Regulation of target proteins by small ubiquitin-like modifiers

Xiao, Z.

Chapter 6
General Discussion

Post-translational modifications (PTMs) are essential to regulate the localization, conformation, interaction and stability of target proteins. PTMs do not play their roles solo but interact with each other[1].

Protein SUMOylation is essential for almost all organisms and regulates many nuclear process[2-4]. It is a highly dynamic process that enables fast and reversible cellular signal transduction. However due to the low modification stoichiometry, highly active proteases and a large remnant after trypsin digestion, functional proteomes of SUMO targets as well as SUMO acceptor lysines are difficult to characterize[2,5-19]. The first three chapters of this thesis focus on deciphering the roles of SUMOs in response to replication stress, how the roles of SUMO and ubiquitin signalling are balanced by the STUbL RNF4 and how SUMOylation crosstalks with phosphorylation during replication stress using global and unbiased proteomic strategies.

UFM1 is a recently identified ubiquitin-like protein that covalently modifies lysine residues of its substrates through a three component enzymatic cascade - UFMylation. UFMylation is conserved among nearly all of the eukaryotic organisms, except yeast, and associated with several cellular activities including the endoplasmic reticulum stress response and haematopoiesis. The last chapter of this thesis describes how ribosomal protein large subunit 26 (RPL26) is modified by UFM1, and we investigated its role in the last step of translation.

1 Dynamic SUMO targets and acceptor lysines in response to replication stress.

SUMOylation was first linked to DNA damage (DNA mismatch repair) by studies on the molecular mechanisms of SUMOylation induced protein transfer[20]. Since then, studies were focused on the roles that SUMO play in response to DNA damage and SUMOylation turned out to be one of the most important processes to modify DNA repair proteins[21]. However, the relevant SUMO target proteins to maintain genome stability during replication have remained largely elusive due to difficulties of purifying and identifying relevant SUMO target proteins. Therefore, we wanted to identify SUMO target proteins involved in counteracting replication stress.

In chapter 2, we have performed a large-scale proteomic screen to identify proteins that are regulated by SUMO-2 upon treatment of cells with Hydroxyurea at two different time points (2 hours and 24 hours). In mammalian cells, agents like hydroxyurea can stall or collapse DNA replication forks and induce homologous recombination to counteract replication stress. It was reported that most DNA replication forks restart after 2 hours of Hydroxyrurea (HU) while 24 hours of HU will cause the collapse of the replication fork[22]. By introducing these two-time points, we could study SUMO-2 conjugates from cells exposed to replication stress for a short period (2 hours) when repair proteins are involved to restart the replication fork or for a longer period (24 hours) when double strand breaks occur and homologous recombination is induced to repair it.

We used optimized purification procedures that were developed in our lab to enrich SUMO target proteins as well as SUMO acceptor lysines[6,23]. The improved approach employed a tag of 10 histidines to the N-terminus of SUMO-2 or SUMO-2 that lacks all its lysines. The use of the His10 tag allows a purification procedure with a relative high concentration of Imidazole to largely get rid of the binding of non-specific proteins and allows the use of denaturing buffers to inactivate proteases. Lysine-deficient version of SUMO-2 protect SUMO-2 from Lys-C digestion and enabled a second round of purification of SUMOylated peptides after Lys-C digest, which reduced the copurification of non-SUMOylated peptides and decreased the complexity of the samples.

Using this method, 566 SUMOylated proteins and 1,043 SUMOylation sites were identified in this project. Among those identified SUMOylated proteins, when cells were treated with HU for two hours, 12 dynamic SUMO-2 targets were identified (for 10 proteins SUMOylation was increased and for 2 proteins SUMOylation was decreased), while for U2OS cells that were treated with HU for 24 hours, 48 dynamic SUMO-2 targets were identified (for 35 proteins SUMOylation was increased and for 13 proteins SUMOylation was decreased).

