Regulation of SUMO Modification

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Regulatie van SUMO modificatie

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Promotor:

Prof.dr. T.K. Sixma

Overige leden:

Prof.dr. R.H. Medema

Prof.dr. C.P. Verrijzer

Prof.dr. J.H. Hoeijmakers

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Chapter 1

Introduction

Introduction

Post-translational modification of proteins, such as phosphorylation, methylation, acetylation or ubiquitination, plays a critical role in many cellular processes due to its ability to rapidly and reversibly change the behavior of the modified protein. Ubiquitination is unusual in that the modifier is not a small functional group but a 76 amino acid polypeptide. Ubiquitin modification of target proteins has many functions, it can target for degradation by chain formation linked through lysine 48, but it also plays a role in DNA repair, signal transduction, endocytosis and other processes by modifying targets with a single ubiquitin or differentially linked chains (reviewed in [1-3]).

Besides ubiquitin there are a number of ubiquitin like proteins (UBLs) that resemble ubiquitin both in fold (Figure 1b) and modification mechanism (Figure 2) but have distinct functional roles (reviewed in [4-7]). SUMO is one of these UBLs and, although it only shares 18% sequence identity with ubiquitin, the three dimensional structure is conserved between the two[8] (Figure 1). The biological functions of SUMO modification are highly diverse as it has been shown to be involved in nuclear transport, DNA repair, regulation of transcription, signal transduction and many other processes[9-15].

There are four SUMO isoforms in mammalian cells that share between 40 and 97% sequence identity (Figure 1a) and only partially overlap in target specificity. SUMO 2 and 3 are almost identical (97%) and differ in cellular localization and distribution from SUMO1. Whereas most SUMO1 is conjugated to targets there is a large pool of free SUMO2 and SUMO3 in cells that gets conjugated upon various stress signals[16-18]. Both SUMO1 and SUMO2/3 form chains on targets in vitro but so far only SUMO 2/3 chains have been found in vivo[19,20].

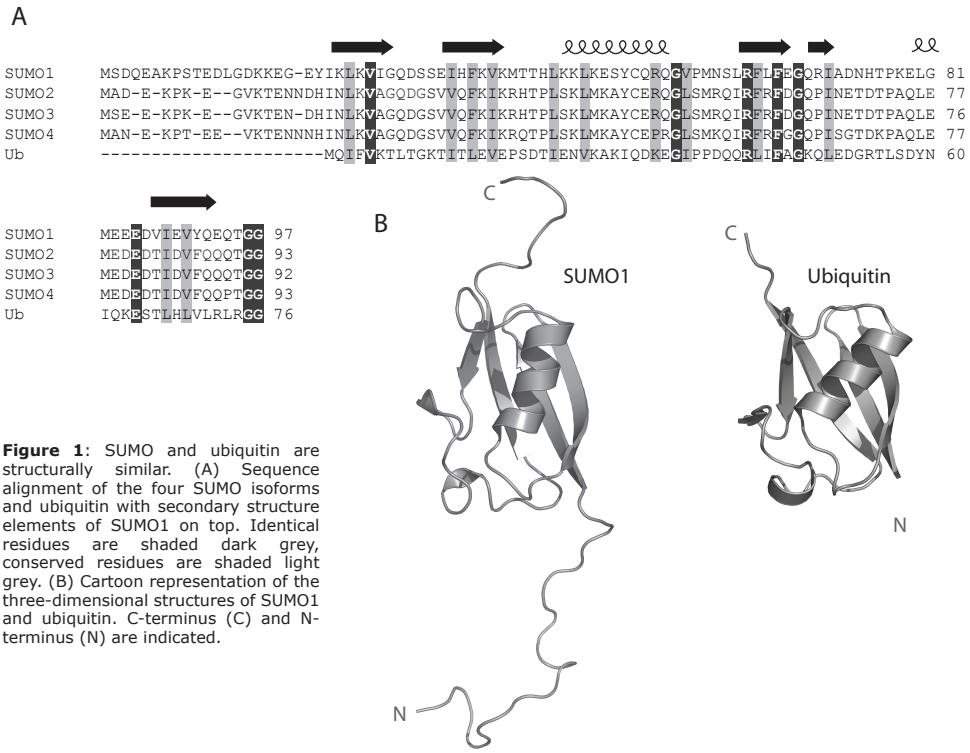

Figure 1: SUMO and ubiquitin are structurally similar. (A) Sequence alignment of the four SUMO isoforms and ubiquitin with secondary structure elements of SUMO1 on top. Identical residues are shaded dark grey, conserved residues are shaded light grey. (B) Cartoon representation of the three-dimensional structures of SUMO1 and ubiquitin. C-terminus (C) and N-terminus (N) are indicated.

SUMO and disease

Since the SUMO pathway is involved in many cellular processes it is not surprising that it has been implicated in diseases as well. A role for SUMO in cancer, neurodegenerative diseases and viral infection is suggested although the exact functional mechanisms are not known.

