A role of SUMOylation in proteostasis, centromere integrity and the DNA damage response

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CHAPTER 6

General Discussion

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Proteins are highly important components of the cell. Due to the fast, flexible and transient nature of post-translational protein modifications, such as SUMOylation, the cell can quickly react to extracellular or intracellular changes. SUMOylation plays an important role in systematic cellular changes such as the cell cycle, but is also pivotal for the adaptation to several stresses such as proteotoxic stress or DNA damage. The studies in this thesis investigate how SUMOylation is regulated and how SUMOylation can influence cellular processes. Mechanistic models and hypotheses derived from the data presented in this thesis are summarized and discussed in this chapter.

GROUP MODIFICATION VERSUS SELECTIVITY

The concept of group-modification describes that the biological function of SUMOylation often depends on multiple interactions between SUMOylated and SUMO-interaction motif (SIM)-containing proteins[1]. Therefore, the overall SUMOylation status of a protein complex matters more than the SUMOylation of a single subunit. It also implies that SUMOylation can have a certain degree of site-promiscuity, as for example demonstrated by the SUMOylation of the oncogenic transcription factor, c-Myc[2]. Since the proposal of group modification, seven years ago, researchers have delivered supporting data that SUMOylation often targets functionally or physically related proteins[3-5]. Mass spectrometry offers an ideal tool to study global SUMOylation events and detect clusters of proteins that are targeted by SUMO simultaneously. In this thesis, mass spectrometry was used to uncover SUMOylated proteins that are regulated by a HSF1-dependent mechanism upon heat shock (Chapter 3), by the poly-SUMO specific protease SENP6 (chapter 4) and in response to ultraviolet (UV)-induced or ionizing radiation (IR)-induced DNA damage (chapter 5). In all cases we identified clusters of highly interconnected proteins and protein complexes in which multiple subunits are modified by SUMO. STRING protein interaction analysis of the SUMOylated proteins that we identified to be regulated in a HSF1-dependent manner showed interconnected clusters of proteins involved thyroid hormone signalling, DNA replication or are components of the proteasome (Figure 1). The SUMO protease SENP6 also seems to regulate multiple clusters of proteins, including the constitutive centromere-associated network (CCAN), DNA damage response proteins, proteins involved in ribosomal RNA biogenesis and proteins that regulate DNA replication (Chapter 4, Figure 3). The classical concept of group- modification proposes multiple synergistic SUMO-SIM interactions, by which SUMOylation further stabilizes the interaction between proteins that already have affinity towards each other. Interestingly, in the case of the CCAN proteins, the opposite seems to be true. The SUMOylation of the CCAN proteins does not act as molecular glue but most probably marks the fraction of the CCAN proteins that are not assembled into the protein complex at the centromere and might even be the cause of the disassembly. Still, it is the highly interconnected group of proteins that is targeted for deSUMOylation by the protease SENP6. A possible and intriguing scenario could be that a balanced SUMOylation of the CCAN proteins is needed for the complex to assemble, with SENP6 carefully controlling SUMOylation levels.

In contrast to the group-regulation of the CCAN, it is less clear whether the SUMOylation of CSB upon UV-irradiation is part of a group-SUMOylation event or an isolated modification that functions independently. Although we did not identify UV-induced SUMOylation of obvious CSB-interaction partners, like CSA or RNA polymerase II, we cannot exclude the possibility of CSB being part of a SUMOylation-driven protein complex. For example, we observed the SUMOylation of multiple components of the transcription initiation factor TFIID upon

UV-induced DNA damage (Chapter 5, Figure 1). Both, TFIID and CSB, are associated with RNA polymerase II[6-8], therefore the SUMOylation of the TFIID components and CSB could potentially be connected in a group-like fashion. Not all SUMOylation events target protein groups and the SUMOylation of CSB could be an isolated modification event. Supporting a selective modification by SUMO, is the observation that an uncommonly large fraction of CSB is SUMOylated upon UV treatment, visible by the appearance of higher molecular weight species on the immunoblot without prior enrichment of SUMO conjugates (Chapter 5, Figure 2). Unfortunately, the precise mechanistic consequences of CSB SUMOylation remain elusive as will be elaborated on later within this chapter.