A plethora of known SUMOylated DNA damage response factors have been identified in our screen, such as MDC1[24], BLM[25], ATRIP[26] and BRCA1[27], which can serve as a proof of the validity of our approach. There are also many DNA damage response related proteins that had no previous known relationship with SUMOylation at the time when the paper was published such as RMI1, BARD1, EME1, CHAF1A. In 2016, there was a paper on RMI1 SUMOylation in yeast, mediated by Smc5/6[28]. This paper showed that the STR complex (Sgs1, Top3, Rmi1) can interact with SUMO and Smc5/6 through Sgs1 and these interactions contribute to the SUMOylation of Sgs1, Top3 and Rmi1. Reduced STR SUMOylation leads to recombination intermediates accumulating and impairment in cell growth. RMI1 is the second most highly upregulated SUMO target protein in our screen in response to 24 hours of HU treatment. In human cells, TOP3α, BLM and RMI1/2 forms the BTR complex that dissolves Double-Holliday junctions. Increased SUMOylation of RMI1 could represent a mechanism to increase interaction of RMI1 with BLM and TOP3α to disentangle double Holliday junctions and to produce non-crossover products. Further studies about SUMOylation of RMI1 in homologous recombination will be of great interest.

We also identified some components of the DNA replisome in our screen such as Proliferating Cell-Nuclear Antigen (PCNA). After two hours of HU treatment, SUMOylation of PCNA was shown to be down-regulated. PCNA is a ring type homotrimer that embraces DNA. PCNA functions as a sliding clamp and is critical for faithful DNA replication and repair. The original modification of PCNA by SUMO was identified in budding yeast on two residues, lysine127 and lysine164. In the presence of DNA damage, the interaction between PCNA and UvrD-like helicase Srs2 is strongly enhanced at both residues to prevent access of the recombination machinery[29-32]. In human cells, PCNA was also SUMOylated to inhibit double strand break initiated homologous recombination and also to prevent

double strand breaks formation at stalled replication forks[33,34]. PCNA SUMOylation was down-regulated at the early time point of replication fork stalling.

Furthermore, SUMOylation of a group of centromeric proteins was identified in our screen after 24 hours of HU treatment, including MLF1IP (CENPU), CENPH and MIS18A, which play a role in the regulation of CENPA deposition[35]. It was reported that the subcellular localization of SUMO-2/3 was at centromeres and kinetochores while the subcellular localization of SUMO-1 was at the mitotic spindle and spindle midzone[36]. Overexpressing SUMO protease SENP2 inhibited the accumulation of SUMO conjugates and led to a prometaphase arrest due to a defect in targeting of CENPE to the kinetochores, which indicates a key role of SUMOylation in regulating the cell cycle[37]. The functional significance of SUMOylation to centromeric proteins could further be explored.

Finally, it is of great interest to note that SUMOylation is involved in regulating other post translational modifications such as ubiquitination, phosphorylation and demethylation, demonstrating extensive crosstalk among different modifications. These proteins include lysine-specific demethylases 5D, 5C and 4A; ubiquitin E3 ligases RAD18 and BRCA1. Regarding crosstalk, mixed SUMO-ubiquitin chains have also been found in our study as well. There are three SUMO-2 acceptor lysines in ubiquitin, which are lysine11, lysine48 and lysine63. Moreover, there are 83 peptides that have been found to be modified by both SUMO-2 and phosphorylation indicating a crosstalk between SUMOylation and phosphorylation in the DNA damage response. Also, the optimized methodology could be broadly applied to study signal transduction by other ubiquitin-like modifiers.

2 Crosstalk between SUMO and other PTMs

2.1 The ubiquitin E3 ligase RNF4 regulates protein group SUMOylation by targeting the SUMO E2 and E3s.

We identified and characterized targets for the human STUbL RNF4 by using two complementary proteomics approaches. Firstly, levels of RNF4 targeted SUMO conjugates in the cell should be increased upon RNF4 knockdown. Secondly they were covalently trapped using RNF4-TULIP constructs. They were enriched in a SIM-dependent manner after treatment of cells with proteasome inhibitor MG132. Targets meeting both requirements were identified as SUMO E3 ligase PIAS1, PIAS2, PIAS3, NSMCE2, ZNF451, Ubiquitin E3 ligase RAD18, RNF216 and other proteins such as SLFN5, IMPDH2, TOP2A, SLX4, MORC3, SMC5, ERCC4. They were regulated by RNF4 with intact SIMs and they were enriched upon proteasome inhibitor MG132 treatment. Thus, we proposed a model of RNF4 to balance SUMO-ubiquitin signalling by targeting the SUMO conjugation machinery.

