Shining light on proteolysis by the ubiquitin proteasome system: fluorescence-based activity profiling of the ubiquitin proteasome system & peptide splicing by the proteasome

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Chapter 5

Summarizing discussion

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In order to maintain protein homeostasis in cells and to ensure proper cell functioning, proteins are continuously synthesized and degraded. The ubiquitin proteasome system (UPS) is the system responsible for the controlled degradation of ubiquitinated proteins in eukaryotic cells. In addition, the UPS is responsible for the generation of antigenic peptides derived from the proteins degraded by the 26S proteasome. These peptides are presented on the cell surface by MHC class I proteins, to be scanned by the immune system for detrimental intracellular content. This thesis shines light on multiple facets of the UPS, both figuratively as literally, as we have used fluorescence as a technique to visualize catalytic activity of the proteasome as well as of deubiquitinating enzymes. Moreover, we enlighten a relatively unknown feature of the proteasome in which peptide fragments derived from protein degradation are merged together, forming a peptide which cannot be predicted from the genetic code and which can be presented by MHC class I molecules to the immune system.

In **chapter 1**, an overview is provided on the ubiquitin-proteasome system, elaborating on the post-translational modification of proteins with ubiquitin, the ubiquitin ligation cascade of E1, E2 and E3 ligases, the catalytic activity and cellular regulation of deubiquitinating enzymes (DUBs), the catalytic activity of the 26S proteasome, the generation of antigenic peptides by the 26S proteasome, the UPS as drug target and developed tools and assay reagents to study the UPS.

Given that the UPS governs numerous crucial cellular functions, both the UPS itself and its enzymatic components, such as DUBs, are emerging as potential drug targets. In addition to inhibition, enhancing proteolytic activity can also be of interest, particularly concerning the treatment or delay of diseases linked to protein accumulation, such as neurodegenerative diseases.

Here, activity-based probes (ABP) can be used for the accurate profiling of enzymatic activity, for the isolation of enzymes as well as inhibitor profiling (e.g. target specificity). Classical DUB ABPs are based on Ub as a DUB targeting motif and comprise a reactive C-terminal warhead such as vinyl methyl ester (VME) or vinylmethylsulfone (VS) for binding to DUBs. Epitope-tagged active-site-directed probes have been widely used in the past to visualize the activity of deubiquitinases (DUBs) in cell extracts (using immunoblotting) to investigate the potency of small-molecule DUB inhibitors, and to isolate and identify DUBs (using affinity pull-down experiments followed by mass spectrometry). These probes have been proven very useful in the search for yet unidentified DUBs and the search for specific and potent small-molecule DUB

inhibitors by making use of activity assays. However, the established method for the generation of DUB probes relied on labor-intensive intein-mediated expression and ligation methods that have inherent limitations concerning the incorporation of unnatural amino acids and the amount of material that can be obtained. In order to address these issues, an alternative procedure was developed to make Ub by total chemical synthesis by Fmoc-based solid phase peptide synthesis (SPPS). This procedure allows for easy incorporation of useful tags for specific applications that cannot be encoded genetically, such as fluorescent reporters, affinity tags, and cleavable linkers. Additionally, the synthetic method can be easily scaled up to provide significant amounts of probe. In **chapter 2**, the development and application of a variety of novel activity-based DUB probes is described which have been generated by total chemical synthesis. **Chapter 2.1** and **2.2** describe the total chemical synthesis of UbVME DUB activity probes, containing a variety of N terminal building blocks, such as fluorescent tags, affinity handles (epitope tag, biotin), spacers and cleavable linkers. I here demonstrate that fluorescently-tagged DUB probes allow for faster, in-gel detection of active DUBs, as compared to (immuno)blotting procedures. In addition, an optimized SDS-PAGE procedure for profiling DUB activity in cell lysate using the fluorescent DUB probe TMRUbVME is described, including an inhibition assay which can be used to determine the specificity and potency of potential DUB inhibitors. Making use of the fluorescent probe TMRUbVME, DUB activity levels were monitored in response to inhibition by small molecules and to overexpression or knockdown of DUBs, showing that genetic and cell-biological manipulation can be successfully followed using the fluorescent DUB activity-probe. Lastly, differential DUB profiles were revealed in a panel of different cancer cells using a fluorescent DUB activity-probe. Some of these cell lines showed remarkable overexpression of a certain DUB, showing that these activity-based DUB probes can potentially find application as a future diagnostic tool or to predict outcome of DUB inhibitor therapy. In addition, **chapter 2.2** describes the synthesis of a biotinylated probe holding a photocleavable linker, which can be used for 'catch-and-release' of DUBs. This probe enabled the affinity pull-down and subsequent mild, photorelease of DUBs. A disadvantage of the activity-based DUB probes described in **chapter 2.1** and **2.2** is that after binding to the DUBs, these probes become irreversibly attached to their active site, thereby rendering these DUBs inactive. Consequently, the activity of these DUBs can no longer be studied after probe binding. In **chapter 2.3** we address this issue by the development and synthesis of novel activity-based DUB probes that comprise of a ubiquitin moiety and a methyl disulfide warhead at the C-terminus. These reagents can bind DUBs covalently by thiol-specific exchange with the active site cysteine residue, thereby forming a disulfide bridge between the active site cysteine residue and the ubiquitin-based DUB activity-probe. We showed that these novel probes can

