Exploring ubiquitin and ISG15 biology with chemical tools

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Chapter 1.

General Introduction

Proteins play an essential role in almost all the processes of a living organism, and post-translational modifications (PTM) can regulate their structure, location, function, and fate. Ubiquitination, one of the most versatile and common PTMs in eukaryotic cells, is a reversible and dynamic process involving linkage of one or more ubiquitin (Ub) molecules to a substrate protein [1]. Ubiquitination of a protein often provides a signal for proteasome degradation [2]. Besides ubiquitination, eukaryotic cells have evolved several ubiquitin-like modifications to regulate biological processes, such as ISGylation [3], SUMOylation [4, 5], Neddylation [6, 7], UFMylation[8], and others. In the years since the discovery of the Ub system, it has been difficult to study the functions and mechanisms of ubiquitination-related processes in detail, due to lack of appropriate technology. The advances in chemical tools, including but not limited to modified model substrates [9], activity-based probes [10-12], and small-molecule inhibitors [13-15], have revolutionized our ability to explore the ubiquitin and ubiquitin-like systems. This thesis describes and discusses the development and application of a series of chemical tools into exploration of ubiquitin and ISG15-related biology.

Ubiquitin and (De)Ubiquitination

Ubiquitin (Ub) is a 76-amino-acid polypeptide (8.6 kDa) that adopts a compact globular β-grasp fold with a flexible C-terminal tail. Its sequence is highly conserved throughout eukaryote evolution. In the ubiquitination process, Ub is first activated by forming a thioester with E1 ubiquitin-activating enzyme in an ATP-dependent manner, and subsequently transferred to the E2 ubiquitin-conjugating enzyme via trans-thiolation and linked to a substrate with E3 ligases mediating the selectivity. The C-terminus of Ub can be ligated to the ε-amino group of a lysine residue via isopeptide bond or α-amino group of the N-terminal amino acid in a substrate protein. Ubiquitination can be reversed by enzymes called deubiquitinases (DUBs) [16] (Figure 1).

Ubiquitin substrates can be modified with a single Ub moiety (mono-ubiquitination), by multiple mono Ub moieties on different lysines within the same substrate (multi-ubiquitination), or by chains of Ubs attached one to another via one of the eight lysine residues of Ub itself (poly-ubiquitination) [17]. Accordingly, eight attachment sites within Ub itself (M1, K6, K11, K27, K29, K33, K48, and K63) can be used for a variety of Ub linkage formations. Different Ub linkages confer different functions or fates to the target protein. For instance, K48 linkage is the most abundant type and has been extensively studied as proteasomal degradation signal (described in detail in **Chapter 2**), while M1-linked linear chains act predominantly in the NF-κB activation processes [18]. On the other hand, the K63 linkage-type plays an essential role in DNA damage responses [19], trafficking [20], and immune responses [21]. Besides homotypic (one linkage type) chains, heterotypic (two or more linkage types) chains are also possible [22]. Furthermore, the complexity of Ub chains can increase along with the supplementation of other PTMs on Ub, such as phosphorylation [23], acetylation [24], or inclusion of ubiquitin-like proteins, like SUMO [25], NEDD8 [26], or ISG15 [27].