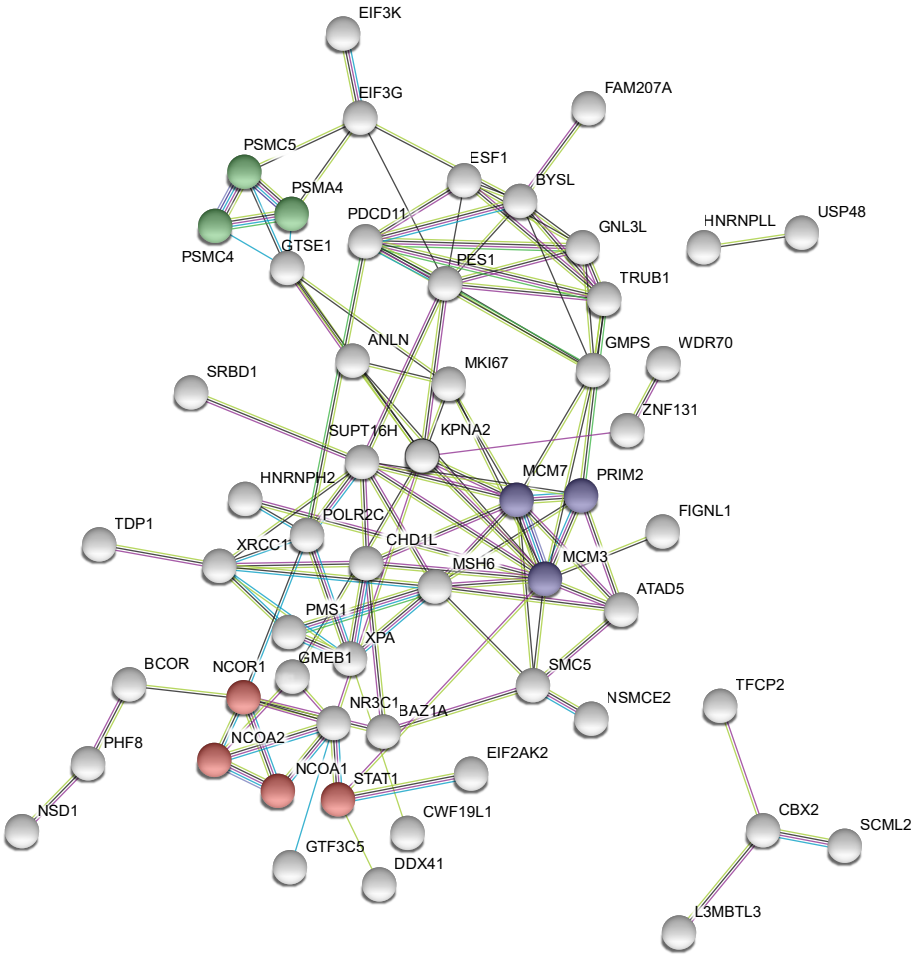

Figure 1. STRING protein-protein interaction network of HSF1-regulated SUMO substrates after heat shock. Each node represents a protein, red nodes indicate proteins involved in thyroid hormone signalling, blue nodes represent proteins involved in DNA replication and green nodes represent proteasome subunits. The edged represent interaction that are based on known interactions from curated databases (blue) or experimentally determined (magenta) or predicted interactions due to gene neighbourhood (green), gene fusions (red) or gene co-occurrence (dark blue). Other types of interactions identified by textmining (light green), co-expression (black) and homology (violet) are depicted as well.

