Chapter 7

General Discussion

Discussion

The 26S proteasome plays a central role in the cell through degradation of the majority of unneeded, damaged and misfolded proteins. Consequently, malfunctioning of this complex is associated with various diseases, such as cancer and neurodegenerative disorders. In this work we aimed to get a better understanding of 26S proteasome functioning and regulation under both normal conditions and stress conditions. We focused on identification of 19S/20S interaction partners, as well as monitoring global proteome and ubiquitinome dynamics under different cellular states. We also studied the function of three proteasome-bound deubiquitinating enzymes RPN11, UCHL5 and USP14. A minor part of this work focusses on the transcriptional and translational regulation of the ecdysone hormone which is important for insect development. A common feature in all chapters of this thesis is a solid detailed quantitative mass spectrometry-based analysis.

Chapter 6 describes how we used a comprehensive quantitative SILAC MS-based approach to study how the *Drosophila* proteome is affected upon treatment with a hormone, ecdysone, which is involved in many different regulatory processes. Cellular responses were monitored at three levels: global proteome dynamics, global transcriptome dynamics and finally interaction partners of the ecdysone receptors were identified. We found that the abundances of the far majority of proteins remained unchanged. There was a small subset of proteins that was up- or downregulated already at early time points, including two known early ecdysone responsive genes, *i.e.*, ecdysone-induced protein 71 (Eip71CD or Eip28/29) and BR-C. Increased abundance of BR-C was confirmed with Western Blot (WB). Other early responsive genes include eater, CG18765, mus309, regeneration (rgn) and glycine N-methyltransferase (CG6188). At later time points, the set of affected proteins expanded, but was still a relatively small fraction of the total (measurable) proteome. It remains to be confirmed whether these effects are direct or indirect.

Furthermore, we have compared changes upstream of the proteome *i.e.,* at the transcriptome level. We observed a substantial overlap in terms of affected targets between the dynamic proteome and transcriptome after ecdysteroid induction. However, effects in the proteome are usually delayed with respect to the changes in the transcriptome. Also, downregulation of mRNAs in many cases did not correlate to downregulation at the proteome level and in some cases there seemed to be no correlation between transcriptome and proteome dynamics whatsoever.

In order get a better understanding about ecdysone signaling induction we purified the ecdysone receptor from *Drosophila* embryo nuclear extracts. Proteins co-purified with EcR include factors involved in RNA Pol II dependent transcription and chromatin modifying enzymes. Also, several proteins previously linked to ecdysone signaling and/or biosynthesis were identified.

However, the overlap between two different antibodies used for the purification of EcR was poor. This might be partly explained by steric hindrance of EcR interactors which mask specific antibody epitopes. Using antibodies against the same epitope or against a tagged form of EcR might increase overlap of results.

Next, we turned towards a biochemical system that is supposed to have a large effect on the proteome and includes protein degradation: the 26S proteasome. Manipulation of this system directly affects the proteome, and therefore proteomic techniques, including mass spectrometry, are the methods of choice for readout.

In **Chapter 3** we analyzed the effect of chemical proteasome inhibition and 26S proteasome subunit knockdown (RPN11, Prosalpha5 and Prosbeta6) on both the *Drosophila* S2 cell proteome and ubiquitinome. We showed that the global proteome and, to an even greater extent, the ubiquitinome were severely remodeled upon both treatments. We observed that increased protein fold changes were in concert with increased ubiquitination for the majority of the proteins, which suggests that these proteins accumulated as a result of proteasome inhibition or knockdown. However, new protein synthesis also led to increased protein fold changes in some cases, as observed by a 5h cycloheximide treatment. Pulsed SILAC would allow to discriminate between protein accumulation and protein synthesis also during longer incubation times (Schwanhäusser *et al.*, 2009). Protein fold changes were generally lower than diGly peptide fold changes, indicating that in general the increase in protein accumulation was much lower than the extent of ubiquitination. The difference between the responses of both (sub)proteomes may first of all be explained by the relatively low stoichiometry of ubiquitinated proteins, hence major changes in the ubiquitinome may not alter total protein levels. Second, ubiquitination is also involved in a variety of regulatory pathways other than protein degradation and may thus not (directly) affect protein levels (Kaiser *et al.*, 2011; Kim, Eric J Bennett, *et al.*, 2011; Komander and Rape, 2012). In several cases we observed differential ubiquitination dynamics at different lysine residues on the same protein, which may indicate that these are regulatory ubiquitination signals rather than signals for protein degradation.