Many active SUMO E3 ligases could undergo auto-SUMOylation[38-40]. Our data indicate that in human cells, RNF4 could target auto-SUMOylated SUMO E3 ligases to limit SUMO signal transduction. In budding yeast, it was reported that the yeast STUbL Slx5/Slx8 mediates regulation of the SUMO E3 ligase Siz1 and affected SUMOylation and nuclear localization of Siz1 *in vivo* to prevent

the accumulation of SUMO conjugates in the nucleus[41]. In fission yeast, the SUMOylated SUMO E3 ligase Pli1 was also found to be targeted by the STUbL and its cofactor Ufd1[42]. It was previously found that there are many closely located SUMO acceptor lysines on SUMO E3 ligases[6], which might be efficiently recognized by RNF4.

The SUMO E2 Ubc9 was also identified in our RNF4-TULIP screen, co-enriched with RNF4 wild type constructs from cells treated with proteasome inhibitor MG132. It has been reported that the mammalian E2-conjugating enzyme Ubc9 could undergo autoSUMOylation at Lys14. SUMOylated Ubc9 provides an additional interface for its SIM-containing targets and this regulates target discrimination[43]. Ubc9 standard activity was severely reduced by SUMOylation but this stimulated SUMO chain formation via noncovalent SUMO interaction. Targeting SUMOylated Ubc9 by the STUbL RNF4 could therefore be a valid way to decrease SUMO polymer formation. In the RNF4 knockdown approach, Ubc9 was also identified in our screen but statistically not picked up because its value was below the cut off value.

Yeast homologs (Rfp1 and Rfp2) of RNF4 are essential for DNA repair[44]. Mammalian RNF4 knockout mice die at the early embryonic stage with higher levels of methylation in genomic DNA[45]. RNF4 has been implicated in arsenic-induced PML degradation[46]. RNF4 also accumulated rapidly at sites of DNA damage and co-localized with γH2AX. RNF4 deficient MEFs showed an increased size and number of phosphorylated H2AX foci.

PIAS1, 2, and 3 identified in our screen is reminiscent to two studies in 2009 that reported that PIAS1 and PIAS4 were responsible for the SUMO accrual at DNA damage foci[27,47]. The BRCA1 ubiquitin ligase activity was reduced in PIAS1/4-depleted cells. Upon RNF4 knockdown, we identified BRCA1 as well as its partner BARD, but they did not show up in the TULIP screen. Therefore we hypothesized that BRCA1/BARD1 could be regulated by RNF4 in an indirect manner with the SUMO E3 ligase PIAS1 as its primary target. BARD1 is a binding partner of BRCA1[48]. Mutations in BRCA1 increase the risk of female breast and ovarian cancer[49]. BRCA1 is a human tumor suppressor protein which helps to repair damaged DNA and acts as a ubiquitin ligase[50]. BRCA1-BARD1 plays an important role in protecting cells from DNA double strand breaks and maintains genome stability via homologous recombination (HR). Interestingly, depletion of RNF4 showed similar defects in homologous recombination, indicating that RNF4 may be functionally related to BRCA1-BARD1 in regulation of homologous recombination repair pathways. Moreover, in the TULIP RNF4 screen, PIAS4 was identified upon proteasome inhibitor-MG132 treatment in a SIM-dependent manner, but its value was below the statistical cut off.

When validating RNF4 targets, we did not observe similar changes in total protein levels. This could be explained by SUMOylation as a low stoichiometry PTM. Only a small portion of target proteins are SUMOylated. However, a small fraction of SUMOylation can have a profound effect. In the ubiquitin field, substoichiometric ubiquitination was also found in a study using an antibody

recognizing the diGly remnant of ubiquitin. It showed that the ubiquitinated portions of many proteins increased in the absence of changes in total protein levels[51].

Although many approaches have been used for the impartial identification of target proteins for ubiquitin E3 ligases, including yeast two hybrid assays, co-immunoprecipitation approaches, Ubait approaches[52], identifying E3 ligase substrates in cells is still very challenging. We have developed TULIP technology to meet this challenge. By using the TULIP technology, we can sufficiently distinguish non-covalent binding partners and covalently bound substrates and the TULIP technology could be widely used to identify substrates of other UBL E3s.

2.2 Proteomics Reveals Global Regulation of Protein SUMOylation by ATM and ATR Kinases during Replication Stress.

In this project, by employing unbiased proteomics approaches, almost 1400 regulated SUMOylated acceptor lysines and 3300 phosphorylation sites were found in proteins upon DNA damaged caused by MMC and HU. This work expands our understanding of the DNA damage response and repair system, especially related to DNA replication stresses. Interestingly, by integrating SUMOylation and phosphorylation datasets for further bioinformatics analysis, our work showed that proteins modified by both SUMO and phosphor, have many sites that are regulated in the response of DNA replication stress. This poses a challenge for the functional research on a given modification site.

