Exploring the Ub/UBL landscape with activity-based probes

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Chapter 7

Discussion and Future Perspectives

Ubiquitination is inarguably one of the most impactful post translational modifications next to phosphorylation. Modulating a plethora of cellular process, the regulation of the Ubiquitin conjugating and deconjugating enzymes is key and has been studied extensively. However, some aspects of Ubiquitin biology remain enigmatic, as appropriate tools to study these enzymes are lacking thus necessitating the development of such reagents. In particular, studying the mechanistic nature of Ubiquitin transfer from the conjugating E2 enzyme to the E3 ligase has long been impeded due to the lack of suitable methodologies allowing accurate recapitulation of the chemical and structural nature of an E2-Ubiquitin thioester intermediate. Although mimicking of the transient E2-Ub thioester complex has been attempted by the use of disulfides, oxyesters, or lysines, these surrogates do not reflect the molecular nature of Ubiquitin transfer to the E3 ligase. To overcome this, we developed a transferrable activity-based probe that is sequentially relayed from the E1 to the E2 and finally to the E3 enzyme, but simultaneously permits the covalent trapping of these enzymes (**Chapter 3**). Concurrently, I also explored whether genetically encoding an unnatural amino acid that would allow generation of stable thioester surrogates (**Chapter 2**). Given the challenges of amber suppression technology, such modification of an enzyme's active site proved difficult due to unforeseen metabolic issues arising from the genetic incorporation of an unnatural amino acid such as L-azidoalanine.

Later, the repertoire of activity-based probes was expanded to the Ubiquitin-like modifiers SUMO and UFM1, by developing facile synthesis methods that allowed the generation of reagents and activity-based probes (**Chapters 4** and **5**). With these tools available, identification of novel conjugating and deconjugating enzymes and their subsequent characterization is feasible opening new avenues for understanding the underlying biology.

Especially the advent of a practical synthetic strategy to obtain fully synthetic UFM1, permitted the generation of ABPs targeting the UFM1 enzyme cascade, allowing insights into UFM1 biology (**Chapter 5**). Despite access to UFM1 ABPs and reagents, many unknowns regarding the design of these tools (i.e. the suitable reactive groups, substrate contexts, etc.) remain to be explored to develop a powerful toolkit akin to that for Ubiquitin enzymes. Another aspect impeding the rapid development of suitable tools is the lack of knowledge of UFM1-modified substrates and their underlying biology. In an attempt to dissect the UFMylated proteome, a proteomics strategy that permits the site-specific identification of UFM1-modified substrates described in **Chapter 6** was devised. Given the low abundance and the rapid turnover of UFMylation, it is unsurprising that only a handful of target proteins were identified with the ribosomal protein RPL26 being the most prominent target upon UFSP2-depletion.

In conclusion, the research outlined in this thesis demonstrates the utility of generating and applying ABPs for Ubiquitin and Ubiquitin-like modifiers to address outstanding questions in the field. Last but not least, we explored UFM1 biology by identifying its substrates and

forayed into dissecting the molecular mechanism of RPL26 UFMylation during protein translocation.

Introduction of unnatural amino acids by amber codon suppression (Chapter 2)

Genetically encoding unnatural amino acids into proteins in cells or even organisms by repurposing the amber stop codon (UAG) has permitted their site-specific introduction and the generation of a myriad of designer proteins. This technology has facilitated new insights into the conformational changes, protein-protein interactions, and has furthered the dissection of signal transduction pathways, and has enabled the site-specific installation of post-translational modifications such as phosphorylation[1-3], methylation[4-6], or even Ubiquitination[7, 8]. In particular, the genetic encoding of an unnatural amino acid allowing the formation of stable enzyme-Ubiquitin complexes is critical to investigating the underlying structural and molecular mechanisms governing Ubiquitin transfer from the E2 to the E3 enzymes[9-11]. To accomplish the site-specific installation of 2,3-L-diaminopropionic acid (Dap), which permits the generation of a stable Ubiquitin-E2 thioester intermediate, we set out to evolve the pyrrolysine tRNA synthetase (PylRS) to recognize the unnatural amino acid L-azidoalanine. Although we identified a PylRS variant that charged L-azidoalanine, further validation by MS/MS revealed that the isostere amino acid L-cysteine had been incorporated instead. As an alternative approach to precisely modify a protein post-translationally, we explored whether chemical mutagenesis would enable the installation of 2,3-diamiopropionic acid (Dap) by utilizing dehydroalanine as a chemical handle and further modify it with an amine. Surprisingly, the elimination of the active-site cysteine of our model enzyme UCH-L3 proceeded effortlessly, but further transformation into the photocaged amine was futile, due to lack of suitable biocompatible reaction conditions.