HSF1-DEPENDENT DEGRADATION OF CO-MODIFIED PROTEINS

Proteostasis describes a cellular state in which the synthesis and degradation of proteins are in equilibrium. Proteostasis can be disturbed by an accumulation of denatured proteins due to extracellular stresses, such as heat stress, or genomic causes including the enhanced expression of oncogenes in cancers or expression of aggregation prone mutant proteins often associated with neurodegenerative diseases. The cell has evolved mechanisms to counteract such disturbances and to re-establish proteostasis. A well-studied mechanism to protect proteostasis is the heat-shock response, which is regulated by its master transcription factor heat shock factor 1 (HSF1). HSF1 is responsible for the transcription of proteins that assist directly in the recovery of proteostasis. A major group amongst the HSF1-induced proteins are the heat-shock proteins (HSPs), molecular chaperones that help refold or degrade denatured proteins, which accumulate due to proteotoxic insults such as increased temperature. In chapter 3, we show that SUMOylation and HSF1-controlled proteostasis are linked. Several publications have previously shown that global SUMOylation is induced in cells exposed to heat shock but decreases to normal levels when cells recover at lower temperatures. We show that this decrease of SUMOylation during the recovery of cells is dependent on the activity of HSF1 and the HSPs. This observation let us to speculate on the role of SUMOylation during the heat-shock response. We hypothesize that SUMOylation in concert with ubiquitination, both being able to quickly change properties of proteins, act as a first aid mechanism before HSF1 transcription is fully activated and HSPs are produced in high quantities to re-establish proteostasis. We propose that an unknown HSF1-mediated mechanism specifically targets proteins that are co-modified, by SUMOylation and ubiquitination, for proteasomal degradation. How and why are co-modified proteins degraded in an HSF1-dependent manner? The established co-modified protein is differentially regulated upon heat shock compared to proteins modified by either SUMO or ubiquitin alone, suggesting a distinct property of the co-modified protein. Ubiquitin most likely serves as signal for proteasomal degradation, but the property added by SUMOylation is less clear. Intriguingly, SUMO has been reported to solubilize proteins and is often used as solubility tag for protein purifications[9]. In Chapter 3, we demonstrate that either recombinant fusion of SUMO to a protein or SUMO conjugation increases the solubility of model target proteins in the in-vitro assay used. It is intriguing to think that the cell could exploit this property of SUMOylation in a similar fashion, by solubilizing proteins and making them more accessible to STUbLs, ubiquitination and degradation and therefore counteracting aggregation of unfolded proteins. In line with this theory and as reported in chapter 2, SUMOylation has been implicated to enhance solubility of the disease associated proteins in several neurodegenerative diseases such as the amyloid precursor protein (APP) in Alzheimer's disease or α-synuclein, which forms aggregates in Parkinson's disease[10,11].

Interestingly, several reports suggest that proteotoxic stress-induced transient aggregates can protect the ubiquitin-proteasome system (UPS) from being overwhelmed. Transient aggregates could prevent overload of the UPS system[12-17]. Another ubiquitin-like modifier, Nedd8 promoted the formation of these transient aggregates, which also contained sequestered ubiquitin and SUMO[18]. While Nedd8 stimulates a transient sequestration of proteins, many of which are already ubiquitinated, SUMO could possibly promote solubilisation at a later time point to increase accessibility when the UPS system is less occupied. This hypothesis would also open up a possible role for the HSF1-transcribed HSPs, as they would facilitate refolding and rescuing of proteins and therefore releasing pressure