It remains unclear which part of the ubiquitinome was covered in our screen, and in general it is not known what the size an entire ubiquitinome could be. We identified diGly peptides of 3077 proteins, on a total pool of 5899 identified proteins, suggesting that at least 52% of all unique proteins carry a ubiquitin modification (not including the stoichiometry of ubiquitinated proteins). Recently it was shown that about 75% of the proteins in HeLa lysates could be phosphorylated (Sharma *et al.*, 2014). This percentage of identified phosphoproteins has increased over the years in concert with improved sample preparation protocols and developments in high resolution quantitative mass spectrometry. In line with this it has been proposed that basically every protein could potentially be phosphorylated. We suggest that it would also not be unlikely that every protein should be able to become ubiquitinated. It is mainly

for technical reasons that we don't have this proof yet to date. To our knowledge, we presented here the first global ubiquitinome study upon proteasome malfunctioning in a fly cell line, and therefore our datasets might serve as a valuable repository of protein ubiquitination sites in *Drosophila*.

Taken together we showed that proteasome subunit knockdown could be used in combination with quantitative proteomics to study proteome and ubiquitinome dynamics upon proteasome malfunctioning. This approach would make it possible to functionally characterize specific proteasome subunits, such as the three deubiquitinating enzymes of the 26S proteasome. Understanding the functional mechanism of the proteasome in more detail will be helpful for the development of next generation proteasome inhibitors which could be used in the clinic. Already, proteome and ubiquitinome analyses have been applied to study the specific mode of action of the novel proteasome inhibitor Capzimin (Li *et al.*, 2017).

Finally, diGly peptide screens in combination with global proteome screens give a wealth of information about protein and ubiquitination dynamics in different experimental settings. Often, additional experiments are required to fully understand the nature of these changes.

In **Chapter 4** we studied the effect of proteasome-bound DUB knockdown on global proteome and ubiquitinome dynamics in order to identify DUB specificity in targeted 26S proteasome-dependent protein degradation. First, we showed by the use of Label Free Quantification-based interaction proteomics that USP14, in contrast to RPN11 and UCHL5, is a weak interactor of the proteasome in *Drosophila* S2 cells, which is in correlation with studies in mammalian cells (Elena Koulich, Xiaohua Li, 2008; Kuo and Goldberg, 2017). Next, we found that depletion of RPN11 destabilized the proteasome holocomplex and resulted in extensive remodeling of both the global proteome and ubiquitinome. Our finding that RPN11 is important for proteasome activity and stability is in agreement with published studies (Maytal-Kivity *et al.*, 2002; Verma *et al.*, 2002; Yao and Robert E. Cohen, 2002; Gallery *et al.*, 2007; Elena Koulich, Xiaohua Li, 2008; Finley, 2009). In contrast we found that depletion of UCHL5, USP14, or simultaneous depletion of both UCHL5 and USP14 did not show any effect whatsoever. These findings suggest that RPN11 plays an important role in proteasome-mediated protein degradation whereas the roles of UCHL5 and USP14 in general proteostasis remain unclear. It remains to be determined whether the effects found upon RPN11 depletion were the result of decreased RPN11 activity or of decreased protein levels, for instance by using RPN11 catalytic mutants. Additionally, proteasome-bound DUB knockdown did not affect levels of any polyubiquitin linkage type.