It is already known that protein SUMOylation plays vital roles in the ATR pathway with the evidence of the SUMOylation of the regulatory partner of ATR, that is ATRIP at lysine 234 and lysine 289. Without SUMOylation, ATRIP failed to accumulate at the DNA damage sites and failed to support ATR activation[26]. Many other ATRIP partners such as RPA70, TopBP1 and the MRE11-RAD50-NBS1 complex display a lower binding ability to ATRIP without SUMOylation. Later on, the SUMO E3 ligase PIAS3 was shown to be indispensable for ATR phosphorylation, activation and ATR downstream checkpoint signalling[53]. Moreover, in budding yeast, RPA coated ssDNA enabled the damage site localization of the yeast SUMO ligase Siz2 and RPA is needed for Siz2 mediated Rad53/59 SUMOylation. In this study, our results showed that ATR and ATM kinases that are known to phosphorylate downstream proteins in responses to replication stress and replication fork breakage, through transmitting DNA damage signals with the ATR-Chk1 and ATM-Chk2 kinase cascade, respectively, also regulate SUMOylation signaling in the response of DNA replication stress derived DNA damage. More interestingly, we find that under conditions of induced replication fork breakage, inhibiting ATM kinase leads to decreased SUMOylation of many target proteins, suggesting that this kinase may play an important role in the maintaining of the physiological balance of SUMOylated proteins.

With the purpose of uncovering the crosstalk between protein phosphorylation and SUMOylation in DNA damage response signaling cascade caused by DNA replication stress, our proteomics analysis identified a large set of central replication stress and DDR responders like BRCA1, BARD1, Fanconi

Anemia proteins, DNA double strand response proteins including MDC1, NBN and CtIP and TOPBP1. These proteins were co-regulated by both phosphorylation and SUMOylation upon DNA damage. SUMOylation and phosphorylation of BRCA1 was proved to be important for its function, and SUMOylation of BRCA1 was reported to increases its ubiquitin ligase activity[27,47]. Further research will be required to analyse the co-dependency of these modifications for the functions of BRCA1 and other co-modified proteins. Especially, in this study, we found that SUMOylation of a main ATR activator, TOPBP1, in response to MMC treatment causing replication stress. TOPBP1 was identified as the most highly phosphorylated and SUMOylated protein in our dataset in response to eight hours of MMC treatment, and using pharmacological inhibitors of ATR and ATM signalling cascade. Our study showed that SUMOylation of TOPBP1 was heavily regulated by ATR during DNA replication stress and ATM upon DNA replication fork collapse. Future studies should point out whether TOPBP1 SUMOylation is required for ATR activation.

The work in this chapter represent an analysis of global protein phosphorylation and SUMOylation in response to replication stress for the first time and presents the largest database in proteomics to date of SUMOylation regulated target proteins under these conditions. With our findings, we propose that SUMOylation increases on specific targets in response to distinct DNA lesions, as shown in our research by unchanged TOPBP1 SUMOylation upon IR-induced DSBs, whereas TOPBP1 is hyper-SUMOylated after replication stress induced DNA breakage. Our data suggest that this SUMOylation response is regulated by two key kinases ATR and ATM. It is of great interest to further investigate the co-regulation of these two modifications, since the induction of DSBs induced by DNA replication stress is increasingly used in killing cancer cells[54-57]. On the basis of the vital role of SUMOs in the maintenance of genomic stability, further research on how SUMOylation perturbation affects global signaling networks will be of great interests. Also the methodology used in this study is applicable to study co-regulation of proteins by other PTMs.

3 Ubiquitin like modifiers (UFM1)

Recently, post-translational protein modification (PTM) with Ufm-1 has gained increasing attention, but its biological function as well as its substrates have remained largely unknown. Although Ufmylation has been connected to biological processes including ER homeostasis, vesicle trafficking, blood progenitor development and differentiation, (G-coupled protein receptor) GPCR maturation, transcriptional control, mitosis and more recently autophagy[58,59], the underlying mechanisms and biological consequences remain obscure.