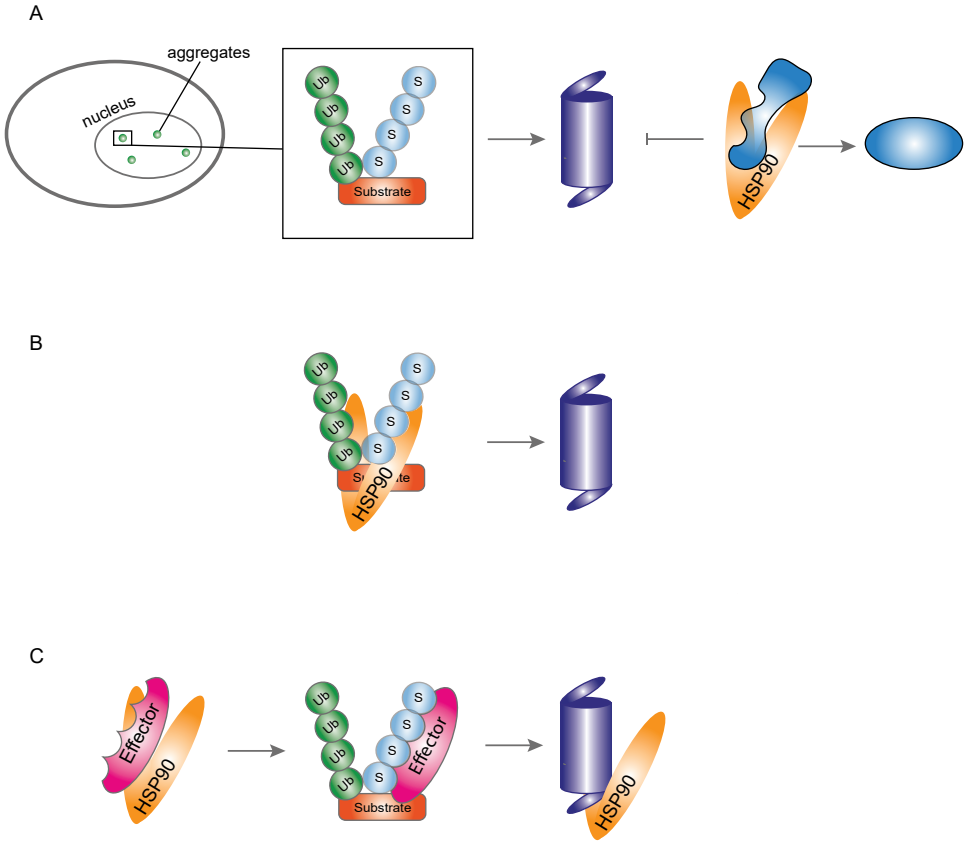

Figure 2. Hypotheses on how HSPs can contribute the proteasomal degradation of co-modified proteins. (A) Upon proteotoxic stress modified proteins are sequestered into nuclear aggregates to prevent overloading of the proteasome. HSP90 contributes to refolding and degradation of unfolded proteins, therefore releasing pressure on the proteasome. SUMO contributes to resolubilize proteins so that they can be targeted for degradation. (**B)** HSP90 selectively binds SUMOylated proteins due to the presence of SIMs and assists in delivery of the substrate to the proteasome. The substrate is marked for degradation by ubiquitin. **C** HSP90 stabilizes proteins which assist in the degradation of co-modified proteins, either proteasomal subunits or unknown SIM-containing effector proteins.

on the UPS and promoting the degradation of the previously sequestered PTM-modified proteins (Figure 2A). Another possibility for the observed HSF1-dependent degradation of co-modified proteins would be that SUMOylation stimulates binding of unidentified effector proteins, facilitating the delivery of the co-modified protein to the proteasome. The HSPs would be interesting effector protein candidates (Figure 2B). We showed that inhibition of HSP90 also prevented the degradation of the co-modified proteins. HSP90, one of the most prevalent molecular chaperones, is mostly reported to protect proteins from degradation rather than promote it, although the opposite was proposed in a few cases[19-21]. HSP90 also has multiple potential SUMO-interaction Motifs (SIMs) making it theoretically possible for it to recognize SUMOylated proteins. HSPs could also be indirectly involved by e.g. stabilizing the unidentified effector proteins that facilitate the degradation of the co-modified proteins (Figure 2C). For example, HSP90 can influence the stability of the proteasome itself[22].