Despite extensive research, the role of USP14 and UCHL5 in proteasome dependent degradation is not yet clear. Proteasomes can efficiently degrade substrates without USP14 (Hanna *et al.*, 2006; Lee *et al.*, 2010; Kim and Goldberg, 2017). In one model, USP14 and UCHL5 could antagonize substrate degradation via their polyubiquitin trimming activity leading to dissociation of substrate from the proteasome prior to degradation (Lam *et al.*, 1997; Lee *et al.*,

2010; M. J. Lee *et al.*, 2011). This model is mainly based on *in vitro* degradation rates, for instance of monoubiquitinated globin peptides and other lower–order conjugates upon isopeptidase inhibition by ubiquitin aldehyde (Lam *et al.*, 1997). Additionally, Finley and coworkers observed enhanced degradation of Cyclin B and of Sic1 upon chemical inhibition of USP14 with IU1 *in vitro,* and additionally, they observed reduced levels of tau, TDP-43 and ataxin-3 in murine embryonic fibroblasts upon IU1 treatment (Lee *et al.*, 2010). These results could however not be reproduced by Ortuno *et al.* (Ortuno, Carlisle and Miller, 2016). In contrast to the trimming hypothesis, it was recently found that USP14 rather cleaves chains en bloc, and specifically left one intact chain on proteasome substrates (Lee *et al.*, 2016). Altogether, our data is not in agreement with the model that describes USP14 and UCHL5 as proteins that regulate substrate degradation, for instance via Ub trimming, as we did not observe major protein abundance dynamics upon knockdown of UCHL5 and/or USP14.

The findings of our study are not in disagreement with the model that describes USP14/Ubp6 as a protein that can facilitate substrate degradation via non-catalytic induction of 19S structural changes. In this model ubiquitin-bound Ubp6/USP14 inhibits degradation-coupled RPN11-mediated *en bloc* deubiquitination of polyubiquitin chains (Hanna *et al.*, 2006; Peth, Besche and Goldberg, 2009; Aufderheide *et al.*, 2015; Bashore *et al.*, 2015). Furthermore, ubiquitin-bound Ubp6/USP14 causes the proteasome to adopt the substrate-engaged conformational state, which is characterized by the coaxial alignment of the RPT base subunits and the channel of the 20S CP, and moreover this state positions RPN11 close to the entrance of this channel (Matyskiela, Lander and Martin, 2013; Unverdorben *et al.*, 2014). Proteasomes which adopt the substrate-engaged state cannot process new substrates (Bashore *et al.*, 2015). Both mechanisms, locking the proteasome in the substrate-engaged state and inhibiting the deubiquitinating activity of RPN11, are mechanisms by which Ubp6/USP14 can delay substrate degradation (Aufderheide *et al.*, 2015; Bashore *et al.*, 2015). These mechanisms do not require the catalytic activity of Ubp6/USP14 but do require its ability to bind ubiquitin. The catalytic activity of Ubp6/USP14, on the other hand, plays a role in ubiquitin recycling. Thus, these results suggest that Ubp6/USP14 acts as a timer to coordinate individual substrate processing steps at the proteasome. This suggests that Ubp6/USP14 facilitates, but not regulates, protein degradation and is important for ubiquitin recycling and maintenance of the free ubiquitin pool (Aufderheide *et al.*, 2015; Bashore *et al.*, 2015). The characteristic of Ubp6/USP14 to just temporarily delay substrate degradation, with just the purpose to correctly process the substrate *e.g.,* deubiquitination of polyubiquitin chains, might explain why we did not observe major global proteome dynamics upon knockdown of this enzyme. However, several studies have shown that either free ubiquitin (David S Leggett *et al.*, 2002; Chernova *et al.*, 2003; Hanna, Leggett and Finley, 2003) or ubiquitinated proteins such as cyclin B (Hanna *et al.*, 2006) undergo accelerated degradation by the proteasome in the absence of Ubp6. We did not focus onto Ub synthesis and degradation in our study, but we observed a stable pool of free ubiquitin monomers as well as a

stable pool of total ubiquitin upon simultaneous KD of UCHL5 and USP14 (2xKD), suggesting that ubiquitin regulation is not affected. It is important to note that the findings of this model with Ubp6 acting as a timer to delay the degradation of subsequent substrates by locking the proteasome in the substrate-engaged state and to inhibit RPN11 activity has been demonstrated in *S. cerevisiae*. This organism does not express an ortholog of UCHL5, and therefore this model might not be directly translatable to higher eukaryotes which express orthologs of both UCHL5 and USP14. Recently in mammalian cells it was shown that proteasome-bound USP14 inhibits ATP hydrolysis, substrate entry into the 20S particle and deubiquitination by RPN11 when no ubiquitinated substrates were bound, thus supporting the mechanism as described above for yeast (Kim and Goldberg, 2017).