Cancer

A growing number of oncogenes and tumor suppressor genes, including PML, MDM-2, c-Myb, c-Jun, p53 and Rb are targets for sumoylation[21-27]. Moreover, SUMO plays a role in many processes involved in cancer like DNA replication and repair, NFκB and MAP kinase signaling, and cell cycle control. One would expect that the regulatory role of SUMO modification of these proteins can influence the role of these proteins in cancer. The fact that the SUMO machinery may be involved in cancer is supported by the fact that both the SUMO E3 ligase Pias3 and the SUMO-conjugating enzyme Ubc9 are upregulated in a number of human malignancies[28-30]. Recently, a link between SUMO and cancer metastasis was found when it was shown that the expression of the metastasis repressor gene KAI1 was affected by sumoylation. The β-catenin-reptin chromatin remodeling complex is responsible for this downregulation of the KAI1 promoter and this is mediated by reptin sumoylation leading to enhanced HDAC1 interaction[31,32].

Neurodegenerative diseases

Several studies have implicated sumoylation in the pathology of neurodegenerative diseases like Parkinson's, Alzheimer and Huntington's. These disorders all involve the accumulation or aggregation of abnormal proteins and in many cases show a malfunction in proteasomal degradation. Huntington's disease is characterized by the accumulation of a pathogenic mutated form of the Huntingtin protein (Htt) and appears to have increased toxicity when sumoylated[33]. Tau and α-synuclein are other examples of proteins that aggregate in neurodegenerative diseases and both are SUMO modified in vitro suggesting a role for SUMO in regulating these proteins[34]. The Parkin gene encodes for a ubiquitin E3 ligase and is mutated in a juvenile form of Parkinson's. There are two aspects that link this protein to the SUMO pathway, the first is that Parkin targets the SUMO E3 RanBP2 for proteasomal degradation, and the second is that Parkin interacts with SUMO and this affects its intracellular localization and ligase activity. The last link between SUMO an neurodegenerative diseases came from a study on amyloid β (Aβ), the peptide that causes neurotoxicity in Alzheimer's disease. Here it was shown that overexpression of SUMO3 dramatically reduces Aβ production while overexpression of a SUMO3 mutant that is not able to form chains increased Aβ production. Although this study does not show that Aβ or its precursor amyloid precursor protein (APP) is modified with SUMO3, it does indicate a role for SUMO3 chain formation in Aβ production in Alzheimer's disease[35].

Viral infection

Several viral proteins can either be covalently modified by SUMO themselves, or, can alter the sumoylation status of host proteins[36]. The first is true for the bovine papillomavirus E1 protein. Upon SUMO modification this protein is transported to the nucleus where it plays a role in viral replication. Mutation of the sumoylation site prevents nuclear targeting and causes a loss of replication capacity of the virus [37]. Altered host cell sumoylation is caused by the Gam1 protein from an avian adenovirus, this protein inactivates the human SUMO E1 activating enzyme causing an overall inhibition of protein sumoylation[38]. Also, it was shown that both the herpes simplex virus (HSV) ICP0 protein and the cytomegalovirus (CMV) IE1 protein can abrogate the SUMO modification of PML and Sp100 causing a disruption of nuclear body formation [39].

The mechanism of ubiquitin(-like) modification

In sumoylation as well as ubiquitination, the C-terminus of the modifier is ligated to a lysine on the target protein using a highly regulated three step enzymatic cascade (Figure 2). The enzymes involved are in many cases related but not identical between ubiquitination and ubiquitin-like modification. The first, ATP-dependent step, is catalyzed by an E1 or activating enzyme, and involves the formation of an intermediate adenylate followed by the formation of a thioester bond between the C-terminus of the modifier and the catalytic cysteine of the E1 enzyme. Then, the modifier is transferred to an E2 or conjugating enzyme again forming a thioester bond with an active cysteine of the enzyme. In the last step, an E3 enzyme promotes the ligation of the modifier to an ε-amino group of a substrate lysine

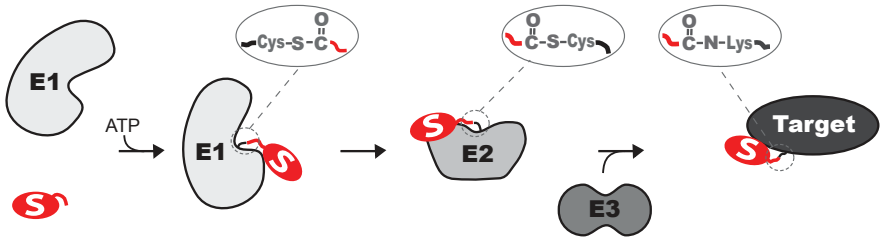

Figure 2: Schematic view of the multistep process of SUMO modification showing the transfer of SUMO (S) from the E1 to the E2, and with the help of an E3 to the target. The chemistry of the created bonds is shown in close-ups.

residue through an isopeptide bond. The removal of the modifier from a substrate is a single step reaction catalyzed by specialized proteases called deconjugating enzymes.