bind to the active site of purified DUBs of different enzyme classes as well as to DUBs present in cell extract. We showed that the catalytic activity of the DUBs was restored after treatment with a mild reducing agent. This principle allowed us to capture and elute active DUBs from cell extract. we here describe the first DUB ABP that allows the release of active enzymes after catch and release under mild conditions. These novel reagents allow for identification of active DUBs involved in a specific biological context or that deubiquitinate specific substrates.

So far, I described Ub reagents (ABPs) that allowed the detailed study of DUBs. However, with the proteasome itself as a valuable therapeutic target for the treatment of for example cancer, reagents that can be used to profile proteasome activity are valuable research tools and hold promise as diagnostic reagents. The first small molecule-based activity probe in its class that could be used for profiling the specificity of proteasome inhibitors in living cells without the need of laborious procedures was a dansylated vinylsulfone based probe. This probe contained a proteasome-targeting motif and a vinylsulfone group that covalently reacted with all catalytic β-subunits of the proteasome. Using antibodies labeled active subunits could be detected *in vitro* and *in vivo*. Replacement of the dansyl group by fluorophores such as Bodipy allowed for direct readout by scanning of the SDS-PAGE gel for fluorescence emission of fluorescently labeled subunits. Moreover, the probe could also be used for confocal microscopy and flow cytometry assays to detect (changes in) proteasome activity in living cells. **Chapter 3.1** describes the synthesis of the Bodipy-based fluorophore Me4BodipyFL-*N*-hydroxy-succinimidyl (NHS) ester in six steps and the synthesis of the proteasome-targeting moiety Ahx_3Leu_3VS and subsequent coupling with Me_4BodipyFL-NHS ester yielding the fluorescent probe Me_4BodipyFL-Ahx_3Leu_3VS. In this chapter, optimized procedures to profile the effects of proteasome inhibitors in cell lysates, living cells, and murine tissues using this fluorescent proteasome activity probe in both SDSPAGE and FACS-based assays are described.

In **chapter 3.2** we utilize the proteasome activity probe Me4BodipyFL-Ahx3Leu3VS in a small molecule modulator screen. Here, we set out a search towards proteasome activators, opposed to compounds that inhibit proteasome activity, that may be of significant therapeutic value for neurodegenerative disorders. These disorders, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS) are characterized by the presence of toxic intracellular protein aggregates that correlate with impaired proteasome function. Enhancing UPS activity, by either increasing the pool of free ubiquitin or overexpressing specific ubiquitin ligases, can reduce toxicity induced by protein aggregates in neurodegenerative disease pathogenesis. We therefore speculated that enhancing proteasome activity by small-molecule

compounds could be of therapeutic value in the treatment of these diseases that are associated with toxic intracellular protein aggregates. As from the reported subset of proteasome activity-enhancing compounds, only very few have been shown to activate proteasomes in vivo we performed a chemical genetics screen of over 2,750 compounds using the proteasome activity probe Me_4BodipyFL-Ahx$_3$Leu$_3$VS as described in **chapter 3.2.** The screen was done using a high-throughput live-cell fluorescence-activated cell sorting-based assay in which we searched for compounds that showed increased proteasome activity. We identified more than ten compounds that increased proteasome activity, with the p38 MAPK inhibitor PD169316 being one of the most potent ones. Genetic and chemical inhibition of either p38 MAPK, but also its upstream regulators, ASK1 and MKK6, and a downstream target, MK2, enhanced intracellular proteasome activity using probe-based assays without changing the cellular proteasome level. Additionally, chemical activation of the 26S proteasome by inhibition of p38 MAPK increased PROTACs-mediated degradation of ubiquitinated proteins as well as degradation of α-synuclein, a protein that can form insoluble fibrils and is associated with Lewy body dementia and Parkinson's disease. Survival of cells overexpressing toxic α-synuclein assemblies was increased in presence of p38 MAPK inhibitors. These findings indicate that enhancement of 26S proteasome activity could be of interest as a novel therapeutic strategy e.g. for diseases associated with toxic protein aggregates.