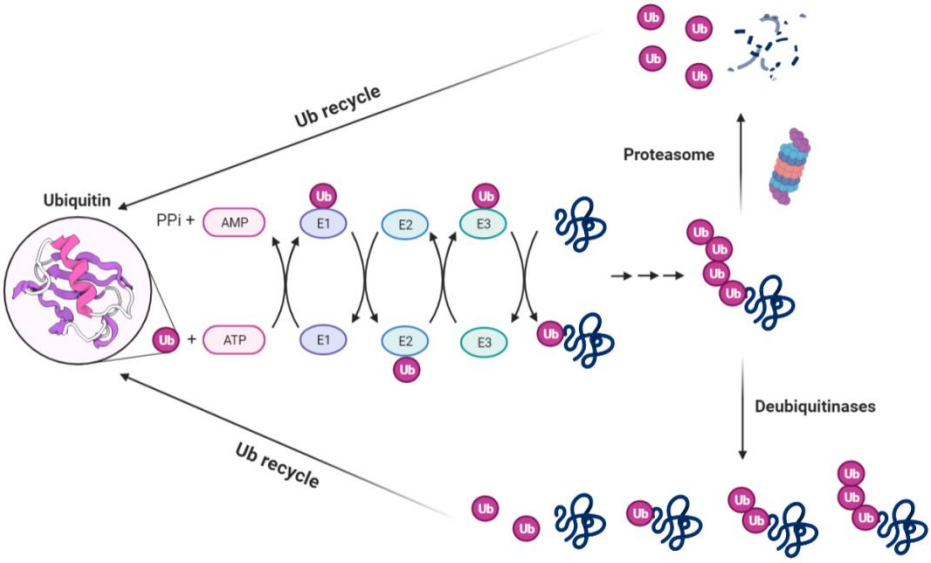

Figure 1. Schematic illustration of the ubiquitin cycle. Ubiquitination is carried out in an E1-E2-E3 enzyme cascade, ATP consumption is required in the first step. Ubiquitinated substrates can be degraded by the 26S proteasome, the attached Ub chains are firstly en bloc removed by the proteasome-associated DUB Rpn11 [28]. The unanchored chains are further dissembled into single Ub by other DUBs, e.g., USP5 [29], while substrates are degraded into peptides by the 20S proteasome. Ubiquitin can be also trimmed or cleaved from the ubiquitinated substrates by various DUBs in a proteasome-independent manner. Both proteasome degradation and deubiquitination processes enable the cycling of the Ub moiety.

Deubiquitinases are a group of proteases that cleave Ub attached to substrates or within Ub chains. Currently, 99 putative DUB genes have been identified in the human genome, but 11 of them are regarded as catalytically deficient enzymes [30]. DUBs are classified into seven families based on their sequence and domain conservation. Six of them belong to cysteine proteases that carry a nucleophilic cysteine thiol in a catalytic triad to mediate the hydrolysis of iso-peptide bond between substrate and Ub, including ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Machado-Josephin domain proteases (MJDs), motif interacting with Ub-containing novel DUB family (MINDYs) and Zinc finger containing Ub Peptidase 1 (ZUP1) [30-34]. The seventh family consists of zinc-dependent metalloproteases, including the JAB1/MPN/MOV34 metalloenzymes (JAMMs) [35, 36]. It is noteworthy here that the main enzymes described in this thesis, USP18, and USP16 are members of the USP family, while OTUB2 belongs to the OTU family.

DUBs need to recognize the correct ubiquitinated substrates for the cleavage of ubiquitin. Numerous DUBs directly bind target proteins and cleave ubiquitin moieties from substrates regardless of the ubiquitin chain linkages; therefore, their substrate specificity appears to be mainly determined by high-affinity protein-protein interaction between DUBs and substrates. For example, USP7 recruits its P/AxxS motif-containing substrates

MDM2 and p53 through its N-terminal TRAF domain [37, 38]. In contrast, some DUBs, especially OTU, MINDY and ZUP1 DUBs, are linkage specific and less concerned with the substrate [33, 39, 40]. A good example is OTULIN, which exclusively targets Met1-linked chains [41]. However, DUBs can also be both linkage and substrate specific. For instance, OTUB2 cleaves Lys11- and Lys48-, and preferentially Lys63-linked chains but is also selective for substrate proteins [42, 43].

Besides targeting ubiquitin, some DUBs have been reported to be cross-reactive towards ubiquitin-like proteins (UBLs). For example, UCHL3 can cleave both NEDD8 and ubiquitin model substrates [44] and USP21 cleaves both ubiquitinated and ISGylated substrates [45]. Several other DUBs have evolved to be specific to only one type of UBL, such as USPL1 being a SUMO-specific isopeptidase [46], and USP18 being an ISG15-specific isopeptidase [47].

DUBs are subject to multiple layers of regulatory mechanisms to fine-tune their abundance, localization, and activity. These mechanisms involve control of expression, substrate interaction, allosteric regulation and PTMs [48, 49]. Expression of some DUBs can be induced in a cell type- and stimulus-dependent manner. CYLD and A20 are induced upon NF-κB activation, and thereby act as negative feedback regulators of NF-κB signaling [50, 51]. The transcription of USP16 was also reported to be enhanced by NF-κB activation [52]. The expression of certain DUBs has been found to be elevated during tumor progression, such as in the case of OTUB2 [42, 43] and USP5 [53]. Moreover, a comprehensive amount of DUBs have been found to be phosphorylated, ubiquitinated, SUMOylated and oxidated, which could affect DUB localization, stability, activity and/or interactor binding [54, 55]. For instance, OTUB2 is found to be poly-SUMOylated on Lysine 233, and this SUMOylation enables OTUB2 to bind YAP/TAZ, thereby stabilizing YAP/TAZ through deubiquitination [42].