Because global SUMOylation increases not only upon heat shock but also upon proteasomal inhibition and ethanol stress, it is not unlikely that SUMO conjugation fulfils a general role upon these stresses. Previous research already established unfolded proteins to be a common trigger for SUMOylation after inhibition of the proteasome by MG132 and heat shock. SUMOylation also seemed to occur on an overlapping pool of proteins[23], potentially representing proteins prone to unfolding. Insoluble protein inclusion that are induced by MG132 treatment, include SUMO, ubiquitin and HSPs, proposing that a link between the proteostasis network and SUMO conjugation is not limited to the response upon heat shock. Another interesting observation was that K63-linked ubiquitin chains accumulated on SUMOylated proteins upon prolonged treatment with MG132. These ubiquitin chains have been linked to the aggresome-autophagy pathway, which is activated when the capacity of chaperones and UPS is overwhelmed by the amount of misfolded proteins[24-26]. This opens up the possibility of co-modified proteins being additionally regulated by alternative degradation pathways during proteotoxic stress.
Unravelling this intriguing network between SUMOylation, other PTMs, HSPs and the UPS would be greatly beneficial for the development of new treatment options for diseases that are characterized by a disturbed proteostasis. Small molecule inhibitors for most of the players exist and are tested in different contexts and diseases, e.g. cancer, but potentially offer strong tools to be combined in the combat against Alzheimer's, Parkinson's or Huntington's disease.

SUMO CHAINS - DIFFERENT LINKAGE? DIFFERENT STRUCTURE? DIFFERENT EFFECTOR PROTEINS?

The increase of SUMO conjugation as reaction towards stresses is characterized by an increase of SUMO chains[4,27,28]. In the ubiquitin field, chain formation has been intensively studied in the last decade and different types of chains were shown to have different functions. Ubiquitin has seven internal lysines and each can be used as acceptor lysine for ubiquitination. Additionally, ubiquitin can be fused to the N-terminus of another ubiquitin, a phenomenon called linear ubiquitination. Each chain linkage has a set of linkage-specific proteins that can write, read and erase the ubiquitin chain.
Similar to ubiquitin, SUMO can also form polymers. It is believed that SUMO chains predominantly consist of SUMO2 and SUMO3, which both have eight internal lysine[29]. SUMO-SUMO linkages have been identified by mass spectrometry for most of the lysines[30]. Even SUMO1, which is proposed to play a limited role in SUMO chain formation, can be modified by SUMO2/3 at multiple positions. Lysine 11 of SUMO2 and SUMO3 is located in a SUMO consensus motif within their flexible N-termini and is thought to be the predominant site for polymerization[29]. The observation that different linkages can be identified in vivo, raises the possibility of an unexplored diversity of SUMO chain functions, including specific proteins that catalyse distinct chain linkages, recognize different chain structures and specifically target different chains for depolymerization, in other words unidentified writers, readers and erasers, similar to the ubiquitin system. In Chapter 4, we identify poly-SUMOylation targets that are regulated by the polySUMO specific protease, SENP6. We show that SENP6 knockdown leads to the SUMOylation of multiple components of the constitutive centromere-associated network (CCAN), which is the most chromatin-proximal group of proteins within the kinetochore. The accumulation of high-molecular weight species of the CCAN proteins upon SENP6 knockdown strongly suggests the formation of