Previously, uS3, uS10, uL16, subunits of the ribosome have been identified by mass spectrometry to be novel UFM-1 targets[60] and more recently, 494 UFMylated proteins also were identified by Pirone et al, of which 82 passed the cut off[64]. However, none of them have been independently validated and no biological functional studies have been conducted to elucidate the role of protein UFMylation[61].

In this project, we employed a site-specific strategy, which was developed by our lab to enrich SUMO target proteins as well as SUMO acceptor lysines, to identify novel Ufm-1 acceptor lysines. We have identified RPL26 (lysine 132, 134, 136, 142), RPL26L1 (lysine 132, 134, 136), TUBA1B (lysine 370), TUBA4A (lysine 355), MCM5 (lysine582), SLC26A7 (lysine120), SCYL2 (lysine 73, 76) and WDR63 (lysine564) as novel Ufm-1 targets. We further confirmed RPL26 as a Ufm-1 modified protein by western blotting.

Ribosomal proteins were reported to be post-translationally modified in many studies. The phosphorylation of rpS6 has attracted considerable attention in numerous labs since its discovery in 1974, RpS6 phosphorylation has no obvious role in protein synthesis or other cellular functions in yeast, however in MEFs, protein synthesis was found to be downregulated by rpS6 phosphorylation[62,63].

The early events in the life of newly synthesized proteins in the cellular environment are remarkably complex[64]. In order to address the biological function of RPL26 and identify its associated factors, we immunoprecipitated ribosomes with UFMylated or non-UFMylated RPL26 using a label free quantitative proteomics approach. We identified the signal recognition particle alpha (SRPR), to be significantly enriched in UFSP2 depleted cells, suggesting that this protein might be preferentially binding UFMylated RPL26. As expected, immunoblot analysis revealed that SRPR co-immunoprecipitated more efficiently with UFM-1 modified RPL26 than with the non-modified RPL26. These results suggest that in the presence of UFM-1 modification, RPL26 interacted with SRPR and seems to mediate direct contact of the exit tunnel of the ribosome with the SRPR during the last step of directing peptides to the Sec61 translocon. Our findings might help to understand how UFM1 regulates protein synthesis by coordinating its target. However, to fully understand the role of UFMylated RPL26 in regulating translation, more studies like ribosome profiling are needed to be performed.

Overall, the results of this thesis showed that SUMOs play a pivotal role in maintaining genome stability. SUMOs do not play their roles alone but in coordination with other PTMs such as ubiquitination and phosphorylation. By identifying substrates of UFM1 as well as their acceptor lysines, we also proved that the site-specific strategy developed in our lab can be applied in identifying substrates of other ubiquitin like proteins. This will help us to better understand the roles of ubiquitin like modifiers in eukaryotic life.

References

1 Uy, R. & Wold, F. Posttranslational covalent modification of proteins. *Science* **198**, 890-896 (1977).

2 Kuehn, M. R. Mouse Ubc9 knockout: many path(way)s to ruin. *Dev Cell* **9**, 727-728, doi:10.1016/j.devcel.2005.11.008 (2005).

3 Constanzo, J. D. *et al.* Pias1 is essential for erythroid and vascular development in the mouse embryo. *Dev Biol* **415**, 98-110, doi:10.1016/j.ydbio.2016.04.013 (2016).

4 Saracco, S. A., Miller, M. J., Kurepa, J. & Vierstra, R. D. Genetic analysis of SUMOylation in Arabidopsis: conjugation of SUMO1 and SUMO2 to nuclear proteins is essential. *Plant Physiol* **145**, 119-134, doi:10.1104/pp.107.102285 (2007).

5 Schimmel, J. *et al.* Uncovering SUMOylation dynamics during cell-cycle progression reveals FoxM1 as a key mitotic SUMO target protein. *Mol Cell* **53**, 1053-1066, doi:10.1016/j.molcel.2014.02.001 (2014).

6 Hendriks, I. A. *et al.* Uncovering global SUMOylation signaling networks in a site-specific manner. *Nat Struct Mol Biol* **21**, 927-936, doi:10.1038/nsmb.2890 (2014).

7 Hendriks, I. A., D'Souza, R. C., Chang, J. G., Mann, M. & Vertegaal, A. C. System-wide identification of wild-type SUMO-2 conjugation sites. *Nat Commun* **6**, 7289, doi:10.1038/ncomms8289 (2015).