ISG15 and (De)ISGylation

Like Ub, ubiquitin-like proteins (UBLs) are also widely distributed in mammalian cells. UBLs usually share structural and evolutionary relationships with Ub [56], and include Small Ubiquitin-like Modifier (SUMO) [4, 5], Neural precursor cell expressed developmentally down-regulated protein 8 (NEDD8) [6, 7], Ubiquitin-fold modifier 1 (UFM1) [8], Ubiquitin-related modifier-1 (URM1) [57, 58], FAT10 (human leukocyte antigen-F adjacent transcript 10 or Ubiquitn D) [59, 60], Interferon-stimulated gene 15 (ISG15) [61], Autophagy-related protein 8 (ATG8) and Autophagy-related protein 12 (ATG12) [62].

ISG15 is composed of two ubiquitin-like domains which are connected by a “hinge” polypeptide sequence [63]. It is expressed as a precursor protein, and needs to be processed by deISGylating enzymes to expose its carboxy-terminal LRLRGG motif before conjugation (ISGylation) [64]. ISG15 exists in three distinct states: intracellular free ISG15, extracellular ISG15, and conjugated to lysine residues of target proteins. Intracellular free ISG15 works as a reservoir to provide modifiers for ISGylation, but was also reported to non-covalently bind to intracellular proteins, thereby modulating their functions [65, 66] or stability [67]. Extracellular ISG15, secreted by fibroblasts, neutrophils, monocytes, and lymphocytes [68], functions as a cytokine to enhance interferon (IFN)-γ secretion by lymphocytes [69] or peripheral blood mononuclear cells (PBMCs) [70], which is vital for the activation of both innate and adaptive immune

responses [69, 71]. Recently, the leukocyte function-associated antigen-1 (LFA-1) has been identified as one of the receptors for extracellular ISG15 [72].

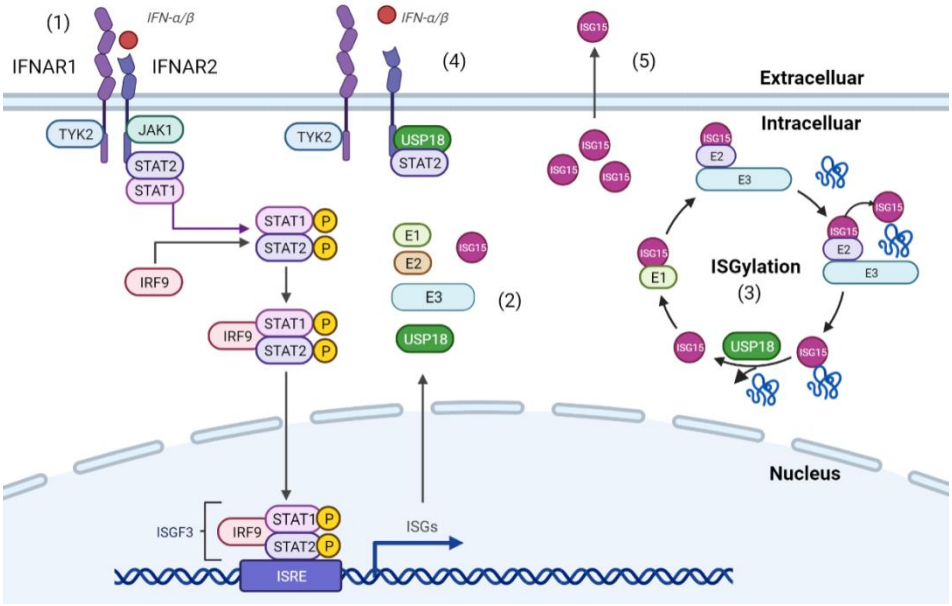

Figure 2. Type-I interferon signaling and the ISGylation cycle. (1) Binding of Type-I interferons leads to dimerization of Interferon alpha/beta receptors IFNAR1 and IFNAR2. The JAK-STAT pathway is subsequently activated, resulting in phosphorylation of STAT1 and STAT2 and formation of the ISGF3 complex (including IRF9 and phosphorylated STAT1 and STAT2). This complex translocates to the nucleus and binds Interferon-sensitive response elements (ISRE) to activate transcription of interferon-stimulated genes (ISGs). (2) Upon induction by interferon, ISG15, E1 (UBE1L), E2 (UBE2L6), E3 (HERC5, TRIM25 and HHARI), and USP18 mRNA and protein accumulate in the cytoplasm, which starts and/or potentiates (3) the ISGylation cycle. (4) USP18 also associates with IFNAR2 and STAT2 to form an inhibitory complex to regulate IFN signal transduction negatively. (5) Free ISG15 can be secreted into the extracellular space as a cytokine.