Current experiments in our lab, which are however not part of this thesis, focus on more efficient depletion of UCHL5 and USP14 *i.e.,* gene knockouts, since it might be possible that the remainder fraction of these proteins after knockdown could still do the job. Furthermore, if only a small fraction of these DUBs is potentially active, then overexpression studies rather than knockdowns might reveal differences in protein or diGly peptide abundances. Another possibility is that the coverage of ubiquitination sites is still insufficient, although all ubiquitination sites of ubiquitin itself were reliably quantified. We are currently improving the coverage and sensitivity of the diGly IP assay in order to identify more diGly peptides and potential UCHL5 and USP14 KD responsive proteins (Van Der Wal *et al.*, 2018).

Chapter 5 describes the characterization of the (dynamic) interactome of 19S/26S proteasome complexes under both stress and non-stress conditions. Using both α-RPN8 and α-RPN10 antibodies we immunopurified 19S particles and interacting protein complexes including intact 26S proteasomes from *Drosophila* S2 cell lysates under non-stress conditions (DMSO/H_2O), ER stress (24h 1μM Tunicamycin), oxidative stress (30 min 1μM H_2O_2 or 24h 1μM H_2O_2), or upon proteasome inhibition (16h 50μM MG132/ 5μM Lact). Label Free Quantification (LFQ) based mass spectrometry was used to identify *bona fide* dynamic interaction partners. Identification of all constitutive 26S proteasome subunits, as well as a variety of known proteasome interactors, such as USP14, SEM1, ECM29 homolog and Thioredoxin-like, showed that we efficiently enriched for both 19S and 26S proteasomes in all purifications, thus independent of the imposed stress conditions. Our data gave novel insights into proteasome and interactome composition dynamics upon non-stress and stress conditions. For instance, Ub shuttle protein RAD23 was identified as a proteasome interactor only under non-stress conditions, while CG7546, a UBL domain containing protein of yet unknown function, interacted with the proteasome exclusively upon proteasome inhibition. CG7546 is structurally related to BAG6, a protein which targets pro-apoptotic proteins to RPN10 for subsequent proteasome-dependent degradation (Kikukawa *et al.*, 2005). Thus, CG7546 might be a shuttle factor involved in targeting substrates to the proteasome. In chapter 3 of this thesis we found that Ref(2)p was newly synthesized and upregulated in response to proteasome inhibition (Sap *et al.*, 2017). In chapter 5 we additionally

demonstrate that the substrate shuttle Ref(2)p interacts with the proteasome only upon treatment with proteasome inhibitors. This suggests that Ref(2)p functions as a substrate shuttle under cytotoxic stress condition when proteasome substrate loads are high. Moreover, proteasome activator PI31 interacted with the proteasome exclusively upon all tested stress conditions, while in non-stress conditions it only co-precipitated in α-RPN10 IP's. It was shown that PI31 can increase the activity of the proteasome when elevated levels of proteasome substrates are present (Bader *et al.*, 2011). Finally, heat shock proteins (HSP's) also interacted specifically upon stress conditions. HSP's function as chaperones that mediate proper folding of substrate proteins and could, for instance, facilitate poteasome-dependent degradation of misfolded proteins (Arndt, Rogon and Höhfeld, 2007; Kettern *et al.*, 2010). Additionally, HSP70 is also involved in both association and dissociation of 26S proteasome complexes, for example shortly after mild oxidative stress (Grune *et al.*, 2011). Finally, chaperones are also able to affect proteasome activity, for instance overexpression of HSP27 increases the activity of the proteasome upon stress induction by inflammatory cytokines and cytotoxic drugs (Parcellier *et al.*, 2003). Additionally, in chapter 3 of this thesis we observed new heat shock protein synthesis upon MG132/lact treatment in S2 cells (Sap *et al.*, 2017). Taken together, our data suggests that chaperones also play a role at the proteasome upon treatment with chemical proteasome inhibitors. Additional experiments are however required to proof this.