8 Yang, W. & Paschen, W. SUMO proteomics to decipher the SUMO-modified proteome regulated by various diseases. *Proteomics* **15**, 1181-1191, doi:10.1002/pmic.201400298 (2015).

9 Filosa, G., Barabino, S. M. & Bachi, A. Proteomics strategies to identify SUMO targets and acceptor sites: a survey of RNA-binding proteins SUMOylation. *Neuromolecular Med* **15**, 661-676, doi:10.1007/s12017-013-8256-8 (2013).

10 Matafora, V., D'Amato, A., Mori, S., Blasi, F. & Bachi, A. Proteomics analysis of nucleolar SUMO-1 target proteins upon proteasome inhibition. *Mol Cell Proteomics* **8**, 2243-2255, doi:10.1074/mcp.M900079-MCP200 (2009).

11 Vertegaal, A. C. *et al.* A proteomic study of SUMO-2 target proteins. *J Biol Chem* **279**, 33791-33798, doi:10.1074/jbc.M404201200 (2004).

12 Tammsalu, T. *et al.* Proteome-wide identification of SUMO2 modification sites. *Sci Signal* **7**, rs2, doi:10.1126/scisignal.2005146 (2014).

13 Tammsalu, T. *et al.* Proteome-wide identification of SUMO modification sites by mass spectrometry. *Nat Protoc* **10**, 1374-1388, doi:10.1038/nprot.2015.095 (2015).

14 Bursomanno, S. *et al.* Proteome-wide analysis of SUMO2 targets in response to pathological DNA replication stress in human cells. *DNA Repair (Amst)* **25**, 84-96, doi:10.1016/j.dnarep.2014.10.011 (2015).

15 Galisson, F. *et al.* A novel proteomics approach to identify SUMOylated proteins and their modification sites in human cells. *Mol Cell Proteomics* **10**, M110 004796, doi:10.1074/mcp.M110.004796 (2011).

16 Matic, I. *et al.* In vivo identification of human small ubiquitin-like modifier polymerization sites by high accuracy mass spectrometry and an in vitro to in vivo strategy. *Mol Cell Proteomics* **7**, 132-144, doi:10.1074/mcp.M700173-MCP200 (2008).

17 Hendriks, I. A. & Vertegaal, A. C. A high-yield double-purification proteomics strategy for the identification of SUMO sites. *Nat Protoc* **11**, 1630-1649, doi:10.1038/nprot.2016.082 (2016).

18 Matic, I. & Hay, R. T. Detection and quantitation of SUMO chains by mass spectrometry. *Methods Mol Biol* **832**, 239-247, doi:10.1007/978-1-61779-474-2_17 (2012).

19 Hendriks, I. A. & Vertegaal, A. C. A comprehensive compilation of SUMO proteomics. *Nat Rev Mol Cell Biol* **17**, 581-595, doi:10.1038/nrm.2016.81 (2016).

20 Baba, D. *et al.* Crystal structure of thymine DNA glycosylase conjugated to SUMO-1. *Nature* **435**, 979-982, doi:10.1038/nature03634 (2005).

21 Jackson, S. P. & Durocher, D. Regulation of DNA damage responses by ubiquitin and SUMO. *Mol Cell* **49**, 795-807, doi:10.1016/j.molcel.2013.01.017 (2013).

22 Petermann, E., Orta, M. L., Issaeva, N., Schultz, N. & Helleday, T. Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. *Mol Cell* **37**, 492-502, doi:10.1016/j.molcel.2010.01.021 (2010).

23 Matic, I. *et al.* Site-specific identification of SUMO-2 targets in cells reveals an inverted SUMOylation motif and a hydrophobic cluster SUMOylation motif. *Mol Cell* **39**, 641-652, doi:10.1016/j.molcel.2010.07.026 (2010).

24 Luo, K., Zhang, H., Wang, L., Yuan, J. & Lou, Z. Sumoylation of MDC1 is important for proper DNA damage response. *EMBO J* **31**, 3008-3019, doi:10.1038/emboj.2012.158 (2012).

25 Ouyang, K. J., Yagle, M. K., Matunis, M. J. & Ellis, N. A. BLM SUMOylation regulates ssDNA accumulation at stalled replication forks. *Front Genet* **4**, 167, doi:10.3389/fgene.2013.00167 (2013).

26 Wu, C. S. *et al.* SUMOylation of ATRIP potentiates DNA damage signaling by boosting multiple protein interactions in the ATR pathway. *Genes Dev* **28**, 1472-1484, doi:10.1101/gad.238535.114 (2014).