Analogous to ubiquitination, ISGylation is mediated by a sequential E1-E2-E3 enzyme cascade including E1 UBE1L [73], E2 UBE2L6 [74, 75], E3s (HERC5 [76], TRIM25 [77] and HHARI [78] in human cells, mHERC6 [79, 80] in murine cells), and deconjugating enzymes (mainly USP18 [47]). The ISGylation components and process can be strongly induced by either pathogenic infection or stimulation by type I interferon (IFN-α and IFN-β), or lipopolysaccharide (LPS) [81]. Proteome-wide ISGylated substrates under specific stimulations have been studied in both mouse and human cells [82, 83]. Interestingly, enhanced levels of ISG15 and ISGylation were also observed in many cancer cells [84, 85], but it still remains elusive whether ISG15 and ISGylation have anti-tumor or pro-tumor functions, as discussed in several reviews [84-86]. However, it is well established

that ISGylation plays a critical role in antiviral responses by modifying both viral and host proteins, which is reviewed in [3, 81, 87].

Ubiquitin-specific peptidase 18 (USP18, also known as UBP43) is the major deISGylase to remove ISG15 from its targets, and does not have reactivity towards ubiquitin [47]. According to the crystal structure of mouse USP18, resolved alone and in complex with mouse ISG15, the C-terminal domain of ISG15 is necessary and sufficient for USP18 binding, and its specificity toward ISG15 is mediated by the interaction between a hydrophobic patch in mouse USP18 (formed by residues Ala138, Leu142, Gln139, Ser192, and His251) and a region of mouse ISG15 (Trp121, Pro128 and His149) [88]. Independent of its deconjugating activity, USP18 also can act as a negative regulator of type I and type III interferon signaling. USP18 can directly bind to IFN-α/β receptor 2 (IFNAR2) by associating with the Signal transducer and activator of transcription 2 (STAT2) to form a ternary protein complex, thus competing with receptor-associated Janus kinase 1 (JAK1) and inhibiting IFN-α/β signaling [89, 90] (Figure 2). Defects in the non-enzymatic function of USP18 can lead to severe type I interferonopathy in both mice [91] and human [92].

In addition to USP18, several ISG15-crossreactive DUBs have been reported, including USP2, USP5, USP13, USP14, and USP21, but the studies on these enzymes only showed ISG15 probe labeling [93], or *in vitro* substrate cleavage [45]. Moreover, several viral deISGylating enzymes were reported, including viral ovarian tumor domain proteases (vOTUs) from Nairovirus and arteriviruses [94], papain-like proteases (PLpro) in coronavirus [95, 96], and leader proteases (Lbpro) in picornavirus [97].

To study the biological relevance of (fine-tuning of) ISG15 conjugation, detailed analysis of the specific roles of the various ISGylation and deISGylation enzymes is needed. Although USP18 is the major deISGylase, a small-molecule inhibitor against USP18 activity has not been reported in the literature so far. Moreover, besides USP18 ISG15-crossreactive DUBs were known, but it remained to established whether their deISGylation functions are relevant in a cellular context. Interestingly, small-molecule inhibitors against USP18 enzymatic activity are now reported in **Chapter 4** of this thesis, while **Chapter 5** describes the identification of USP16 as a so-far unreported ISG15-crossreactive DUB with a deISGylation function both *in vitro* and in cells.

Chemical tools to study Ub signaling

In recent years, use of chemically prepared tools, including modified enzyme substrates, has been a helpful strategy to decipher the intricacies of the ubiquitin-proteasome system (UPS) and ubiquitin signaling. Specific tools for proteasome components, deconjugating enzymes, and E1-E2-E3 cascade enzymes have been developed in various laboratories. The proteasome-related tools will be described extensively in **Chapter 2**. For the E1-E2-E3 cascade-related tools I refer to several recent reviews [12, 98], since they are not used in the studies described in this thesis. Here, I will focus on the chemical reagents developed for deconjugating enzyme-related studies, in particular on the tools developed and utilized in my own Ph.D. work.