Conclusively, the misincorporation of L-cysteine instead of L-azidoalanine by the evolved PylRS variant underscores that while the binding cavity had been modified to facilitate the recognition of this unnatural amino acid, other factors ranging from inefficient acylation activity of the evolved PylRS to metabolic alterations arising from intermediates prevented the introduction of L-azidoalanine to the genetic lexicon. Moreover, the identification of an improved PylRS variant capable of effectively charging this unnatural amino acid may facilitate the translational incorporation of L-azidoalanine. Given that laboratory evolution is a slow and tedious process inherently limited to allowing only a few selection rounds, recent technological advances such as phage assisted continuous evolution (PACE) to rapidly evolve an aminoacyl synthetase highly specific and catalytically efficient for a given unnatural amino acid[12] would potentially lead to the identification of an efficient PylRS variant. This innovative methodology, which connects aminoacylation activity

to phage infectivity, facilitates the mutation, selection, and replication of variants over hundreds of generations, and thus rapid convergence at the best possible variant[12]. Since evolved tRNA synthetases typically exhibit dramatically reduced acylation activities[12-14], this inefficiency has been compensated for by overexpression using multi-copy plasmids for both the tRNA-synthetase and the cognate tRNA[15]. To resolve this shortcoming, *E. coli* strains with chromosomal integration of aaRS libraries including corresponding selection markers enabling multiplex automated genome engineering (MAGE)-based contextual evolution of the translation system, have been introduced[15]. Utilizing this sophisticated expression system in addition to substitution of all amber (UAG) stop codons by opal (UAA) stop codons together with a release factor (RF1) knockout[15-17] and modification of the core translation machinery[15] may yield optimized variants capable of efficient L-azidoalanine incorporation. In addition to these aforementioned techniques, innovative genome editing technologies such as CRISPRi can be employed to rationally re-design metabolic pathways or to fine-tune metabolic fluxes to circumvent the toxicity of L-azidoalanine[18].

Moreover, profounder understanding of essential genes and corresponding cellular responses regulating the introduction of a modified genetic code is urgently needed to enable a broader application of this technology[19, 20]. Given the recent advances in amber codon suppression technology, this technique is of particular interest for introducing expedient unnatural amino acids to enable the targeted capture of the interactors and substrates of Ubiquitin conjugating and ligating enzymes (**Chapter 2**). However, achieving this necessitates further evolution of the tRNA synthetase variant described in **Chapter 2** possibly in conjunction with engineering of the translational machinery as well as the bacterial metabolism.

Developing a sequentially transferable Ubiquitin ligase probe (Chapter 3)

Ubiquitination of target proteins is orchestrated by a cascade consisting of two E1, ~ 30 E2, and more than 600 E3 enzymes, of which some are highly specialized, while others are promiscuous. Reversibility of this modification is conferred by ~ 80 proteases known as DUBs, permitting specific regulation of highly dynamic cellular events[21]. Given the pivotal importance of E3 ligases in virtually any cellular pathway, it is unsurprising that deregulation of these enzymes gives rise to cancer, neurodegeneration, and inflammatory diseases[22, 23]. Thus, the development of reagents and activity-based probes (ABPs) targeting the Ubiquitin enzyme cascade is of utmost importance. Although most efforts have focused on developing ABPs for the deUbiquitinating enzymes, advances in developing appropriate counterparts targeting E3 ligases have only been made in recent years. Nonetheless, the earliest designs for ABPs targeted only the E1 enzyme primarily

through mimicry of the Ub/Ubl-AMP intermediate, severely limiting the utility of these reagents to monitoring a single enzyme at the apex of the Ubiquitin cascade[24-26].

To address this shortcoming, we developed a mechanistically engaged Ub-ABP which is relayed through the Ubiquitin enzyme cascade akin to that of the native trans-thioesterification, but concurrently forming the covalent thioether adduct[27]. This unique feature of UbDha, in addition to being inert towards lysine residues of substrates renders it amenable for proteome-wide profiling of the Ubiquitin enzyme cascade. Moreover, introduction of UbDha into living cells by electroporation permits the monitoring of these enzymes in living cells in response to genetic or chemical perturbations. However, as our ABP design relies on the active site cysteine and sequential thioester intermediate formation, it is constrained to detecting HECT- and RBR- E3 ligases. While this limitation could be partially overcome by increasing selectivity for HECT/RBR E3 ligases through introduction of specific Ub-variants generated by phage display[28], the inherent lack of direct labelling of the scaffolding RING E3 ligases still needs to be addressed. Ironically, the vast majority of the ligases pivotal to cancer development and progression are characterized by a RING architecture[29], excluding them from ABP reactivity. Devising ABPs endowed with the ability to selectively engage with RING E3 ligases requires the a priori knowledge of the specific interfaces between the E2 and the RING-E3 enzyme. Although Pao *et al.*, previously developed a modular E2-Ub probe utilizing a TDAE (tosyl-substituted doubly activated ene)- modified Ubiquitin to capture RING- and RBR E3 ligases[30-32], this probe lacks residues implicated to be critical for enzymatic recognition by E3 ligases. However, to amend this, a conceivable approach towards capturing RING-E3 ligases interacting with E2-Ub conjugates would be to generate stable E2-Ub thioether complexes using UbDha. This approach, foreshadowed in **Chapter 3**, would potentially permit the identification of interacting RING-E3 and RBR-E3 ligases in the context of its cognate E2 enzyme. This methodology would enable chemo-proteomic approaches to identify the Ubiquitin ligase associated with specific cellular perturbations, such as diseased states, infection, or modulation of expression levels. Moreover, having access to a facile method of generating stable E2-Ub conjugates akin native thioester intermediates, would advance both structural and biochemical knowledge of E2-E3 interacting enzyme pairs providing valuable insights for drug discovery efforts for E2 conjugating enzymes and E3 ligases[33].