27 Morris, J. R. *et al.* The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. *Nature* **462**, 886-890, doi:10.1038/nature08593 (2009).

28 Bonner, J. N. *et al.* Smc5/6 Mediated Sumoylation of the Sgs1-Top3-Rmi1 Complex Promotes Removal of Recombination Intermediates. *Cell Rep* **16**, 368-378, doi:10.1016/j.celrep.2016.06.015 (2016).

29 Pfander, B., Moldovan, G. L., Sacher, M., Hoege, C. & Jentsch, S. SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. *Nature* **436**, 428-433, doi:10.1038/nature03665 (2005).

30 Burkovics, P. *et al.* Srs2 mediates PCNA-SUMO-dependent inhibition of DNA repair synthesis. *EMBO J* **32**, 742-755, doi:10.1038/emboj.2013.9 (2013).

31 Armstrong, A. A., Mohideen, F. & Lima, C. D. Recognition of SUMO-modified PCNA requires tandem receptor motifs in Srs2. *Nature* **483**, 59-63, doi:10.1038/nature10883 (2012).

32 Papouli, E. *et al.* Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p. *Mol Cell* **19**, 123-133, doi:10.1016/j.molcel.2005.06.001 (2005).

33 Gali, H. *et al.* Role of SUMO modification of human PCNA at stalled replication fork. *Nucleic Acids Res* **40**, 6049-6059, doi:10.1093/nar/gks256 (2012).

34 Moldovan, G. L. *et al.* Inhibition of homologous recombination by the PCNA-interacting protein PARI. *Mol Cell* **45**, 75-86, doi:10.1016/j.molcel.2011.11.010 (2012).

35 Fujita, Y. *et al.* Priming of centromere for CENP-A recruitment by human hMis18alpha, hMis18beta, and M18BP1. *Dev Cell* **12**, 17-30, doi:10.1016/j.devcel.2006.11.002 (2007).

36 Brown, P. W., Hwang, K., Schlegel, P. N. & Morris, P. L. Small ubiquitin-related modifier (SUMO)-1, SUMO-2/3 and SUMOylation are involved with centromeric heterochromatin of chromosomes 9 and 1 and proteins of the synaptonemal complex during meiosis in men. *Hum Reprod* **23**, 2850-2857, doi:10.1093/humrep/den300 (2008).

37 Zhang, X. D. *et al.* SUMO-2/3 modification and binding regulate the association of CENP-E with kinetochores and progression through mitosis. *Mol Cell* **29**, 729-741, doi:10.1016/j.molcel.2008.01.013 (2008).

38 Ritterhoff, T. *et al.* The RanBP2/RanGAP1*SUMO1/Ubc9 SUMO E3 ligase is a disassembly machine for Crm1-dependent nuclear export complexes. *Nat Commun* **7**, 11482, doi:10.1038/ncomms11482 (2016).

39 Gocke, C. B., Yu, H. & Kang, J. Systematic identification and analysis of mammalian small ubiquitin-like modifier substrates. *J Biol Chem* **280**, 5004-5012, doi:10.1074/jbc.M411718200 (2005).

40 Schmidt, D. & Muller, S. Members of the PIAS family act as SUMO ligases for c-Jun and p53 and repress p53 activity. *Proc Natl Acad Sci U S A* **99**, 2872-2877, doi:10.1073/pnas.052559499 (2002).

41 Westerbeck, J. W. *et al.* A SUMO-targeted ubiquitin ligase is involved in the degradation of the nuclear pool of the SUMO E3 ligase Siz1. *Mol Biol Cell* **25**, 1-16, doi:10.1091/mbc.E13-05-0291 (2014).

42 Nie, M. & Boddy, M. N. Pli1(PIAS1) SUMO ligase protected by the nuclear pore-associated SUMO protease Ulp1SENP1/2. *J Biol Chem* **290**, 22678-22685, doi:10.1074/jbc.M115.673038 (2015).

43 Knipscheer, P. *et al.* Ubc9 sumoylation regulates SUMO target discrimination. *Mol Cell* **31**, 371-382, doi:10.1016/j.molcel.2008.05.022 (2008).

44 Kosoy, A., Calonge, T. M., Outwin, E. A. & O'Connell, M. J. Fission yeast Rnf4 homologs are required for DNA repair. *J Biol Chem* **282**, 20388-20394, doi:10.1074/jbc.M702652200 (2007).