In mammalian cells there is only one E1 enzyme for ubiquitin conjugation, Uba1, and also a single E1 for SUMO, the heterodimer Aos1-Uba2[40,41]. In contrast to ubiquitination where there are roughly 30 E2 enzymes identified[42], sumoylation uses a single E2 enzyme, Ubc9, which is essential in both yeast and mammals[43-46]. Ubiquitin modification requires an E3 enzyme belonging to one of three different classes, the HECT, RING or U-box proteins. SUMO modification can take place without the help of an E3 since Ubc9 interacts directly with many targets (see below). However, there are factors that enhance SUMO modification, three different types of these SUMO E3 ligases have been identified: the Siz/Pias SP-RING family [47-51], the nucleoporin RanBP2/Nup358[52,53] and Pc2 (polycomb group protein 2)[54,55]. SP-RING SUMO ligases resemble the RING ubiquitin ligases and are thought to function similarly by bringing the E2 and target together. The RanBP2 E3 ligase has no similarity to any other E3 ligase, it does not directly interact with the target but functions by positioning the Ubc9-SUMO thioester for efficient SUMO transfer to the target[53,56]. The E3 function of Pc2 involves two domains of the protein, one that directly recruits both Ubc9 and the target, and one that comprises the E3 ligase activity[55].
Deconjugating enzymes perform two functions, they process the nascent modifier to its mature form, and they cleave the isopeptide bond between modifier and target. A large number of deubiquitinating enzymes (DUBs) have been identified belonging to two different classes, the ubiquitin C-terminal hydrolases (UCH) or the ubiquitin-specific proteases (USP)[57,58]. In mammalian cells the presence of 6 SUMO deconjugating enzymes or SUMO isopeptidases has been predicted, of these, 4 have been shown to possess SUMO isopeptidase activity, Senp1, 2, 3 and 6. SUMO deconjugating enzymes are highly target specific due to their catalytic properties as well as their variable physical location[59,60].

SUMO consensus sequence

The SUMO E2 enzyme, Ubc9, differs from ubiquitin E2 conjugating enzymes in that it directly interacts with a specific site on many targets. This site consists of a hydrophobic residue, followed by the SUMO acceptor lysine, any amino acid, and a glutamic or aspartic acid, creating the traditional consensus site for SUMO modification: ΨKxE/D[61]. The consensus site is usually found on an unstructured region of a protein and the details of the interaction are shown in the crystal structure of RanGAP1 in complex with Ubc9[62]. The presence of a consensus site is not a strict requirement for sumoylation of a target since several proteins have been found to be modified on non-consensus sites[10,63]. Possibly, there are additional consensus sequences that are not identified yet.

Ubiquitin and SUMO binding motifs

Although many SUMO targets have been identified, the direct consequence of both ubiquitination and sumoylation is in many cases still not precisely known. One popular view is that ubiquitination causes a signal by changing the interaction surface of a protein. This newly created surface can in its turn be recognized by a downstream receptor protein carrying a ubiquitin binding domain (UBD) probably combined with a target-specific binding domain. Many UBDs have been identified recently, like the UIM, UBA, CUE, GAT and NZF domains, and each of them seems to have a preference for either mono-ubiquitin or poly-ubiquitin chains linked through specific lysines[64,65]. One obvious example of receptor proteins carrying a UBD are

the proteasomal receptors that are responsible for targeting of poly-ubiquitinated proteins to the destruction machinery[66-68].
In analogy with ubiquitin, also for SUMO an interaction motif (SIM) has been found. Several groups have identified a similar sequence [69-72] and the consensus appears to be a hydrophobic stretch (V/I)(V/I)X(V/I/L) flanked by acidic residues or possible phosphorylation sites. Structural studies of SUMO bound to a SIM have revealed that the hydrophobic residues form a beta strand that is added to SUMOs beta-sheet[56,72]. Interestingly, this binding motif was identified in SUMO targets but also in enzymes of the SUMO cascade like the SUMO E1 and several SUMO E3s [56,70,71,73].

Structural information in the SUMO pathway
Structural and biochemical studies in the SUMO pathway have revealed many aspects of the mechanism of SUMO modification. The structure of the SUMO E1 heterodimer Aos1-Uba2 in complex with SUMO and ATP shows that the enzyme needs to undergo multiple conformational changes to facilitate the three phases of its mission; adenylating, thioester formation and thioester transfer to the E2[74]. The ubiquitin-like domain (Ubl) of the E1 plays an important role in the recruitment of the E2 as is also shown for the E1 enzyme of another ubiquitin like molecule, Nedd8[75]. The crystal structure of the SUMO E2, Ubc9 is very similar to ubiquitin E2s showing the characteristic 150 amino acid E2 α/β fold[76,77]. The specificity of Ubc9 for SUMO instead of ubiquitin seems to be predominantly caused by the discrimination in E1 interaction due to a different surface charge[76]. Another important piece of structural information came with the Ubc9-RanGAP1 structure showing how Ubc9 recognizes a target by interacting with the ΨKxE/D consensus site sequence located on a loop-region in RanGAP1[62]. This structure was the basis for the structure determination of a larger complex comprising SUMO modified RanGAP1, the catalytic fragment of the RanBP2 E3 ligase and Ubc9[56]. This structure, together with the biochemical work that is presented in chapter 2 of this thesis, has shown how the SUMO E3 ligase RanBP2 most probably works, by positioning the SUMO E2-thioester in an optimal orientation to enhance conjugation[53,56]. Even though this structure has provided many pieces of valuable information, it does not show a true E2-E3-SUMO-target intermediate complex since RanGAP1 is already SUMO modified and it is not a target for the E3 RanBP2. A structure of a bonafide RanBP2 substrate in complex with the Ubc9~SUMO thioester intermediate and RanBP2 would be needed to fully confirm this mechanism.
The structures of several SUMO modified targets have shown that there is a large variation in the degree of interaction between the target and the modifier. NMR studies on both SUMO modified RanGAP1 and Ets-1 have shown that, besides the covalent bond that links the modifier to the substrate, there is virtually no interaction between the two[78,79]. However, in case of SUMO-modified TDG the target and modifier interact extensively and this is, to a lesser extent, also true for both E2-25K and Ubc9 ([80] and chapter 3 and 4 of this thesis).

