Safeguarding genome integrity with small ubiquitin-like modifiers

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Chapter 6

General discussion

SUMOylation is a posttranslational modification critical for regulating a variety of predominantly nuclear processes. In this thesis, we have set out to gain a better understanding of how the SUMO system governs the integrated network of nuclear signalling pathways, with a specific focus on genome maintenance. We have done this by exploring both SUMO conjugation and deconjugation **(chapter 2** and **3)**, and noncovalent SUMO interactions **(chapter 4** and **5)**, within this context.

Expanding the SUMO landscape

To date, thousands of SUMO target proteins and their SUMO sites have been identified with mass spectrometry-based proteomics [1, 2]. The SUMO target proteome has been analysed under a variety of different physiological conditions, including during the cell cycle and in response to DNA damage [3-6]. These approaches have relied and mostly still do on the exogenous expression of tagged SUMO for sufficient enrichment and reliable detection of the modified protein fraction. In **chapter 3**, we use our established His10-SUMO2 purification methodology for this purpose [7, 8]. With the emergence of improved strategies for endogenous protein tagging and endogenous SUMO enrichment, as well as continued improvement in detection methods, researching covalent SUMOylation under endogenous conditions should become more feasible and standard practice in the field in the near future.

Over the past years, increasing effort has been directed towards identifying substrates of specific components of the SUMO enzymatic machinery, including SUMO E3 ligases and SUMO proteases of the SENP family, and the SUMO-targeted ubiquitin ligase (STUbL) RNF4 [7, 9-13]. Together with work from Wagner et al., we have provided novel insights on the target proteome of the SUMO protease SENP6 in **chapter 3** [7, 11, 12]. Initially, reports on individual proteins identified some as substrates of SENP6 [14-16]. We have expanded significantly on this by performing an unbiased mass spectrometry screen combined with knockdown of SENP6. This led to the identification of 180 potential SENP6 substrates and multiple interconnected clusters of functionally and/or physically related proteins involved in a variety of nuclear processes, including the cell cycle [12] and the DNA damage response **(chapter 3)** [7]. In these SUMO-based proteomic screens, it is important to keep in mind potential indirect effects. For example, the SUMO E2 enzyme UBC9 and the SUMO E3 ligases PIAS1, PIAS2, PIAS3, ZNF451 and NSMCE2 have been identified as RNF4 ubiquitination targets, which can have downstream effects on the SUMOylation levels of proteins [9]. PIAS1-4 and NSMCE2 were also identified as potential SENP6 substrates [12].

Besides the SUMO target proteome, mass spectrometry-based proteomics is also being

used to explore the SUMO interactome. Our understanding of noncovalent SUMO binding is more limited than our knowledge on covalent SUMO modification. In the last few years, we and others have independently captured SUMO-interacting proteins in cells using different approaches, including microarray and pulldown strategies **(chapter 4** and **5)** [17-21]. Collectively, this has led to the identification of ~400 SUMO-binding proteins, making the SUMO interactome less vast than the SUMO target proteome. Notably, direct crosstalk appears to exist between covalent SUMO modification and noncovalent SUMO interactions. This is explored in **chapter 5**, where we determined the dependency of the SUMOylation of proteins in cells on SUMO-SIM interactions.

Further increasing the complexity of the proteome is the yet mostly unexplored microproteome. Microproteins are small proteins (<100 amino acids) that originate from short open reading frames that were not known to encode for proteins until recently [22]. Interestingly, the microprotein pTINCR, involved in epithelial differentiation and tumour growth, was shown to bind to SUMO through a SIM [23]. It is tempting to speculate that the SUMO interactome and perhaps also the SUMO target proteome might expand further than the conventional proteome.

Protein group modification

For the majority of SUMO substrates, it is still unknown what the physiological relevance and functional role is of their SUMOylation. The SUMO field has tried to tackle these questions by making SUMOylation mutants of proteins by mutating the involved lysine residue(s). This is often a courageous endeavour since the modification can jump to a nearby lysine. Thus, creating a defective SUMOylation mutant of a protein can require a substantial number of mutations, which also increases the risk of hampering protein function beyond its SUMOylation. Moreover, SUMOylation mutants of individual proteins often lack notable phenotypes. To explain this redundancy of single SUMOylation events, Psakhye and Jentsch introduced the concept of group modification [24]. In this model, SUMO acts synergistically on multiple proteins that are functionally or physically connected within a cellular pathway to exert its function.