The deconjugating enzyme tools used in this thesis involve three types of reagents: modified substrates, activity-based probes (ABP), and small-molecule inhibitors.

Modified substrates for deconjugating enzymes

The adapted substrate reagents for deconjugating enzymes consist of one or more Ub moieties attached to reporter molecules. Figure 3A shows a typical fluorogenic substrate, in which Ub is conjugated to a quenched fluorogenic molecule at the C-terminus via an amide bond. The cleavage of the amide bond by a DUB uncages the fluorophore causing an increase of fluorescent intensity that is proportional to the DUB activity and can be measured at certain wavelengths depending on the fluorogenic molecule. These substrates include ubiquitin C-terminal 7-amido-4-methylcoumarin (Ub-AMC) [99], Ubiquitin-Rhodamine110Gly [100], and Ubiquitin-Rhodamine morpholine [101]. In theory any DUB can cleave these substrates, so they lack DUB specificity, and are mostly utilized to measure the cleavage activity of purified (e.g., recombinant) DUBs *in vitro*. The specificity of these substrates can be improved by modifications and/or specific mutations in the Ub part. For instance, non-hydrolyzable DiUb-AMC was developed to characterize the Ub linkage-specific reactivity of OTUD2 and OTUD3 [102], and the Ub-M20-AMC substrate was published to monitor endogenous USP16 enzymatic activity in cell lysates due to the high specificity of the engineered Ub mutant M20 towards USP16 [103]. Another issue with these substrates is high background noise in the assays when a high substrate concentration is used to measure low-affinity DUBs. This problem was circumvented by replacing the fluorophore with luciferin (Figure 3B). In this case DUB cleavage releases the free amino-luciferin which is not luminescent by itself, and background noise is therefore almost absent. Detection of the DUB activity can only occur after addition of luciferase, which oxidizes luciferin to release photons in the presence of ATP and Mg^{2+} [104].

The isopeptide-linked fluorescence polarization (FP) substrates, in which the C-terminus of Ub is linked to the lysine residue of substrate peptides via an isopeptide bond (Figure 3C), represent a second type of substrates. A fluorophore (such as TAMRA) is generally attached to the N-terminus of the peptide, and can be excited by polarized light. The value of fluorescence polarization / fluorescence anisotropy (FP/FA) can be calculated based on the measured parallel and perpendicular polarized fluorescence intensity. The FP value reflects the degree of polarization of a fluorophore, which is inversely related to its molecular rotation. The fluorophore rotation is largely driven by Brownian motion [105], the smaller it is, the faster it rotates, and the lower FP value is. Therefore, DUB cleavage results in the release of the fluorescent substrate peptides from substrates leading to a decreased FP value [106, 107]. This type of substrate distinguishes itself from the previous with the advantage that it contains an isopeptide bond for DUB cleavage, which is more related to a real physiological substrate. Moreover, its affinity for DUBs can be greatly enhanced by functionalizing the linked peptides with substrate-derived elements around the iso-peptide linkage. For example, recombinant USP7 efficiently hydrolyses Ub FP substrates containing TAMRA-labelled 17-amino-acid $PTEN_{5\text{-}21}$ rather than $PTEN_{281\text{-}297}$ [107]; and recombinant OTUB2 does not cleave TAMRA-K(Nedd8)G [39], but shows cleavage toward Biotin-VKAK(Nedd8) IQD (a short peptide from $Ub_{26\text{-}32}$) [108].

A third group is formed by the Fluorescence Resonance Energy Transfer (FRET) assay-based substrates (Figure 3D), in which Ub and substrate are labeled by two different fluorophores, and linked via a native iso-peptide bond. DUB cleavage results in the dissociation of the two fluorophores, resulting in decrease of the FRET signal, which can be used to quantify the enzyme kinetics [109]. Importantly, these FRET assay substrates were further developed to specific DiUb reagents, in which the two Ub residues are linked via either M1, K6, K11, K27, K29, K33, K48 or K63, which became valuable tools for analysing the ubiquitin linkage specificity of DUBs [110-112].