Besides the characterization of stress and non-stress specific 19S interactomes, we also analyzed the dynamics of these interactomes upon stress. Some of the treatments used could affect proteasome stability. We used relatively mild oxidative stress conditions in order to prevent destabilization of the 26S proteasome (Reinheckel *et al.*, 1998; Wang, Kaiser and Huang, 2011). We however purified more intact 26S proteasomes upon proteasome inhibition, which might be the result of improved proteasome stability through the use of proteasome inhibitors (Kleijnen *et al.*, 2007). We like to note that consequently enhanced enrichment of interacting partners upon proteasome inhibition could be the result of either enhanced recruitment or enhanced 26S proteasome levels.

Interestingly, more UCHL5 was recruited to the proteasome under stress conditions. In general, the role of UCHL5 at the proteasome has not been clearly defined yet but it is proposed to edit proteasome substrate-bound polyubiquitin chains (Lam *et al.*, 1997), deubiquitinate proteasome subunits (Jacobson *et al.*, 2014), or remove unanchored polyubiquitin chains *en bloc* (Zhang *et al.*, 2011). For more information see Chapter 4 of this thesis. These are different ways in which UCHL5 might influence proteasome-mediated degradation and our data suggests that stress conditions could enhance these activities by recruiting more UCHL5 enzymes to proteasomes.

In this study we analyzed the response of the 19S/26S proteasome interactome after several different stressors. The majority of the interactors associated with the proteasome during all conditions, several were interacting upon all stress conditions, while we found only few

interactors which were specifically interacting upon specific stress conditions. There may be several explanations for the latter case. First, cross-talk between the different stress conditions makes it difficult to distinguish specific responses for each individual condition (Bush, Goldberg and Nigam, 1997; Lee *et al.*, 2003; Fribley, Zeng and Wang, 2004; Obeng *et al.*, 2006; Oslowski and Urano, 2013). Second, proteins which respond to specific stress conditions may be transient interaction partners which were not captured using IP and LFQ-based proteomics but could be picked up with techniques such as BioID (Varnaite and MacNeill, 2016) and APEX (Kim and Roux, 2016). Thirdly, specific responders might interact with other proteasome caps than 19S caps that were the focus of this study, such as PA28αβ, PA28γ, PA200, PI31, or with solely the standard 20S proteasome or the immunoproteasome.

Finally, our data show that classical immune purifications in combination with LFQ-based quantitative proteomics is a powerful approach to specifically detect (sub)stoichiometric interaction partners, as well as dynamic interactors, of a large and important cellular machinery such as the proteasome. We identified several proteasome interactors, which showed a different interaction behavior upon different stress conditions, such as UCHL5, RAD23, CG7546 and Ref(2)p. Differential proteasome interactors upon specific stressors may be potential therapeutic targets for the treatment of diseases in which cellular stress and homeostasis misbalance play a role. Further research is required to elucidate their function in proteasome-dependent degradation in conditions with and without stress.

Concluding remarks

A large part of this work includes global proteome and ubiquitinome analyses following proteasome inhibition or proteasome subunit/DUB knockdown. These types of analyses could be the basis for further research, for instance in the field of drug development. In principle, a global ubiquitinome screen of cells treated with and without protein knockdown of a specific DUB may reveal the target proteins for this enzyme, *i.e.,* more diGly peptides derived from the target proteins would be found upon DUB knockdown as compared to samples which were not treated this way. Additionally, knockdown of a ubiquitin ligase would result in a decreased amount of diGly peptides derived from its substrate proteins. Unfortunately, we were unable to identify targets for UCHL5 and USP14 with this method, however, a combination with protein knockout (instead of knockdown) appears to be more promising (unpublished data of our lab). Several examples for DUB or Ub ligase target discovery by means of protein knockdown/knockout/overexpression and quantitative proteomics could be found in the literature (K. A. Lee *et al.*, 2011; Thompson *et al.*, 2014; Potu *et al.*, 2017). Vectors used for protein knockdown or protein knockout are relatively easy to design, since these are based on the DNA sequence which is available for all standard model organisms. Design of very specific small molecules and compounds faces extra challenges due to the diverse nature of proteins in terms of splice variants, PTM's, conformational changes and complex compositions. Hence, with the use of protein knockdown or knockout we could in principle target all single proteasome