Consequence of SUMO modification
While hundreds of SUMO targets have been identified so far, only for a limited number the biochemical consequence of modification has been studied in detail. This is the case for mono-sumoylation, for poly-sumoylation even less is known about the functional consequence. In chapter 5 we describe details on the regulation of SUMO chain formation.
While ubiquitination mostly functions as an adapter creating a new binding interface, sumoylation seems to have more diverse consequences. These can be roughly separated in four categories: SUMO can affect enzymatic activity, it can change protein localization, it can modulate the interaction with other proteins, or it can stabilize proteins. Only few targets for each category have been studied in detail and these are discussed here.

Affecting enzymatic activity or protein function
Thymine DNA glycosylase (TDG) is an enzyme that recognizes irregular base pairs between a guanine and a uracil or thymine (G•U or G•T), excises the mismatched base and creates an abasic site that can then be processed by downstream enzymes. TDG has been shown to be a target for SUMO modification in vivo and also interacts with SUMO non-covalently through a sequence resembling a SIM[81]. SUMO modification is required for the release of TDG from the DNA after excision of the mismatched base and thus enhances the turnover rate of the enzyme[81,82]. The crystal structure of SUMO modified TDG confirmed that sumoylation causes a conformational change in the protein that interferes with DNA binding[80]. For the release of DNA both non-covalent and covalent interaction

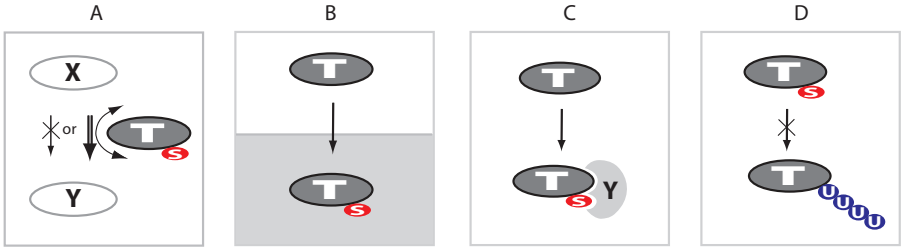

Figure 2: Sumoylation of a target protein (T) can have several consequences. (A) It can affect the activity of the protein, (B) it can change the location of a protein, (C) it can modulate the interaction with other proteins, or (D) it can prevent ubiquitination of a protein.

with SUMO are necessary. Here, the function of sumoylation is to release the abasic site in order for base excision repair to proceed.

E2-25K is a ubiquitin conjugating enzyme that can be regulated by SUMO modification. As is described in chapter 3, SUMO does not influence the active site of E2-25K directly but it prevents interaction with the ubiquitin activating enzyme thereby inhibiting thioester formation of E2-25K[63]. Another, very abundantly, modified protein is the plasma membrane potassium leak channel K2P1[83]. Despite expression of this protein in heart, brain and kidney, it had never been shown to be active as a K+ channel, until it was found that it is fully sumoylated. Mutation of the target lysine, or overexpression of a SUMO-deconjugating enzyme, turned this protein into a functional pH-sensitive potassium channel. How SUMO alters the physical properties of this protein is not clear yet.

Localization

The Ran-GTPase activating protein RanGAP1 is the most abundantly SUMO modified protein in mammalian cells and, upon sumoylation, binds to RanBP2, a protein that forms the cytoplasmic filaments of nuclear pore complexes (NPC) and has SUMO E3 ligase activity[12,84,85]. The fact that the SUMO isopeptidase Senp2 localizes to the nuclear site of the NPC, suggests a role for sumoylation in nuclear–cytoplasmic transport which is further supported by the fact that several SUMO targets depend on a nuclear localization signal to get sumoylated [12,86]. During mitosis, when nuclear pore complexes disassemble, sumoylated RanGAP1 remains associated with RanBP2 and enriches at kinetochores and mitotic spindles where it is responsible for stable microtubule-kinetochore association[87,88].

The PML protein was first identified as part of the oncogenic PML-RARalpha chimera in acute promyelocytic leukemia (APL). PML is the main component of nuclear structures called PML nuclear bodies (NBs), which seem to function in regulation of transcription, genome stability, response to viral infection, apoptosis, and tumor suppression. PML itself is a target for sumoylation and its SUMO modification is a prerequisite for its localization to the NBs where it subsequently recruits other sumoylated and non-sumoylated proteins like Sp100, Daxx, p53, BLM and others[25,89,90]. Recently it was shown that PML contains a SUMO interaction motif and a model was suggested where sumoylated PML creates a network of noncovalent and covalent interactions between SUMOs and SIMs on PML but also on other proteins in the NBs[91,92]. PML seems to play an active role in the formation of this network and its recently confirmed SUMO E3 ligase activity may play a crucial role in this process[93].