SUMO Activity-based Probes (Chapter 4)

Posttranslational modification with SUMO, a small Ubiquitin-like modifier, regulates genome stability, transcription, as well as the immune response rendering it critical for preserving cellular homeostasis following stressor exposure[34]. Given its participation in fundamental cellular processes, SUMOylation contributes to the pathogenesis of a variety of diseases ranging from cancer to neurodegenerative diseases, viral and bacterial infections[35-37].

Although SUMOylation has been studied intensely in the context of DNA damage and genomic instability, its role in other cellular processes such as RNA editing, protein folding, lipogenesis, cytoskeletal organization, and autophagy are beginning to emerge[38-40]. Additionally, the role of SUMOylation enzymes modulating this posttranslational modification in these contexts is poorly understood and necessitates the development and application of suitable reagents and ABPs. To this end, we developed a facile approach to generate SUMO fully synthetically enabling the generation of a variety of ABPs and reagents (**Chapter 4**). With the introduction of ABPs targeting SUMO-specific proteases (SENPs)[41] , not only their linkage specificities, but also their contribution to disease pathogenesis and progression can be unraveled. The value of ABPs targeting SUMO proteases to explore their preferences is showcased by the discovery that SENP3, known to cleave SUMO2/3[42], preferentially reacts with a K11-linked diSUMO ABP[41]. Given that SENPs regulate deSUMOylation and thus promote disease progression[43], developing reagents to monitor their activity are critical to not only to elucidate the underlying biology, but also to enable inhibitor discovery. Despite these advances, innovative SUMO-based activity-based probes and reagents are needed to identify and interrogate the SUMO conjugating and ligating enzymes as well as to dissect the role of poly-SUMO chains and SUMO hybrid chains[41]. Recently, Zhang *et al.*, reported a synthetically generated E2-SUMO conjugate in which the active-site cysteine has been substituted by 2,3-diamniopropionic acid permitting the covalent trapping of cognate E3 ligases[44]. This concept, delineated in Chapter 2 using amber codon suppression, permits the facile formation of a stable ternary E2-SUMO-E3 complex potentially enabling the identification of novel E3 ligases.

Although some progress has been made in developing SUMO-based tools and reagents akin to those available for Ubiquitin activating, conjugating, ligating, and deconjugating enzymes, a comprehensive toolkit for interrogating the SUMOylation enzymes is still in its infancy. Moreover, the advent of our practical synthesis strategy for generating SUMO-based reagents, introduces new possibilities to generate tailored tools for studying emerging players in SUMO and Ubiquitin biology—SUMO-Ubiquitin hybrid chains.

UFM1 Activity-based Probes (Chapter 5)

In contrast to Ubiquitination and SUMOylation, posttranslational modification with UFM1 is less well understood, primarily due to lack of appropriate tools and reagents. Initially, the UFM-1 specific proteases UFSP1 and UFSP2 have been discovered by the use of UFM1-VME, which had been generated by semi-synthesis[45]. Later, a facile synthesis approach to access UFM1 and equipping it with various warheads targeting either the conjugating (UFM1-Dha) or the deconjugating enzymes (UFM1-PA) has been developed[46], as described in **Chapter 5**. Access to a practical synthesis method for generating virtually any UFM-1 based reagent expedites not only the discovery of previously undiscovered enzymes, but also enables the characterization of their enzymatic activity providing insights into UFMylation dynamics. Thus, methodologies to entrap E3 ligases using E2-UFM1 conjugates generated using UFM1-Dha, might unlock access to potentially novel UFM1 specific ligating enzymes. Having devised a facile method to access synthetic UFM1, ABPs targeting both the proteases and ligases can be generated exemplified in **Chapter 5** as a proof of concept. However, unlike Ubiquitin and SUMO enzymes, relatively little is known about the architecture and reactivity of UFM1 specific proteases and ligases thus warranting the quest for suitable reactive groups and ABP strategies. With such tools becoming readily accessible, potentially novel UFMylation enzymes can be discovered and characterized to unlock UFM1 biology.