We show that the SUMO protease SENP6 also regulates proteins by group modification **(chapter 3)** [7, 12]. Using our His10-SUMO2 pulldown strategy in combination with knockdown of SENP6, we identified 180 potential substrates by mass spectrometry [12]. Among these were ten out of the sixteen constitutive centromere-associated network (CCAN) proteins. Moreover, we also identified a set of DNA damage response proteins, including proteins involved in DNA double-strand break repair **(chapter 3)**. This is in line with another

unbiased mass spectrometry screen identifying CCAN proteins and proteins involved in genome maintenance as SENP6 targets [11], as well as several reports on individual proteins involved in these processes [14-16, 25-28]. Collectively, both our research and that of others provide an extensive set of data supporting regulation of genome stability by SENP6. In this context, it is important to emphasize that SENP6 is involved in several nuclear processes. Knockdown of SENP6 leads to a broad phenotype, including a range of defects in cell cycle progression, genome stability **(chapter 3)**, PML body formation and composition **(chapter 3)**, and the nuclear lamina [7, 11, 12, 14, 15, 29-31]. It is therefore not surprising that SENP6 is overall critical for cellular survival; CRISPR-Cas9-mediated knockout of SENP6 is lethal in 1070 out of 1100 cell lines included in the depmap database. Recently, regulation of proteins by group modification was also shown for the SUMO proteases SENP3 and SENP5 [13]. Here, SENP3 and SENP5 regulate an interconnected network of ribosomal proteins and ribosome biogenesis factors.

SUMO proteases in genome maintenance

SUMO proteases of the SENP family play important roles in the DNA damage response. Knockdown of all SENPs, except SENP3, impairs repair by non-homologous end-joining (NHEJ) and homologous recombination (HR) in double-strand break reporter assays [29]. To date, there is some mechanistic insight on how deconjugation by SENP2, SENP3, SENP6 and SENP7 contributes to DNA repair [7, 11, 15, 16, 29, 32-35]. We have summarized and discussed these reports in **chapter 2**. In **chapter 3**, we have provided novel mechanistic insights into the role of SENP6 in the DNA damage response [7]. We found that SENP6 deconjugates SUMO2/3 chains on a group of DNA damage response proteins, including the BRCA1-BARD1 heterodimer, BLM, 53BP1 and the ERCC1-XPF endonuclease. We observed a build-up of polymeric SUMO2/3 chains on these proteins in the absence of SENP6. In contrast, under steady-state conditions, in the presence of SENP6, SUMO2/3 chains on these proteins are virtually absent, suggesting that their conjugation and deconjugation is a highly dynamic process. Moreover, this suggests that under unstressed conditions SENP6 normally maintains these proteins in a hypo-SUMOylated state. There was an overlap of our identified SENP6 targets with previous mass spectrometry screens using different types of genotoxic stress and showing increased SUMOylation of proteins involved in the DNA damage response [3, 5, 6]. Indeed, we confirmed increased SUMOylation of several SENP6 targets in response to hydroxyurea-induced stress.

Posttranslational modification of DNA damage response proteins, including SUMOylation, typically regulates their recruitment, interaction and retention at damaged chromatin. Indeed, SUMO1, SUMO2/3 and components of the SUMO machinery (UBC9, PIAS1 and

PIAS4) accumulate at sites of DNA damage to SUMOylate substrates involved in the repair response, and disappear again over time [36, 37]. We have shown that depletion of SENP6 leads to altered kinetics of SUMO2/3 and several DNA damage response proteins at sites of DNA damage. This is consistent with findings for SENP2 and SENP7, which have been shown to regulate DNA repair through the timely deconjugation of SUMOylated proteins at damaged chromatin [29, 32]. We had difficulties reliably detecting SENP6 at sites of DNA damage, probably due to the local and transient nature of the response. Therefore, it is not yet entirely clear where and when deSUMOylation of proteins by SENP6 happens in response to DNA damage.