45 Hu, X. V. *et al.* Identification of RING finger protein 4 (RNF4) as a modulator of DNA demethylation through a functional genomics screen. *Proc Natl Acad Sci U S A* **107**, 15087-15092, doi:10.1073/pnas.1009025107 (2010).

46 Lallemand-Breitenbach, V. *et al.* Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. *Nat Cell Biol* **10**, 547-555, doi:10.1038/ncb1717 (2008).

47 Galanty, Y. *et al.* Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. *Nature* **462**, 935-939, doi:10.1038/nature08657 (2009).

48 Wu, L. C. *et al.* Identification of a RING protein that can interact in vivo with the BRCA1 gene product. *Nat Genet* **14**, 430-440, doi:10.1038/ng1296-430 (1996).
49 Miki, Y. *et al.* A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* **266**, 66-71 (1994).
50 Hashizume, R. *et al.* The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *J Biol Chem* **276**, 14537-14540, doi:10.1074/jbc.C000881200 (2001).
51 Kim, W. *et al.* Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell* **44**, 325-340, doi:10.1016/j.molcel.2011.08.025 (2011).
52 O'Connor, H. F. *et al.* Ubiquitin-Activated Interaction Traps (UBAITs) identify E3 ligase binding partners. *EMBO Rep* **16**, 1699-1712, doi:10.15252/embr.201540620 (2015).
53 Wu, C. S. & Zou, L. The SUMO (Small Ubiquitin-like Modifier) Ligase PIAS3 Primes ATR for Checkpoint Activation. *J Biol Chem* **291**, 279-290, doi:10.1074/jbc.M115.691170 (2016).
54 Benada, J. & Macurek, L. Targeting the Checkpoint to Kill Cancer Cells. *Biomolecules* **5**, 1912-1937, doi:10.3390/biom5031912 (2015).
55 Rundle, S., Bradbury, A., Drew, Y. & Curtin, N. J. Targeting the ATR-CHK1 Axis in Cancer Therapy. *Cancers (Basel)* **9**, doi:10.3390/cancers9050041 (2017).
56 Manic, G., Obrist, F., Sistigu, A. & Vitale, I. Trial Watch: Targeting ATM-CHK2 and ATR-CHK1 pathways for anticancer therapy. *Mol Cell Oncol* **2**, e1012976, doi:10.1080/23723556.2015.1012976 (2015).
57 Zhang, Y. *et al.* Targeting radioresistant breast cancer cells by single agent CHK1 inhibitor via enhancing replication stress. *Oncotarget* **7**, 34688-34702, doi:10.18632/oncotarget.9156 (2016).
58 Joassard, O. R. *et al.* Regulation of Akt-mTOR, ubiquitin-proteasome and autophagy-lysosome pathways in response to formoterol administration in rat skeletal muscle. *Int J Biochem Cell Biol* **45**, 2444-2455, doi:10.1016/j.biocel.2013.07.019 (2013).
59 Zhang, Y., Zhang, M., Wu, J., Lei, G. & Li, H. Transcriptional regulation of the Ufm1 conjugation system in response to disturbance of the endoplasmic reticulum homeostasis and inhibition of vesicle trafficking. *PLoS One* **7**, e48587, doi:10.1371/journal.pone.0048587 (2012).
60 Simsek, D. *et al.* The Mammalian Ribo-interactome Reveals Ribosome Functional Diversity and Heterogeneity. *Cell* **169**, 1051-1065 e1018, doi:10.1016/j.cell.2017.05.022 (2017).
61 Pirone. L. et al. A comprehensive platform for the analysis of ubiquitin-like protein modifications using in vivo biotinylation. Sci Rep 7 , 40756, doi: 10.1038/srep40756 (2017).
62 Grundy, M. *et al.* Early changes in rpS6 phosphorylation and BH3 profiling predict response to chemotherapy in AML cells. *PLoS One* **13**, e0196805, doi:10.1371/journal.pone.0196805 (2018).
63 Roux, P. P. *et al.* RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. *J Biol Chem* **282**, 14056-14064, doi:10.1074/jbc.M700906200 (2007).
64 Kramer, G., Boehringer, D., Ban, N. & Bukau, B. The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. *Nat Struct Mol Biol* **16**, 589-597, doi:10.1038/nsmb.1614 (2009).