi-synthetic LC3-PE labeled with a small fluorophore was validated in confocal microscopy and was shown to be a useful replacement for GFP-fused LC3. Dysfunction in autophagy has been linked to several diseases, including, *Legionella pneumophila* infection. *Legionella pneumophila* delivers an effector protein called RavZ which irreversibly deconjugates LC3B from PE and thereby inhibits the autophagy process. In 2017, Yang et al. synthesized LC3-PE functionalized with different PEs and discovered that RavZ first extracts LC3-PE from the membrane before cleavage. Furthermore, three LC3-interacting regions (LIR) motifs where

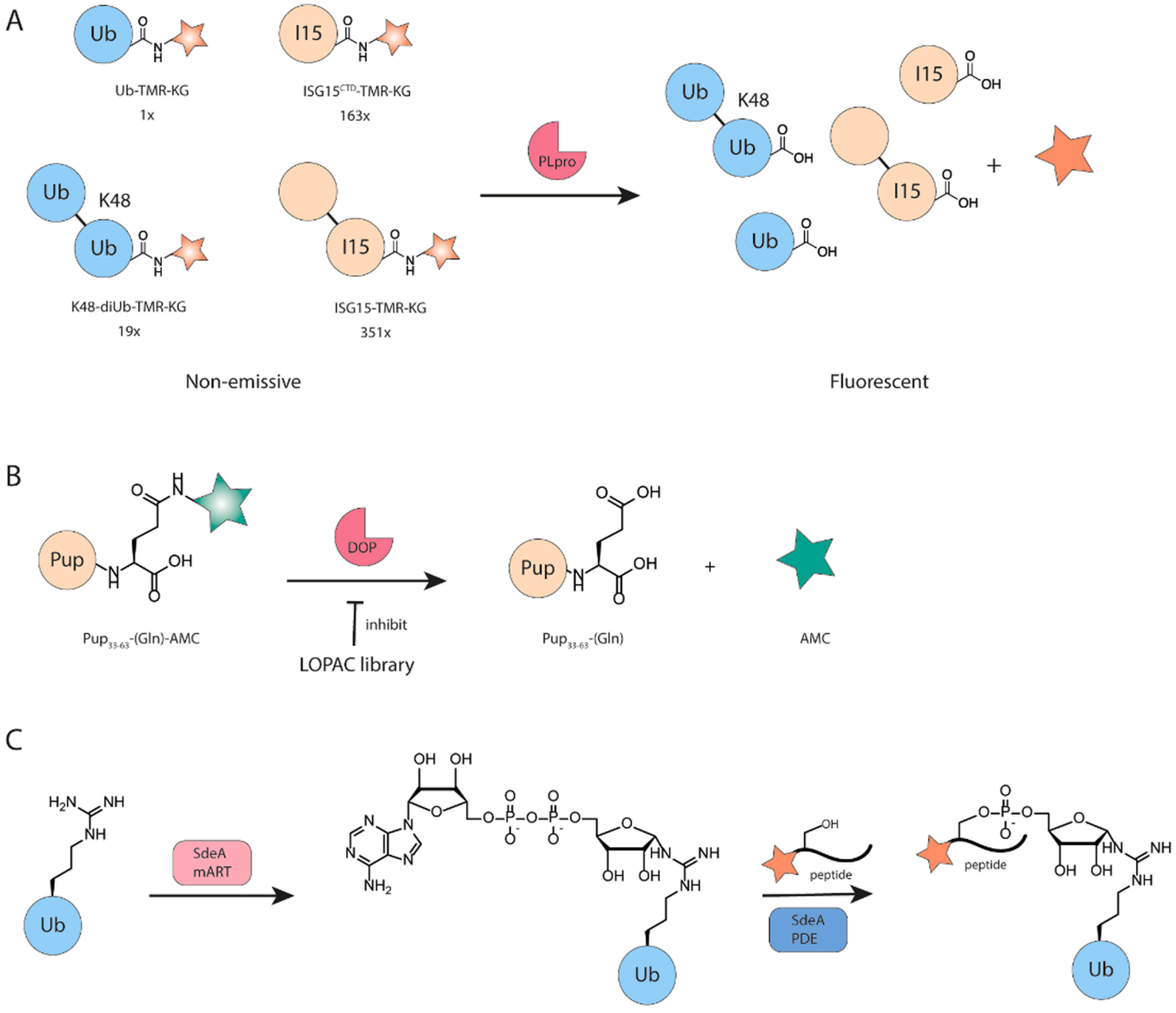

Fig. 6. A) Overview of FP probes used to derive the catalytic efficiencies of PLpro. Substrate preference is indicated by x-fold activity relative to Ub-TMR-KG cleavage. B) Fluorogenic assay to monitor Dop activity and screen for inhibitors. C) SdeA ubiquitination assay using a fluorescently labelled peptide.

identified on RavZ, of which LIR2 (residue 27–32) was shown to be essential for the RavZ:LC3 interaction and extraction from the membrane of autophagosomes [117]. More recently, a set of LC3-PE probes was synthesized containing mutations in the C-terminal region (residues 116–120) to investigate ATG4B and RavZ specificity.

Besides interfering with autophagosome formation, *Legionella pneumophila* interferes with the host cells ubiquitin system to increase intracellular replication efficiency, by ubiquitinating host proteins in an unconventional manner [118]. In this non-canonical ubiquitination process the first step is ADPribosylation of arginine 42 in Ub followed by coupling of a serine residue in the substrate protein, while releasing adenosine-mono-phosphate, to give rise to a phosphoribosyl ubiquitinated substrate. A chemical method was developed by Liu et al., using click chemistry, to synthesize Ub^{ADPr} analogues. These non-hydrolyzable triazole analogues were validated in an auto-ubiquitination assay and proved to behave comparable to the native linkage [119]. This methodology was extended by the preparation of several Ub^{ADPr} analogues on all four arginine positions in Ub that were used to investigate the (de)conjugating enzymes involved in this process. [120]. Besides the synthesis of Ub^{ADPr} analogues FP-assay reagents were developed by Puvar et al. that can be used to screen for inhibitors against the activity of the *Legionella* enzymes involved in this unusual ubiquitination process (Fig. 6.) [121].

4. Conclusion

Ub(l) based probes are of great significance in elucidating the complex and fascinating biology of the ubiquitination machinery. Chemical probes to capture and monitor enzymes or Ub interactors have evolved over the recent years and are capable of targeting enzymes previously not accessible, e.g. the conjugation machinery. The development of specific DUB- and cell permeable small-molecules probes opens the possibility to profile enzyme activity within the complex environment of the cell. Moreover, the ligation machinery has been underexplored for many years due to the lack of suitable probes, which are emerging now, opening a whole new aspect in Ub research. Furthermore, the increase in the development of HTS methods could lead to novel and specific inhibitors as potential therapeutics. The ongoing progress and innovation in chemical protein synthesis will without a doubt lead to the development of new Ub(l) chemical probes in the future, enabling the dissection of many of the still enigmatic aspects of ubiquitination.

We do apologize to all colleagues who's work we were unable to cite do to scope or size limitations of this manuscript.

Acknowledgements

YH acknowledges funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 765445 (ITN UbiCODE) and GJvdHvN acknowledges funding by Netherlands Scientific Organisation (NWO), Netherlands (VIDI grant VI.192.011).

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