Despite the new wealth of knowledge obtained over the last few years regarding the substrates and nuclear functions of SENP6, this is possibly still the tip of the iceberg. We have focussed our mechanistic studies on a subset of the DNA damage response proteins, but there are still many other interesting candidates for follow-up within this group of proteins, as well as proteins involved in other nuclear processes. For example, we and Wagner et al. both identified nuclear lamin proteins as potential SENP6 substrates, and Wagner et al. also confirmed increased SUMOylation of Lamin B [11, 12]. Recently, Liczmanska et al. validated the lamin proteins in a slice-by-slice mass spectrometry approach as SENP6 targets, showing a large SUMO-modified shift in molecular weight upon SENP6 depletion [31]. They also connect regulation of these proteins by SENP6 to laminopathy-type phenotypes observed in SENP6-depleted cells. Other interesting avenues for follow-up are condensin (SMC2/SMC4) and the SMC5/6 complex. In yeast, the SENP6 homolog Ulp2, counteracts SUMO-chain-targeted turnover of SMC complexes by the STUbL slx5/slx8, essential for processes like sister chromatid cohesion, chromosome condensation and DNA repair [38]. It would be interesting to investigate whether in mammalian cells the SMC proteins are regulated by SENP6 and RNF4 in a similar fashion.

SUMO-SIM interactions in genome maintenance

Besides SUMO conjugation and deconjugation, noncovalent SUMO interactions also play an important role in the DNA damage response. A prime example of a SUMO-binding protein is the STUbL RNF4 [39, 40]. RNF4 contains four SIMs in tandem which efficiently bind to polymeric SUMO2/3 chains [41], although it can also bind monomeric SUMO1 and SUMO2/3. Therefore, it is often used as positive control in SUMO interactomics screens or *in vitro* SUMO binding assays. Indeed, we identified RNF4 as a top interactor in our noncovalent SUMO interaction screens in **chapter 4** and **chapter 5** [17]. Moreover, in **chapter 5**, we have shown that RNF4 does not bind to monomeric SUMO2/3 in which the SIM-binding groove is mutated.

RNF4 plays important roles in the DNA damage response (reviewed in [42]). RNF4 localizes to sites of DNA damage and many DNA damage response proteins are regulated by RNF4, including MDC1, BRCA1-BARD1, RPA70 and the Fanconi Anemia ID complex [9, 16, 43, 44]. STUbLs typically function in compartmentalized environments together with SUMO proteases and deubiquitinases, providing spatiotemporal regulation of their substrates. RNF4 binding to polymeric SUMO2/3 chains on a substrate induces its activation, which is followed by substrate ubiquitination as well as RNF4 autoubiquitination and degradation [45]. Consequently, one of the main functions of SENP6 is thought to be antagonizing the targeting of proteins by RNF4. Indeed, in **chapter 3**, we have shown that SENP6 antagonizes targeting of BRCA1, BARD1 and BLM by RNF4. Another consequence is that SENP6 depletion in cells leads to the degradation of RNF4. This strong correlation between SENP6 and RNF4 levels makes it challenging to dissect any potential regulation of proteins by SENP6 that is independent of RNF4.

In **chapter 4**, we have provided an example of how noncovalent SUMO binding of a DNA damage response protein through a SIM facilitates DNA repair [46]. Using mass spectrometry, we identified the NHEJ protein XRCC4 as a preferential binder for SUMO2 trimers rather than monomeric SUMO2. It was also recently identified as a polySUMO2 interactor in a microarray-based screening [20]. We identified a SIM in XRCC4 that is required for this binding. We found that mutating this SIM hampers its binding to DNA ligase IV and its recruitment to damaged chromatin. S320 phosphorylation of XRCC4 by DNA-PKcs was also completely abrogated when the SIM was mutated. Another DNA damage response protein identified in our mass spectrometry screen was SLX4, binding both to monomeric SUMO2 and SUMO2 trimers. Our lab has previously identified three SIMs in SLX4, and mutating these abrogates its binding to SUMO2 as well as its covalent SUMOylation [46]. Moreover, this mutant is no longer recruited to PML bodies and stabilized at sites of DNA damage. Many more DNA damage response proteins were identified as noncovalent interactors of monomeric SUMO2 and/or SUMO2 trimers, including ATRX, BLM and ERCC1. In **chapter 3**, we also confirm binding of BRCA1, BLM, XPF, RAP80, MUS81 and ERCC1 to a SUMO2 trimer [7]. However, how noncovalent binding to SUMO regulates these proteins and their functions in the DNA damage response remains largely unexplored. Moreover, proteins involved in RNA regulation were also identified as noncovalent SUMO binders. Future research should continue to focus on exploring noncovalent SUMO interactions of proteins on a functional and mechanistic level.