SUMO chains. We observed that the depletion of SENP6 led to the failure of CCAN proteins to localize to the centromere. Previously, one CCAN subunit, CENP-I was already reported as target for deSUMOylation by SENP6. It was suggested that accumulation of SUMO chains on CENP-I, due to SENP6 depletion, let to the recruitment of the human StUbL RNF4 and the subsequent ubiquitination and proteasomal degradation of CENP-I, explaining the decreased accumulation of CENP-I foci at the centromere[31]. Although, we also observed reduced centromeric accumulation of other CCAN subunits, including CENP-A, CENP-T and CENP-W, at the centromere upon SENP6 knockdown (KD), the underlying cause of this observation seems to be independent of degradation and RNF4. Surprisingly, upon proteasomal inhibition we failed to observe an accumulation of the SUMOylated or the ubiquitinated forms of the CCAN proteins, including for CENP-I. Also, combined knockdown of RNF4 and SENP6 did not result in the expected stabilization of poly-SUMOylated CCAN proteins. These observations led us to believe that the CCAN proteins are not targeted for degradation in a RNF4-dependent manner and their reduced presence at the centromere upon SENP6 KD must be explained by an alternative mechanism. One possibility is that the bulky SUMO chain itself sterically hinders the CCAN proteins to assemble into the tight structure at the centromere. Inhibition of protein interaction by SUMOylation has been reported before and such obstructions would only be enhanced with increasing length of a SUMO polymer[32,33]. The accumulated SUMO polymer on the CCAN proteins could alternatively induce binding to unidentified SIM-containing proteins that prevent their assembly into the CCAN.
Which mechanism prevents the CCAN proteins to efficiently assemble at the centromere, is still to be determined, but the observation that they are not targeted by RNF4 raises the question of what distinguishes the poly-SUMOylated CCAN proteins from poly-SUMOylated proteins that are recognized by RNF4 and degraded, like PML or Mis18BP1[34-37]. Although, we identified the canonical K11-linked SUMO chain on the in vitro SUMOylated CENP-T, we cannot exclude the presence of different linkages nor can we guarantee that the in vitro approach mirrors the situation in vivo. Therefore, it would be interesting to investigate and compare SUMO chain linkage structure of the CCAN subunits and RNF4-targets upon SENP6 depletion in vivo. The recognition of specific chain linkages by STUbLs would be an intriguing explanation for the observed RNF4 specificity. The second human STUbL, RNF111, was shown to mediate K48-linked ubiquitin chains that facilitated degradation and K48-linked and K63-linked ubiquitin chains with non-degradative role[38-40]. These findings demonstrate that the fate of proteins can depend on which STUbL is recruited and which ubiquitin chain is assembled. Different SUMO chain linkages would be an intriguing explanation for differential signalling.

Interestingly, Gärtner et al, recently demonstrated that in addition to the canonical K11-linked SUMO chain the non-canonical K5- and K7-linked SUMO chains can also stimulate RNF4-dependent degradation of PML. Additionally, a RNF4-derived poly-SIM module could be used to purify non-canonical chains from heat shocked cells expressing a SUMO K11R mutant, demonstrating that RNF4 can recognize different chain-linkages[41]. Cells expressing SUMO2 K11Q, an acetylation mimic mutant, show an increased formation of K5- and K35-linked chains upon heat stress and may be able to compensate for the loss of the K11 chain. Also, mutational analysis the yeast Smt3 demonstrated no requirement of specific SUMO chain linkages[42]. These observations indicate at least a partial redundancy of SUMO chain linkages, but do not conclusively exclude distinct roles of different linkages.