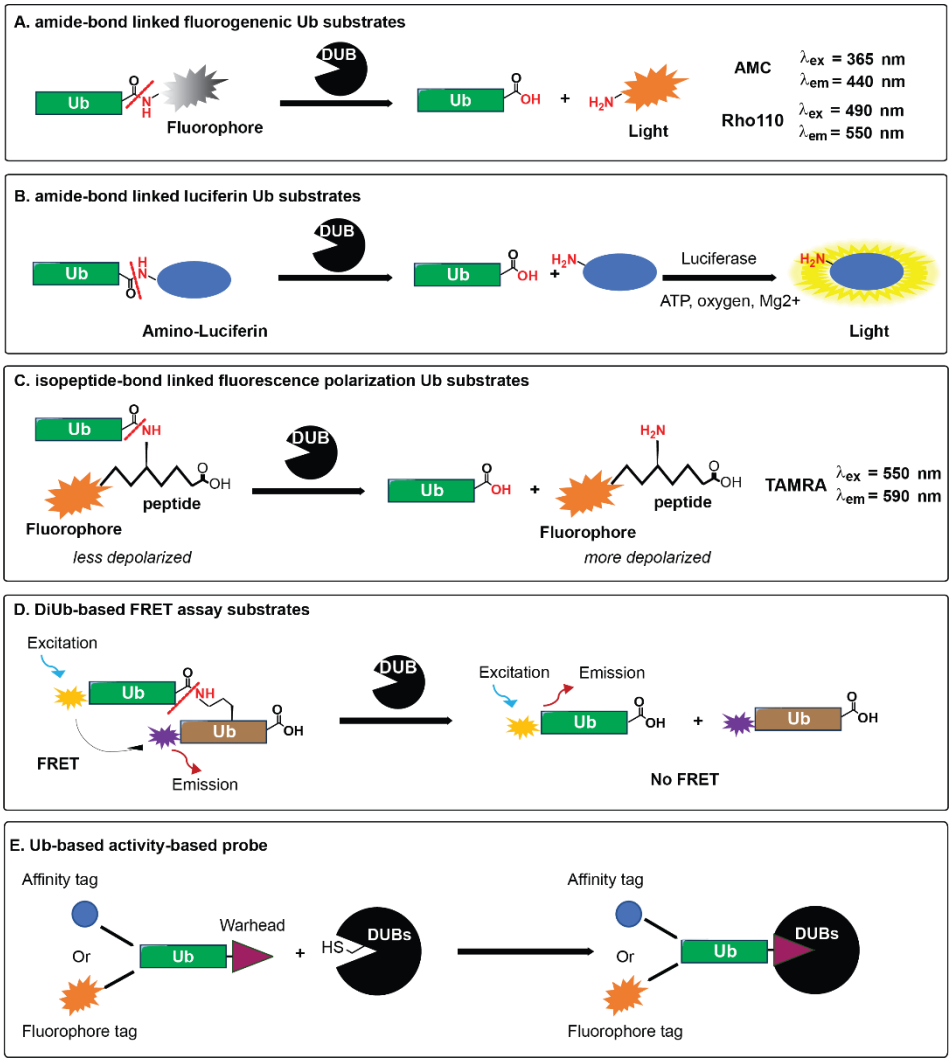

Figure 3. Schematic illustration of Ub-based chemical reagents for DUB research.

All of the above substrates have been widely utilized within *in vitro* settings, and have boosted high-throughput screening (HTS) to identify small-molecule inhibitors for a specific DUB, and/or allowed the functional characterization of DUB cleavage specificity. Improving the specificity of substrates toward cleavage by a single DUB is supposed to be one of the directions for future developments.

Activity-based probes for deconjugating enzymes

The Ub-based chemical probes (Figure 3E) usually comprise a reactive group, a recognition element, and a reporter tag. The reactive group contains an electrophile warhead that forms a covalent adduct with the active site cysteine residue of the DUBs. During the development of the Ub-based probes, a comprehensive of reactive groups has

been explored [113, 114]. Of these, the propargylamide (PA) remained the most cysteine-selective, and most widely used the reactive group for Ub/UBL-based ABPs [115]. Besides cysteine-active warheads, a zinc chelator 8-mercaptoquinoline (8-MQ) was exploited as a warhead to capture metalloprotease DUBs [116]. The recognition element of ABPs confers selectivity toward the deconjugating enzymes. It can be either a monomeric Ub [115], Ub variants [103, 117], a UBL [88, 118, 119], a Ub chain [102, 120], or even a ubiquitinated substrate protein [121, 122]. The reporter group is used to detect probe labeled enzymes; a fluorescent tag (such as Rhodamine fluorophore) allows for rapid and sensitive detection, while an affinity tag (such as HA or biotin) facilitates the isolation and enrichment.