SUMO-SIM interactions and phase separation

In recent years, liquid-liquid phase separation (LLPS) and the formation of molecular condensates have gained a lot of attention and are increasingly recognized for their involvement in various cellular processes, including the DNA damage response. Currently, there is an increasing body of evidence supporting the involvement of SUMO in LLPS and the dynamics and compositional regulation of molecular condensates (reviewed in [47]). PML bodies are a classical example of nuclear condensates that assemble through LLPS and recent *in vitro* studies show that this is driven by SUMO polymers and SUMO-SIM interactions [48]. In **chapter 3**, we have shown that the SUMO protease SENP6 is involved in this process [7]. SENP6 was previously shown to regulate the formation and composition of PML bodies [30]. We found increased colocalization of several DNA damage response proteins in PML bodies after SENP6 knockdown. We found that this was induced through the build-up of SUMO2/3 polymers on these proteins and their ability to noncovalently bind SUMO and thus participate in multivalent SUMO-SIM interactions. Interestingly, we found that these DNA damage response proteins also formed nuclear bodies independently of PML in SENP6-knockdown cells. Thus, by controlling the SUMOylation levels of proteins, SENP6 can participate in the formation and dissolution, as well as compositional control, of molecular condensates. This is supported by another recent study, where the assembly and disassembly of SLX4 DNA repair condensates on chromatin, that do not overlap with PML bodies, were found to be regulated by SENP6 and RNF4 in a SUMO-SIM dependent fashion [33].

Another example pointing towards the involvement of the SUMO system in protein phase separation, including SENPs and RNF4, is the formation and dynamics of cytoplasmic stress granules [49]. Moreover, nuclear condensation of the transcription factor NELF in response to stress was shown to be regulated by SUMOylation [50]. This direction of research in the SUMO field remains relatively new and unexplored, and it will be interesting to further investigate the involvement of the SUMO system here.

Novel modes of noncovalent SUMO interactions

So far, our discussion of noncovalent SUMO interactions has concerned SUMO-SIM interactions in multiple contexts, including the STUbL pathway, the DNA damage response and phase separation. We are still limited in our knowledge on noncovalent SUMO interactions beyond the typical SUMO-SIM interaction. In **chapter 5**, we performed a noncovalent SUMO interaction mass spectrometry screen with SIM-binding groove SUMO2/3 mutants to specifically capture proteins that interact with SUMO2/3 in a different manner. Many proteins

were able to bind both SUMO2/3 wildtype and the mutants, suggesting that the prevalence and relevance of SUMO-SIM-independent modes of interaction is currently underestimated in the field. To date, only one other SUMO interactomics study was designed to enrich for other modes of interactions, specifically class II SUMO1 interacting proteins that interact with a surface on SUMO opposite of the SIM-binding groove [18]. Our screen also allows for the selection of proteins that bind through SUMO-SIM interactions with higher confidence (i.e., proteins binding only to wildtype SUMO2/3 and not the SIM-binding groove mutants).

The proteins capable of binding the SIM-binding groove SUMO mutants must interact with another region on SUMO and through other domains or sequence motifs than the typical SIM. Using domain enrichment analysis and *in vitro* binding assays, we identified a group of WD40 repeat domain containing proteins as novel class of SIM-independent SUMO interactors. How the WD40 repeat domain facilitates binding to SUMO and which region in SUMO is involved in the interaction remains to be determined. The WD40 repeat domain proteins comprise of around 8% of the proteins binding to the SIM-binding groove mutants, suggesting that the other identified proteins might interact through yet uncharacterized binding domains or sequence motifs. Approaches such as NMR spectroscopy could further aid in the identification of residues involved in the interaction in both the interaction partner and SUMO. The N-terminal tail of SUMO was recently shown to inhibit SUMO-SIM binding by interactions with SUMO's core [51], further implying that many aspects of noncovalent SUMO interactions are yet to be unravelled.