Modulating binding interfaces

Proliferating cell nuclear antigen (PCNA) is an essential processivity factor that loads DNA polymerases onto the DNA and associates with many other proteins involved in replication-associated events. PCNA can be modified with ubiquitin and, at least in yeast, also with SUMO, and this leads to the recruitment of specific factors. When the DNA is damaged, PCNA ubiquitination facilitates the bypass of replication-blocking lesions either in a mutagenic way by promoting the association of error-prone polymerases, or in an error-free way by using the undamaged sister chromatid[10,14]. PCNA can be modified with SUMO independent of DNA damage and this recruits the Srs2 helicase in order to inhibit recombination. Here, SUMO modification causes a direct interaction between SUMO modified PCNA and Srs2 to prevent unwanted recombination during replication [94,95].

The SUMO-conjugating enzyme, Ubc9, can also be sumoylated and functions as a modulator of target selection as is described in chapter 4 of this thesis. While modification of some targets is not affected by Ubc9 sumoylation, others are strongly impaired or enhanced in modification. The enhancement of Sp100 sumoylation by SUMO-modified Ubc9 is due to enhanced interaction mediated by a SUMO interaction motif present in Sp100. Therefore, SUMO modification of the target Ubc9 likely serves to recruit a subclass of SUMO targets.

Several transcription factors like p73α, p53, LEF1, c-Jun and c-Myb, p300, Elk-1 are targets for SUMO modification[9,11,21,50,70]. In most cases sumoylation has a repressive effect on transcription but there are also cases where the SUMO can stimulate gene expression[9,96-98]. In many of these cases the functional role of sumoylation is not known. However, there are a few examples where SUMO modification promotes the interaction with co-repressors like HDACs. Sumoylation of the transcription factor Elk-1 stimulates the association with HDAC2 thereby repressing expression from an Elk-1 regulated promoter[99]. Also, SUMO modification of the repression domain of the co-activator p300 was shown to promote binding to HDAC6[9] and mutation of the SUMO-site resulted in an increased p300-mediated transcriptional activity. However, the role of HDACs in SUMO-mediated repression seems to be more complicated since HDACs can both regulate sumoylation of targets and are also SUMO targets themselves[100,101]. Moreover, it is likely that there are also other proteins that contribute to SUMO-dependent inhibition of transcription and regulation.

Stabilization

Protein stabilization was one of the first functions found for SUMO modification. It was based on the fact that in some cases SUMO and ubiquitin compete for the same lysine and thus, SUMO can prevent the proteasomal degradation of targets. This mechanism has be proposed for several targets but in most of these cases it is not as simple as pure competition[102]. The best known example is IκBα, the inhibitory protein that retains the transcriptional activator NF-κB in the cytoplasm. Even though in this case ubiquitination and sumoylation take place on the same residue, several other regulatory events are needed for either modification and only a very small fraction of IκBα seems to be modified in the cell. Another example is MDM2, the ubiquitin E3 ligase for p53. This protein can be both ubiquitinated or sumoylated but it is not clear whether this takes place on the same residue. However, sumoylation of MDM2 seems to inhibit its self-ubiquitination and prevents degradation[103].

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Chapter 2

The RanBP2 SUMO E3 ligase is neither HECT- nor RING-type

Andrea Pichler[1], Puck Knipscheer[2], Hisato Saitoh[3], Titia K. Sixma[2] and Frauke Melchior[1]*

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The RanBP2 SUMO E3 ligase is neither HECT- nor RING-type

Andrea Pichler[1], Puck Knipscheer[2], Hisato Saitoh[3], Titia K. Sixma[2] and Frauke Melchior[1]*

[1] Max-Planck Institute for Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany
[2] Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands
[3] Institute for Molecular Embryology and Genetics, Kumamoto University, Japan
* Corresponding author: e-mail: melchior@biochem.mpg.de

Post-translational modification with the ubiquitin-related protein SUMO1 requires the E1 enzyme Aos1-Uba2 and the E2 enzyme Ubc9. Distinct E3 ligases strongly enhance modification of specific targets. The SUMO E3 ligase RanBP2 (also known as Nup358) has no obvious similarity to RING- or HECT-type enzymes. Here we show that RanBP2's 30-kD catalytic fragment is a largely unstructured protein. Despite two distinct but partially overlapping 79-residue catalytic domains, one of which is sufficient for maximal activity, RanBP2 binds to Ubc9 in a 1:1 stoichiometry. Identification of nine RanBP2 and three Ubc9 side chains that are important for RanBP2-dependent SUMOylation indicates largely hydrophobic interactions. These properties distinguish RanBP2 from all other known E3 ligases, and we speculate that RanBP2 exerts its catalytic effect by altering Ubc9's properties rather than by mediating target interactions.

Introduction

Post-translational modification with ubiquitin-related proteins of the SUMO family is an important mechanism for regulating protein function. The consequences of SUMOylation range from changes in protein interactions or activity to changes in subcellular localization or stability[1–5]. The basic principle of conjugation resembles ubiquitination[6–9]. Activation of the SUMO C terminus requires ATP and leads to thioester bond formation with the E1 activating enzyme heterodimer Aos1-Uba2. In consecutive steps SUMO is transferred to the single E2-conjugating enzyme Ubc9, and then forms an isopeptide bond with the ε-amino group of a lysine in the acceptor protein.