subunits and analyze the effect of loss of individual subunits on protein degradation. Also, global proteome and ubiquitinome screens (or other PTM's) of samples treated for protein knockdown or knockout could be compared to similar screens obtained from samples treated with new compounds or small molecules targeting the same protein of interest. In this way the specificity and off-target effects of the compounds could be investigated. Obviously, in the field of drug development small molecules and compounds are preferred over methods to target the DNA or RNA level due to the possibility of oral administration.

Over the past two decades, we saw rapid developments in mass spectrometry instrumentation and analysis strategies. Several years back it was an achievement to identify ~5000 proteins in a single experiment, while specialized labs with the use of state-of-the-art equipment (and extensive sample preparation) could now identify > 12.000 proteins in a single run (Hosp *et al.*, 2017). It is estimated that the depth of proteome coverage by mass spectrometry could reach the comprehensiveness of transcriptome coverage as analyzed by next generation sequencing (Richards, Merrill and Coon, 2015). Increased proteome coverage will open up new possibilities for mass spectrometry-based research, such as the study of proteoforms *i.e.*, the different molecular forms of proteins such as alternative splice variants, single nucleotide polymorphisms, or post-translational modifications (PTMs) (Nedelkov, 2017). Also, the proteogenomics niche, *i.e.,* the integration of genomics, transcriptomics and proteomics will benefit from enhanced proteome coverage. The proteogenomics field studies the relationship between DNA sequence and the global proteome, for instance the effect of DNA mutations, genetic variation and genetic diseases on the global proteome (Barbieri *et al.*, 2016; Menschaert and Fenyö, 2017). Proteomics research would also be a valuable application in the clinic, for instance for the purpose of precision medicine, in which treatment strategies will be based on patient-specific characteristics derived from different 'omics' platforms instead of a 'one disease – one treatment' strategy. Comparative proteomics of the proteome of the patient versus a database average, or in the future, of a patient's recorded healthy proteome and its current proteome during disease has the potential to greatly increase diagnostic accuracy. Hurdles that have to be overcome in realizing the goal of precision medicine include improving high throughput mass spectrometry, while maintaining robustness, reproducibility and sensitivity. Speeding up the data analysis is necessary, for instance by using automated analysis pipelines, and effective handling and storing of big data is required. Lastly, funding should become better available for multidisciplinary, multi-institutional and often multinational research groups (Nice, 2016). We expect that proteomic research will take a more prominent position in the clinic within the next decade.

For this project we performed a substantial number of large scale global mass spectrometry-based analyses. More than once the extent of changes measured between different samples appeared to be lower than we expected, for instance in our first study in which cells were treated for different durations with the ecdysone hormone (Chapter 6). We then changed the focus of our research to the process of proteasome-dependent protein degradation, a central pathway in

the cell which operates directly at the protein level. Upon inhibition of this important pathway we could measure extensive dynamics of both the global proteome and ubiquitinome, approving the suitability of the used method. We also showed that we could induce extensive changes in the global proteome and ubiquitinome by the use of protein knockdown, first with simultaneous knockdown of RPN11, Prosalpha5 and Prosbeta6, and later with RPN11 alone. However, these extents of changes appeared to be rather exceptional as other treatments, such as knockdown of USP14, UCHL5 or knockdown of both simultaneously did not seem to have much effect on global proteome or ubiquitinome dynamics. We also did not observe many changes in the 26S proteasome interactome upon different stress conditions. Thus, overall global proteome changes as measured by such mass spectrometry-based analyses were in many cases rather stable. From a biological point of view this may however be advantageous. This should be kept in mind when considering the application of these type of large time-consuming and expensive projects. Also, often interdisciplinary collaborations are required to answer mechanistic oriented biological questions.

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