Several enzymes of the SUMO and ubiquitin machinery required the SIM-binding groove for SUMO binding, including the previously validated STUbLs RNF4 and RNF111 [41, 52]. We also found that the ubiquitin E3 ligases RNF2, RNF216, RAD18 and the SUMO E3 ligase TOPORS require the SIM-binding groove (RNF216, RAD18 and TOPORS were also identified as SUMO interactors in **chapter 4**). The SUMO E3 ligase TOPORS was recently shown to also be a STUbL [53], suggesting that RNF2, RNF216 and RAD18 could also potentially be novel STUbLs.

Crosstalk between covalent SUMOylation and noncovalent SUMO binding

We extended our understanding of the SUMO interactome to also include covalent SUMO modification in the context of the SIM-binding groove. In **chapter 5**, we explored the SIM-dependency of the covalent SUMO modification of proteins through noncovalent interactions between SUMO and the SUMOylation machinery, including the SUMO E2 enzyme UBC9, SUMO E3 ligases and SUMO proteases of the SENP family. We and others have previously shown that SUMOylation of USP25, BLM, HIPK2, DAXX and SLX4 is dependent

on SIMs and binding to SUMO [46, 54-56]. A proposed model for this SIM-dependency is through the recruitment of SUMO thioester-charged UBC9 to these substrates, thereby facilitating lysine modification [54]. Another possibility is through the requirement of SUMO E3 ligases to noncovalently interact with SUMO for efficient SUMO discharge from the SUMO-UBC9 thioester followed by substrate modification. Both mechanisms could explain the drastic loss in covalent SUMOylation that we observed in cells expressing a SIM-binding groove SUMO2/3 mutant. Proteins that are still SUMOylated in cells expressing the SIM-binding groove SUMO2/3 mutant perhaps do not require the aid of a SUMO E3 ligase for their covalent SUMOylation. Altogether, **chapter 5** is a unique resource for the field and offers valuable new insights on both noncovalent SUMO interactions and covalent SUMO-modification in the context of the SIM-binding groove.

Inhibiting the SUMO system as study tool and for therapeutic targeting

With the development of selective SAE1 inhibitors that block SUMOylation (TAK981 and ML792) [57] and the emerging role of SUMO in cancer development, the SUMO field is expanding from predominantly fundamental research questions to clinical applications. In **chapter 3** and **chapter 4**, we illustrated the importance of SUMO deconjugation and noncovalent SUMO interactions in maintaining genome integrity. Genome instability is frequently associated with tumour development. Indeed, SENP6 was recently implicated in the development and progression of tumours in MYC-driven B-cell lymphoma [34]. The potential of SUMO inhibitors in the therapeutic targeting of cancer is reviewed in [58].

The SUMO inhibitors are also a useful research tool to study SUMOylation. In **chapter 3**, we have used ML792 to show that the genomic instability that spontaneously occurs in SENP6-knockdown cells can be reversed by treatment with the inhibitor, illustrating that this phenotype is induced by excessive SUMO conjugation [7]. Moreover, we have used it to show that the accumulation of DNA damage response proteins in nuclear bodies is also reversible and induced by excessive SUMO conjugation. Others have also used ML792 to study a variety of processes. For example, treatment of human induced pluripotent stem cells with ML792 was shown to reduce pluripotency markers [59]. Moreover, in a recent CRISPR-Cas9 genetic knockout screen, the BLM-TOP3A-RMI1-RMI2(BTRR)-PICH pathway and NIP45/NFATC2IP became indispensable when SUMOylation was inhibited, and this is due to their role in resolving toxic DNA catenanes to prevent mitotic failure [60].

Besides the SUMO inhibitor, increasing efforts are being directed towards the development of SENP inhibitors. In **chapter 2**, we have summarized the emergence of SENPs as important players in human disease, specifically cancer, and discussed their potential as therapeutic

targets. However, the development of SENP inhibitors is still in its infancy. One of the biggest challenges remaining ahead is the development of isoform-selective inhibitors, before progressing to (pre-)clinical testing and implementation. Selective SENP inhibitors would also provide useful research tools. Currently, experiments looking at SENP function predominantly involve overexpression or knockdown of the entire protein which can lead to indirect effects, not reflecting their physiological functions. To address this issue, the field would benefit from SENP inhibitors capable of selectively and temporally targeting SENP catalytic activity.

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