UFM1-modification of the ribosome—relocating protein translation to the ER (Chapter 6)

While the structural and the biochemical features of some of the UFM1 conjugating and deconjugating enzymes are gradually being unraveled, the physiological role of UFMylation remains enigmatic primarily due to lack of knowledge of its substrates. Yet, in contrast to Ubiquitination and SUMOylation, where techniques such as diGly proteomics and affinity-capture methods[47], have propelled the discovery of their substrates, analogous approaches are nonexistent for UFM1. Given the lack of appropriate tools and the low abundance of UFM1, identification of only a few substrates has been accomplished, primarily by affinity capture using epitope-tagged UFM1[48]. However, the cellular function as well as the underlying molecular mechanisms of UFMylation still remain obscure thus necessitating the development of a suitable proteomics strategy. In an attempt to gain insights into the cellular function of UFM1, we adapted the proteomic method originally employed for site-specific mapping of the SUMO proteome[49], in combination with CRISPR-Cas mediated depletion of UFSP2 to enhance UFMylation.

This approach allowed us to identify several UFM-1 modified proteins involved in DNA replication, vesicle trafficking and protein translation, with the ribosomal protein RPL26 (uL24) and its paralog RPL26L1 being the most prominent targets. Given the close proximity of RPL26 to the ribosomal tunnel exit, we hypothesized that this post-translational modification might evoke the recruitment of specific interactors. In addition to promoting membrane association of ribosomes, we discovered that UFMylation at such a strategic position evokes the direct interaction of the ribosome with (signal recognition particle receptor) SRα, implying the participation of UFM-1 in SRP-dependent protein translocation. Unexpectedly, perturbation of ribosomal function with a variety of translational inhibitors induced RPL26-UFMylation, strongly suggesting that UFM-1 is involved in translation itself

as well as modulating the ribosomal interaction with the SRα during protein translocation[50, 51] (**Chapter 6**). Exploring the translational status of ribosomes, upon modulation of the UFM1 system (i.e. knockdown of UFM1 or UFSP2) revealed that protein translation is dependent on UFM1. However, to advance our understanding of the casual mechanisms further experiments such as global translatome profiling will need to be undertaken. While some aspects of the UFMylation are slowly being deciphered, a myriad of questions still remain unsolved and require novel chemical tools as well as proteomic approaches. Based on our observations, UFMylation seems to be a highly dynamic PTM, as evidenced by the visualization of UFMylation only upon UFSP2 depletion. From the subsequent pulldown of UFMylated substrates, the comparison between the unperturbed and the UFSP2-knockout cells, reveals differences, implying the existence of potentially more UFM-1 specific proteases (**Chapter 6**). Moreover, so far only one UFM1 specific ligase (UFL1) has been reported, necessitating innovative approaches to enable the identification of UFM1 specific ligases. Despite the pioneering work of attempting to uncover UFM1 substrates and to establish a cellular function for UFMylation described within the last chapters of this thesis, the ultimate physiological role remains enigmatic.

Does UFM1 only serve as a ‘’anchor’’ for mediating protein-protein interactions, as illustrated by the UFM-1 dependent contact between the ribosome and the SRα? To what extent is UFMylation involved in translation? Or is UFM1 akin to Ubiquitin and Ubiquitin-like modifiers amenable to forming polymers or further modification by PTMs? What other physiological aspects require UFMylation? While the work described in **Chapter 5** (UFM1 probes) and **Chapter 6** (UFMylation of the ribosome) is at the advent of new insights and discoveries of the UFM1 system, much more research is required to understand UFMylation at the same level as Ubiquitination and SUMOylation.

Final conclusion

Collectively, the research presented here not only describes the attempt to incorporate an unnatural amino acid by amber codon suppression to permit covalent substrate capture, but also the development an activity-based probe that is sequentially relayed through the Ubiquitin cascade. Moreover, the introduction of a practical synthesis approach for both SUMO and UFM1 and have been utilized for ABPs reactive towards protease or ligases. Finally, the identification of potential UFMylation substrates has been undertaken by modification of a proteomics approach successfully employed for SUMO proteomics. Yet, unraveling the physiological function of the most prominent UFMylation substrate—RPL26—led to the discovery that it promotes UFM1-mediated interaction of the ribosome with its cognate SRP receptor and the role of UFM1 in protein translation. With the introduction of facile synthesis strategies as well as innovative ABPs in combination with advanced proteomics

approaches, this work has paved the way for original insights not only for Ubiquitination, but also for dissecting the underlying biology of SUMOylation and UFMylation.

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