ABPs enable monitoring of deconjugating activity changes; inhibition of an enzyme usually corresponds to a reduction of probe labeling. Because of this, ABPs are widely applied to study the potency and selectivity of small molecule inhibitors within a cellular context. When the probe is fluorescently tagged, the labeled enzymes can be easily resolved on an SDS-PAGE gel and visualized by fluorescence scanning or immunoblots against target proteins [101, 123]. In this thesis, such kind of ABPs are exploited to assess the target engagement of small-molecule inhibitors in a cellular context (USP18 inhibitors in **Chapter 4** and OTUB2 inhibitors in **Chapter 6**).

In an advanced strategy, probe labeled deconjugating enzymes are first enriched by immunoaffinity purification, after which analysis by label-free liquid chromatography-tandem mass spectrometry (LC-MS/MS) can identify and quantify the cellular active DUBs bound to the probe [124]. This technique has for instance been applied to identify UCHL1 as a highly active DUB in aggressive breast cancer [125], and for the development of small-molecule inhibitors against USP7 [126], USP9X [127], and USP28 [128].

The Ub / UBL-based ABPs are also versatile tools in the elucidation of the enzymatic mechanisms and identification of novel Ub / UBL deconjugating enzymes. In the crystal structures of mouse USP18 bound to the mouse ISG15-PA probe, a hydrophobic patch in USP18 was found to mediate the specificity of USP18 toward ISG15 [88]. Identification of SUMO-deconjugating enzymes was enabled by activity-based profiling in HeLa cell extract with an HA-SUMO-VME probe, which led to the identification of USPL1 as a new SUMO protease [46]. Ub-PA probe profiling in cell extract enabled the identification of ZUP1 as a member of novel DUB class with high Ub Lys63-linkage specificity [33]. ISG15-vinyl sulfone (ISG15-VS) probe profiling on *in vitro* transcription and translation (IVT) generated DUBs identified USP2, USP5, USP13, and USP14 as ISG15-crossreactive proteases [93].

Nowadays it is becoming popular to apply total chemical synthesis to generate the above-mentioned ABPs. The synthetic concepts established for Ub-based ABPs [129] has expanded to the generation of Ub variants [103, 130], SUMO [118], UFM1 [119], and ISG15-based ABPs. Synthetic ABPs for mouse ISG15 are discussed in **Chapter 3**. It is quite promising that more specialized ABPs can be generated based on these synthetic approaches, such as hybrid Ub/UbL chain-based ABPs, post-translational modified Ub (e.g., phosphorylated Ub)-based ABPs, and ABPs with additional useful chemical handles (e.g., Ub-based ABP installed with photo-crosslinkers to capture the binders of Ub-DUB complex). A shortcoming of the Ub-based ABPs is that they are not cell-permeable and that their usage is in general limited to purified DUBs or cell lysates. Several attempts to address this cell-penetration issue have been reported, including cell membrane micropore formation with either toxin [131] or electroporation [118, 119], and addition of cell-penetrating peptides (eg TAT peptide and cyclic polyarginine) onto the ABPs [132]. It is

foreseeable that DUB research within the context of an intact live cell or organism will be greatly potentiated by the development and application of cell-permeable ABPs in the near future.

Small-molecule inhibitors for deconjugating enzymes

Biological research on protein fate and stability has strongly benefitted from the generation of specific small-molecule inhibitors, such as cycloheximide to block protein translation [133], MG132 to inhibit proteasome-mediated protein degradation [134], and Bafilomycin A1 to inhibit endosomal acidification and autophagy [135]. Small-molecule inhibitors hold several advantages over genetic manipulation: 1) they act on their target protein immediately, giving cells limited time to compensate for protein inhibition; 2) they allow transient blockage of proteins or biological pathways that are essential for cell survival; 3) the inhibition is reversible; 4) small-molecule inhibitors are more applicable across different cells types or tissues [136].