Ubiquitination always depends on a third class of enzymes, the E3 ligases. These proteins recognize both a specific E2 enzyme and the acceptor protein. Two different classes of ubiquitin E3 ligases can be distinguished: HECT E3s such as E6-AP form an ubiquitin thioester intermediate before transferring ubiquitin to the target, while RING-finger E3s and the structurally related U-box proteins serve as adaptors between the E2-Ubiquitin thioester and the target[7,10–13]. RING-finger E3s exist in two flavors, single-subunit proteins such as Mdm2, and multi-subunit complexes like APC (Anaphase promoting complex) or SCF (Skp cullin F-box protein)[12,14,15].

In principle, modification with SUMO can be carried out just by Aos1-Uba2 and Ubc9. This is because target recognition is accomplished (at least in part) by Ubc9, which can bind to the SUMO acceptor site (ΨKXE/D; Ψ is a bulky hydrophobic residue and X is any residue) present in most known SUMO targets[1,2]. However, for most targets, transfer efficiency in the presence of E1 and E2 enzymes is very poor, and can be strongly stimulated through SUMO E3 ligases. The Siz/PIAS family of SUMO E3 ligases shares a conserved domain with similarity to ubiquitin RING-finger proteins. This 'SP-RING' (Siz/PIAS-RING) is required for their function as SUMO E3 ligases (reviewed in ref. 16). The polycomb protein Pc2 has recently been described as an E3 ligase for the transcriptional corepressors CtBP1 and CtBP2[17], but its mode of action is currently unclear. Finally, we have shown previously that the nuclear pore complex protein RanBP2 (Nup358) catalytically enhances SUMOylation of the nuclear body component Sp100 and the histone deacetylase HDAC4, but not of the tumor suppressor p53 *in vitro*[18,19]. RanBP2 is a multidomain protein with interaction sites for proteins including nuclear transport receptors,

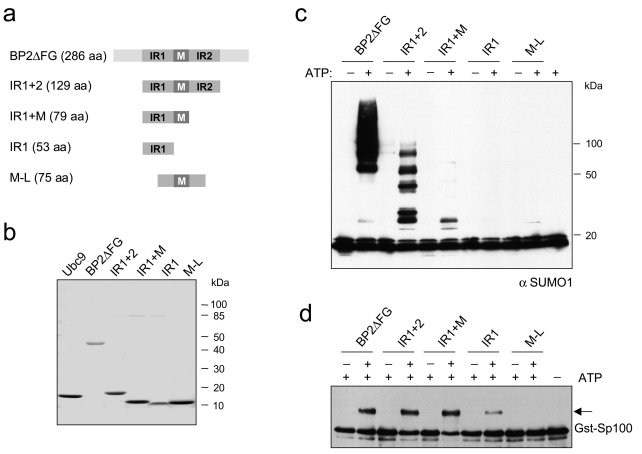

Figure 1 Auto-modification and E3 ligase activity of RanBP2 deletion fragments. **(a)** Schematic representation of selected RanBP2 fragments. BP2ΔFG, the SUMO E3 ligase domain described previously, consists of two internal repeats (IR1 and IR2) separated by the 25-residue M domain, and N- and C-terminal flanking regions. BP2ΔFG: residues 2553–2838; IR1+2: residues 2633–2761; IR1+M: residues 2633– 2711; IR1: residues 2633–2685; M-L: residues 2661–2735. **(b)** Selected recombinant RanBP2 fragments separated on a 5–20% (w/v) SDS gel and stained by Coomassie blue. **(c)** AutoSUMOylation of RanBP2. Indicated RanBP2 fragments (1.5 μM each) were applied as substrates in an *in vitro* SUMOylation assay using 10 ng Ubc9 (28 nM), 150 ng Aos1-Uba2 (68 nM), 1.7 μg SUMO1 (7.5 μM) and ATP, and incubated for 30 minutes at 30°C. Analysis was by immunoblotting using anti-SUMO1. **(d)** RanBP2 catalysis of Sp100 SUMOylation. 250 ng (196 nM) GST-Sp100 was tested for modification in the absence or presence of RanBP2 fragments (20 ng each; 31 nM BP2ΔFG, 67 nM IR1+2, 158 nM IR1, 109 nM IR1+M, 115 nM M-L) and 10 ng Ubc9 (28 nM), 150 ng Aos1-Uba2 (68 nM), 0.5 μg SUMO1 (2.2μM) and ATP. After incubation for 30 minutes at 30°C samples were analyzed by immunoblotting with anti-GST. Arrowhead indicates SUMOylated form.

the GTPase Ran, Ubc9, and SUMOylated GTPase-activating protein RanGAP1[20–24]. Its E3 activity resides in a 33-kDa fragment without substantial homology to any other protein[18]. This suggested that the mechanisms by which E3 ligases function are more diverse than previously anticipated.

To address the question of how RanBP2 functions in SUMOylation, we set out to further dissect its catalytic domain. The data presented here are consistent with the model that RanBP2 enhances SUMOylation at least in part by altering Ubc9's catalytic properties, rather than as a HECT type enzyme or a simple adaptor. RanBP2 is thus the first clear example for an E3 ligase that is neither a HECT enzyme nor a RING-finger (related) protein.