SUMOYLATION REGULATES THE INNER KINETOCHORE

The centromere is a pivotal component of eukaryotic chromosomes and necessary for faithful cell division. It marks the position onto which the proteinaceous macromolecular structure of the kinetochore is assembled. The kinetochore forms the microtubule attachment site of the mitotic and meiotic spindle. Chromosomal segregation relies on these bipolar attachments as the sister chromatids are pulled apart during anaphase to ensure that the genetic material is equally divided between the two daughter cells. Most eukaryotes have regional centromeres, which are defined epigenetically by the presence of the histone H3 variant CENP-A. Notable exceptions are centromeres of some budding yeasts, including Saccharomyces cerevisiae, that are defined by specific DNA sequences and are collectively called point centromeres. CENP-A does not only mark the position of regional centromeres but is also necessary for the assembly of all kinetochore components[43-46]. During DNA replication CENP-A is equally divided between the sister chromatids and in human cells new CENP-A molecules are not deposited into the chromatin until G1 phase[47-49]. The incorporation of CENP-A during G1 phase is dependent on the Holliday junction recognition protein (HJURP) and the three subunit Mis18 complex, consisting of Mis18 binding protein 1 (Mis18BP1), Mis18α and Mis18β. CENP-A deposition is directed to sites of pre-existing centromeres by the direct interaction of Mis18BP1 and CENP-A with CENP-C, one of the components of the pre-existing centromere[50,51]. The deposition of CENP-A is additionally regulated by cyclin-dependent kinases (CDKs) and Polo-like kinase mediated phosphorylation events of CENP-A itself, HJURP and the Mis18 complex[52-55].

The constitutive centromere-associated network (CCAN) forms the most chromatin-proximal part of the kinetochore (also called inner kinetochore) and some of its components directly interact with CENP-A. The CCAN contains 16 subunits that can be divided into five subcomplexes, CENP-C, CENP-L-N complex, CENP-H-I-K-M complex, CENP-O-P-Q-U-R complex and CENP-T-W-S-X complex and their localization at the centromere is mostly cell cycle independent[43,56]. In Chapter 4, we describe that 11 of the 16 CCAN subunits are modified by SUMO chains upon knockdown of the polySUMO specific protease SENP6. Although one of the subunits, CENP-I, was identified to be regulated by SENP6 and SUMOylation in an earlier report we uncovered an unexpected and striking group-regulation of the CCAN[31]. SENP6 depletion was shown previously to result in a complete loss of CENP-I, CENP-H and CENP-O at the mitotic centromere[31]. Another recent report showed that SENP6 depletion affected the correct localization of CENP-A, which was mediated indirectly by the RNF4-dependent degradation of poly-SUMOylated Mis18BP1[37]. Our data confirms a reduced CENP-A accumulation at the centromere upon SENP6 depletion and additionally shows a decreased accumulation of CENP-T and CENP-W. Notable here is that whereas we failed to detect any SUMOylation of CENP-A upon SENP6 knockdown, supporting the previously reported indirect regulation by SENP6, the polySUMOylated forms of most of the CCAN proteins highly increased. This observation indicates that the CCAN proteins are directly regulated by SENP6. The recruitment of all CCAN proteins depend on CENP-A but vice versa CENP-C, CENP-N and CENP-I can influence the positioning of CENP-A to the centromeric chromatin[50,51,56-58]. Further CCAN assembly does not follow a linear hierarchy but depends on a complicated network of dynamic interactions between the subcomplexes, CENP-A and centromeric DNA[59,60]. It is therefore difficult to conclusively identify the underlying cause of the reduced centromere accumulation of the CCAN proteins upon SENP6 knockdown. Double knockdown of RNF4 and SENP6 was reported to result in a substantial rescue of the SENP6

depletion phenotype, demonstrating a clear involvement of RNF4-dependent regulation[37]. However, we demonstrate that the polySUMOylated CCAN proteins are not regulated by RNF4. Therefore, a combination of the RNF4-mediated processes, like the degradation of Mis18BP1, and a poly-SUMO-induced sterical restriction of the CCAN proteins is a possible scenario. Whatever the mechanism, it has become clear that the deSUMOylation of proteins plays an important role in the regulation of the CCAN assembly. Future studies will also have to investigate why these proteins are SUMOylated in the first place.