The physiological and pathophysiological roles of DUBs have been intensively studied in the past decades [137-140]. Since pharmacological targeting of DUBs is supposed to have therapeutic potential, the development of potent and selective small-molecule inhibitors against DUBs is an increasingly explored research area. Several reviews have summarized the DUB inhibitors that are currently in development [13, 139]. Although most DUB inhibitors are still in a preclinical stage, some of them have emerged as valuable tools in advancing the biological research on their DUB targets. One of the aspects is to identify DUB substrates. Many DUBs are supposed to stabilize a subset of target proteins from proteasome degradation by removing Ub chains, and in those cases DUB inhibition can reduce target protein abundance. Such DUB substrates can be identified by treatment of cells with selective and potent inhibitors and subsequent analysis by proteome-wide quantitative mass spectrometry approaches. This strategy has benefited substrate identification for USP7 [141] and USP9X [127]. When the small molecule DUB inhibitor binds its target active site covalently it can be used as a cell-permeable and target-specific ABP when labeled with a fluorophore. For example, UCHL1 small-molecule ABP 8RK59 was derived from inhibitor 8RK64 by installing a Rhodamine fluorophore, and was used to selectively and spatiotemporally detect UCHL1 activity during the development of zebrafish embryos [101]. Nonactive site-targeting small-molecules can be further employed to be DUB-targeting chimerae (DUBTACs) to counteract cellular ubiquitination. For instance, small-molecule EN523, which targets OTUB1 Cysteine 23 without inhibiting deubiquitination activity, was linked with CFTR binding ligand lumacaftor to be a DUBTAC, which recruits OTUB1 to ΔF508-CFTR to stabilize the mutant CFTR protein in cells [142].

Traditional strategies in biological research on DUBs rely on genetic depletion and/or ectopic (over) expression of wild type and (domain) mutated proteins, or genetic inactivation of DUB enzymatic activity. These techniques have become much more powerful when combined with specific small molecule inhibitors and probes. Importantly, according to the latest review, DUB inhibitors can be classified into five types by their mechanism of action [143]: 1) inhibitors binding within the active site and stabilizing the active conformation; 2) inhibitors binding within the active site and stabilizing an inactive conformation; 3) allosteric inhibitors in Ub-binding sites of a DUB; 4) allosteric inhibitors outside of Ub-binding sites; 5) bivalent inhibitors simultaneously binding two regions of a DUB. It is known that many DUBs exist in an equilibrium of different conformations, and each conformation may correspond to a unique regulation or protein interactome in cells.

Thus, by applying appropriate inhibitors to lock the conformational dynamics of a specific DUB within a cellular context novel regulatory mechanisms and/or binding proteins of this DUB might be revealed.

Aim and outline

In this thesis, novel ubiquitin and ISG15-related chemical tools are described and discussed. Part of these tools were utilized to unravel novel functions and regulatory mechanisms of specific deconjugating enzymes. These efforts deepen our understanding of the complexity of the Ub and ISG15 systems and provide essential insights for the development of therapeutics in the future.

Chapter 2 gives an overview of various fluorescence-based tools and methods to study proteasome function and activity. It highlights the use of chemically synthesized activity-based probes to study proteasome localization, dynamics, and activity in living cells.

Chapter 3 provides a method for total chemical synthesis of full-length mouse ISG15. This synthetic mouse ISG15 could efficiently react with the ISG15 E1 enzyme. The derived activity-based probe Rho-mISG15-PA reacted well with mouse USP18 protein.

Chapter 4 describes the development of small-molecule inhibitors against murine USP18, enabled by the development of the mISG15 ABP described in chapter 3. The identified inhibitors enhanced cellular ISGylation in mouse EL4 cells and therefore can serve as research tools to investigate USP18 regulation and biochemical mechanisms. They also appear good starting points for USP18-targeting therapeutics.

Chapter 5 shows the suitability of the developed ISG15-PA ABP to profile deISGylases in cell lysates. USP16 was hereby identified as a novel ISG15-crossreactive DUB. The putative ISG15 hydrolase function of USP16 was validated with several in vitro substrate cleavage assays. In addition, a cellular USP16-dependent ISG15 interactome was identified by an ISG15 IP-MS study.

Chapter 6 describes the improvement of small-molecule inhibitors against human OTUB2. With the use of the most promising inhibitor, novel regulatory mechanisms of OTUB2 were revealed, both with respect to post-translation modification and regulation of expression.

Acknowledgements

Images in Figure 1 and 2 were created with BioRender (https://biorender.com). Images in Figure 3 were created based on the templates from Dr. Dharjath Ahamed Shahul Hameed.

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