Results

We previously mapped the SUMO E3 activity of the 358-kDa RanBP2 to a 33-kDa, 286-residue fragment termed BP2ΔFG[18]. This fragment contains two ~50-residue internal repeats (IR1 and IR2) separated by a 25-residue middle domain (M)[20,21,25]. To understand the catalytic mechanism of BP2ΔFG, we wanted to map its Ubc9-binding site, identify a minimal catalytic domain, and test for automodification. We generated deletion fragments (**Fig. 1a)**, each of which was expressed as a GST-fusion protein in *Escherichia coli*, enriched on GST-sepharose, and cleaved on the column by thrombin. This resulted in near-homogenous fragments (**Fig. 1b**).

AutoSUMOylation

We first tested the deletion fragments for automodification, as we had previously observed extensive SUMO chain formation on BP2ΔFG at high concentrations[18]. We incubated 1.5 μM fragments with recombinant Aos1-Uba2 and Ubc9, SUMO1 and ATP (**Fig. 1c**). Efficient chain formation was observed only for the longest fragment, BP2ΔFG . IR1+2 gives rise to several discrete modified species, consistent with the addition of up to four SUMO1 moieties per fragment. A similar pattern, albeit at much reduced levels, was also observed with IR1+M. Very faint modification of IR1 with several SUMO1 residues and a very low level of a monoSUMOylated M-L species were detectable only at very long exposure (data not shown). Consistent with extensive chain forming activity only for the largest fragment, a lysine residue in the N-terminal flanking region (Lys2592) was identified by mass spectrometry as the major SUMO acceptor site (P.K. and T.K.S., unpublished data).

The IR1+M fragment has full activity

The catalytic activity of the RanBP2 fragments was next tested in Sp100 modification. In a pilot experiment, equal amounts (20 ng) of BP2ΔFG,

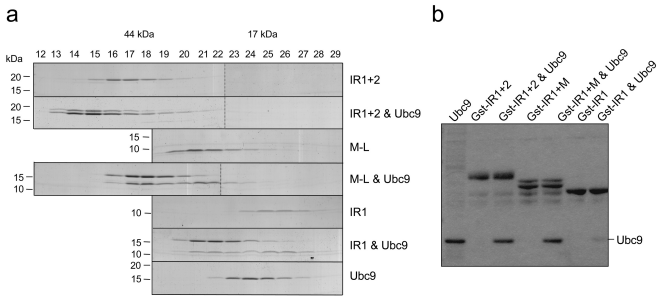

Figure 2 Ubc9 interacts with IR1 and more stably with IR1+M. **(a)** Indicated proteins (50 μg each at final concentrations of 14 μM for Ubc9, 17 μM for IR1+2, 40 μM for IR1, and 29 μM of M-L) were incubated for 1 h on ice and applied to gel filtration on Superdex 75. Fractions were analyzed by SDS-PAGE. **(b)** IR1+M binds Ubc9 more stably than IR1. Bacterial lysate containing recombinant Ubc9 was incubated with immobilized GST-IR1+2, GST-IR1+M and GST-IR1 in PBS plus 0.1% (v/v) TritonX100 for 30 min at room temperature. After extensive washing, bound samples were analyzed by 5–20% (w/v) SDS-PAGE.

IR1+2, IR1+M, IR1 and M-L were added to basic Sp100 SUMOylation reactions with limiting concentrations of Ubc9. Notably, reduction of the RanBP2 fragment from 286 residues to the 79 residue IR1+M fragment did not reduce its catalytic activity, indicating that flanking regions and IR2 are dispensable for activity. Further deletion of 26 residues severely impaired activity, but the resulting IR1 clearly had residual activity when compared to reactions in its absence (**Fig. 1d**, lanes 7 and 8). The M-L fragment, which contains about half of IR1 and half of the related IR2 fragment, and is thus comparable in length and amino acid composition to the highly active IR1+M fragment, was completely inactive. A 29-residue fragment containing just the M domain was also inactive (see below). Taken together, these findings suggest that IR1+M represents a catalytic core domain.

IR1 is sufficient for Ubc9 binding

Previously, we showed that BP2ΔFG coelutes with Ubc9 in gel filtration[18], and that GST-IR1+2 binds to Ubc9 in pull-down experiments[25]. Considering that IR1 had some E3 ligase activity, we wanted to test its ability to stably interact with Ubc9. We applied untagged IR1 and a preincubated mixture of IR1 and Ubc9 to gel filtration on Superdex 75 in a physiological buffer. The highly active IR1+2 and the inactive M-L fragments were also included in this analysis (**Fig. 2a**). As evidenced by a change in elution volume, Ubc9 comigrated with IR1+2, IR1 and the catalytically inactive M-L fragment. Notably, the 29-residue M domain did not stably interact with Ubc9 in gel filtration experiments or pull-down assays[25] (data not shown). These data suggest that IR1 (or the C-terminal half of IR1 that is also present in M-L) binds stably to Ubc9 under conditions comparable to those used in enzyme assays. We next applied a more stringent assay to compare IR1, IR1+M and IR1+2 binding. This involved Ubc9 pull-down on immobilized GST fragments in the presence of 0.1% (v/v) Triton X100 and extensive washing of the beads after binding (**Fig. 2b**). Under those conditions, comparable amounts of Ubc9 bound to both GST-IR1+2 and GST-IR1+M, whereas binding to GST-IR1 was substantially reduced. Taken together, these data suggest that the observed difference in activity for IR1 and IR1+M may be due to differences in Ubc9 binding.