CSB SUMOYLATION MIGHT INFLUENCE PROTEIN INTERACTIONS

In chapter 5 we identified CSB as the most dynamically SUMOylated target upon UV-induced DNA damage. We show that CSB SUMOylation is dependent on transcription and that other DNA lesions, possibly interfering with the progression of elongating RNA polymerase, also cause CSB SUMOylation. Also, we observed reduced efficiency of recruitment and/or retention of SUMO-deficient CSB at the lesion site, suggesting altered binding properties of CSB to other proteins or to the chromatin. A few protein interactions seemed to be influenced by the SUMOylation of CSB (Chapter, Figure 4). For example, two subunits of the polymerase-associated factor (Paf) 1 complex, Paf1 and Leo1, showed enhanced binding to the SUMOylated CSB N-terminus. The Paf1 complex influences multiple steps of RNA polymerase II (RNAPII) transcription, including promotor-proximal pausing, elongation and termination. The functionality of the Paf1 complex is multifaced and can have positive as well as negative influence on transcription levels[61]. A connection between CSB and Paf1 complex has not been reported before and it would be highly interesting to study this interaction in the context of the fate of stalled RNA polymerase II upon transcription-obstructing DNA lesion. Interestingly, we also identified Pol I and transcript release factor (PTRF) to preferentially bind SUMOylated CSB. PTRF regulates the termination of RNA polymerase I transcription[62,63]. Therefore, our findings link the SUMOylation of CSB to regulatory processes of both, RNA polymerase I and II. Proteins that were identified binding preferentially to unmodified CSB included the proliferating cell nuclear antigen (PCNA), which recruits DNA polymerases for the gap filling step during NER, and PolR2H, a subunit of RNA polymerase I, II and III. The significance of these interactions and the role of SUMOylation need to be further evaluated but these findings indicate a possible new layer of PTM regulation during transcription-coupled nucleotide excision repair.

BALANCE BETWEEN INHIBITION AND STIMULATION

SUMOylation has evolved as important regulator of many nuclear processes, some of which have been investigated in this thesis, including proteostasis[64,65], cell cycle control[66] and DNA damage[67]. The SUMOylation pathway is essential and mouse embryos lacking the SUMO E2 conjugating enzyme Ubc9 die at the early implantation stage[68]. Knockdown of Ubc9 or the SUMO E1 activating enzyme subunit SAE1 in human cells, led to a severe reduction of cell proliferation without affecting any specific cell cycle phase[69,70], demonstrating the importance of SUMOylation for each cell cycle phase. Cancer cells, as being highly proliferative, especially depend on SUMOylation[71,72]. This is reflected by the finding that components of the SUMO conjugation cycle are upregulated in many cancers, and drugs targeting the SUMO machinery to block SUMOylation are currently under investigation as anti-cancer treatments[66]. For example, the SUMO E1 conjugating enzyme inhibitor ginkgolic

acid was demonstrated to be effective in Notch-driven breast cancer cells[73,74] and a recently developed potent and selective SAE1 inhibitor demonstrated reduced proliferation of multiple cancer cell lines, by causing mitotic defects [75]. In chapter 4, we show that depletion of SENP6 leads to reduced proliferation with a subtle but reproducible G2-M arrest. SENP6 is essential for cell survival and mitotic progression[31,76,77]. Similar to the overexpression of SUMO conjugation enzymes, the overexpression of deSUMOylation enzymes has been connected to multiple cancers, demonstrating that the equilibrium of SUMOylation is important for cell viability[66]. Tipping the scale towards one or the other direction could be a promising strategy against cancer cell proliferation since cancer cells appear to be especially dependent on SUMOylation. As discussed in chapter 2 and 3, SUMO can have a potentially beneficial role during cellular stresses and neurodegenerative diseases. Inhibition of SUMO proteases could be a potential strategy to increase the neuroprotective role of SUMOylation or in cases where SUMO was shown to have a disadvantageous effect, inhibitors like the SAE1 inhibitor could be of great interest. In conclusion, SUMO affects multiple cellular pathways and hundreds of proteins and consequences of SUMOylation or deSUMOylation inhibition are difficult to predict and have to be carefully investigated in the context of the particular subjective.

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6

GENERAL DISCUSSION