In **chapter 4** of this thesis I describe my initial work on another cellular function of the UPS; the generation of (precursors of) antigenic peptides derived from the proteins degraded by the 26S proteasome. These peptides are presented on the cell surface by MHC class I proteins, enabling scanning for changes in intracellular content by the immune system, e.g. by malignant transformation or viral infection. These antigenic peptides are derived from proteins degraded by the 26S proteasome. It was shown by others that not only contiguous antigens, but also non-contiguous antigens were found to be presented to the immune system, which could not be predicted from the genetic code. These non-contiguous peptides consisted of two post-translationally fused peptides. The fusion of peptides by enzymes can be caused by either reverse proteolysis or transpeptidation. In **chapter 4.1** we reviewed under which conditions proteases catalyze the fusion of peptides. In addition, the consequences of these phenomena for immunity are discussed. This fusion of peptides inside the proteasome, referred to as peptide splicing, is thought to be catalyzed by the proteasome via a transpeptidation mechanism. Transpeptidation occurs when in the active site of enzymes aminolysis is faster (or comparable to) the hydrolysis reaction. This is dependent on the competition between nucleophilic components, such as the amino group of a nearby peptide, and water for the nucleophilic attack on the

acyl-enzyme intermediate. When amino-donating components are in close proximity to the acyl-enzyme intermediate in the active site, this will facilitate its nucleophilic attack and consequently a new peptide bond will be formed, resulting in a spliced peptide. All peptides produced inside the 20S catalytic core have a free N-terminus and can therefore theoretically serve as a ligation partner. However, since only nearby peptides can compete with water for the nucleophilic attack, we postulated that there could be splicing rules, which predict sequences that are more likely to promote splicing events. These rules could help predict and identify noncontiguous antigens, as they cannot be predicted form the genetic code. In **chapter 4.2** short peptides were used for the identification of peptides sequences that are likely to promote peptide splicing. We showed that splicing does not occur at random but follows distinct rules and occur particularly efficiently when the C-terminal ligation partner contains a basic amino acid, such as a lysine or arginine residue at the site of ligation. We showed that splicing can occur efficiently *in vitro* and in cells, and that many splicing products could be formed. This suggests that peptide splicing may play a more important role in immunity than is generally thought.

Next, in **chapter 4.3**, we showed that the proteasome can form a novel type of spliced peptide, containing an isopeptide linkage, in which the two fragments are ligated via the ε-amine group of a lysine residue instead of the N-terminus. These isopeptide linkage-containing peptides form a novel class of post-translationally modified peptides that can be presented on the cell surface by MHC-class I proteins. It was shown that ε-amine ligation occurs at a rate 10 times lower than α-amine ligation, for which we postulate that this could still trigger an immune response. Interestingly, peptides containing the ε-amine isopeptide linkage were found to be more stable toward proteasomal trimming and bind to MHC class I proteins HLA-A2.1 and HLA-A3 with high affinity. In addition, we demonstrated *in vivo* that these ε-amine linked peptides are immunogenic. In summary, these discoveries imply that isopeptide-linked peptides might enhance the repertoire of peptides loaded onto MHC class I proteins, thereby broadening the array of antigens available for presentation to the immune system. Yet, demonstrating the existence of spliced isopeptides on the cell surface is crucial to affirm their biological significance, but this task is intricate due to its difficult detection. Given the potential use of these antigens as targets for cancer immunotherapy[1], additional research is warranted to determine the prevalence of spliced isopeptides and whether these epitopes are exclusive to cancer cells.

To summarize the thesis, I have delved into various aspects of the UPS, presenting innovative tools and insights that advance our comprehension of this adaptable and finely regulated system. Considering UPS modulation as a promising path for treating various significant pathologies like cancer and neurodegeneration, I've outlined a multi-faceted approach toward exploring therapeutic possibilities that target the UPS. As the range of available reagents for studying the UPS continues to grow, the development of future drugs targeting this system for therapeutic use is expected to become more feasible and accessible.

1. Nagel, R., Pataskar, A., Champagne, J. & Agami, R. Boosting Antitumor Immunity with an Expanded Neoepitope Landscape. *Cancer Res* **82**, 3637-3649 (2022).