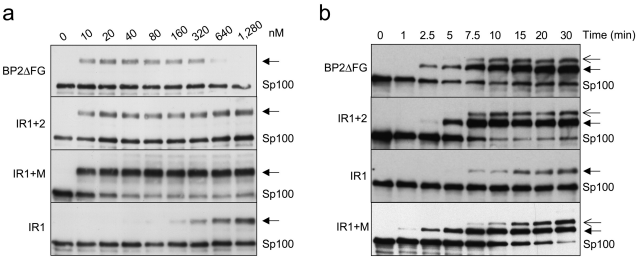

Figure 3 IR1+M catalyzes Sp100 SUMOylation efficiently. **(a)** Concentration dependence of different RanBP2 fragments. GST-Sp100 (250 ng; 196 nM) GST-Sp100 was tested for modification in the absence or presence of increasing concentrations of RanBP2 fragments. After 30 min samples were analyzed by immunoblotting with antibodies against GST. **(b)** RanBP2 fragments (20 ng each: 31 nM BP2ΔFG, 67 nM IR1+2, 109 nM IR1+M, 158 nM IR1) were compared for E3 activity on GST-Sp100 in a time course experiment. Thick and thin arrows indicate mono- and diSUMOylated forms of Sp100, respectively.

The M domain strongly enhances RanBP2's activity

The experiment described above suggested that the M domain enhances IR1's intrinsic E3 ligase activity by increasing its affinity for Ubc9. This model predicts that IR1 and IR1+M differ substantially in their dose-response curves. We therefore analyzed the concentration dependence for each of the fragments (**Fig. 3a**). As shown previously, BP2ΔFG worked at very low concentrations (10 nM), but lost efficiency at high concentrations. This is probably due to its competing reaction, the SUMO chain formation (see **Fig. 1c**). Indeed, IR1+2, which does not form chains efficiently, enhanced Sp100 modification even at the highest concentrations used. Notably, there was a marked difference in dose dependence between IR1 and IR1+M. Whereas IR1+M had full activity at approximately equimolar concentration to Ubc9, IR1 had to be used at substantially higher concentration (~ 1 μM) to modify Sp100 efficiently. All fragments stimulated Sp100 SUMOylation in a time-dependent manner (**Fig. 3b**). In this experiment, fragments were used at the same amounts (20 ng) to compensate for the different concentration requirements. Taken together, Ubc9-binding and activity assays are consistent with the interpretation that the catalytically inactive 25-residue M domain contributes to binding and serves to target the catalytic domain (IR1) to Ubc9.

IR1+M has reduced target specificity

We previously showed that BP2ΔFG exerts target specificity[18]. The mechanism of this was however unclear, as we could not detect BP2ΔFG-target interactions. We were therefore interested in testing the target specificity of the shorter fragments. First, we tested BP2ΔFG, IR1+2 and IR1 for E3 activity on four different SUMO substrates, Sp100, HDAC4, p53 and Lef-1. Sp100 and HDAC4 are efficiently modified *in vitro* in the presence of BP2ΔFG[18,19], p53 is an *in vitro* substrate for the E3 ligases PIAS1 and PIASxβ[26,27], and Lef1 modification has been shown to be stimulated by PIASy[28]. All substrates were subjected to *in vitro* SUMOylation in the absence and presence of different RanBP2 fragments, or for comparison with PIAS1 (**Fig. 4a**). The inactive M-L fragment was included as a negative control. Consistent with our previous findings, BP2ΔFG stimulated SUMOylation of Sp100 strongly, in fact much better than did PIAS1 (compare lanes 3 and 7). HDAC4 and Lef1 modification was enhanced by both PIAS1 and BP2ΔFG. IR1+2 and IR1 modified Sp100, HDAC4 and Lef1 to the extent expected from the analysis above (IR1+2 functioned with high efficiency, IR1 was much less active). As expected, PIAS1 strongly enhanced p53 SUMOylation, whereas BP2ΔFG was inactive. In contrast, IR1+2 and especially IR1 stimulated SUMOylation of p53 to some extent. This effect was even clearer when we repeated the experiment with IR1+M (**Fig. 4b**). Whereas IR1+2 had only a minimal effect on p53, IR1+M clearly enhanced p53 SUMOylation. To exclude that IR1+M activated modification nonspecifically, we also tested IR1+2 and IR1+M on an Sp100

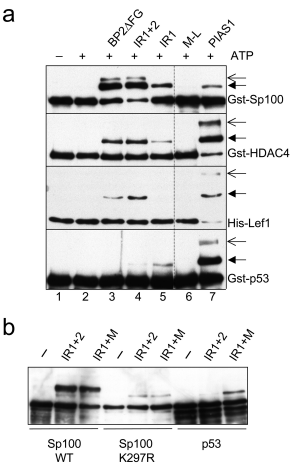

Figure 4 Shorter RanBP2 fragments have reduced target specificity. **(a)** Indicated SUMO substrates (250 ng each of 196 nM GST-Sp100, 127 nM GST-HDAC4, 278 nM His-Lef1 or 166 nM GST-p53) were tested for modification in the absence and presence of RanBP2 fragments (20 ng each; 31 nM BP2ΔFG, 67 nM IR1+2, 158 nM IR1, 115 nM M-L) or PIAS1 (1 μg). Samples were analyzed by immunoblotting with anti-GST or anti-His. **(b)** Substrate specificity of IR1+M (40 nM) and IR1+2 (40 nM) was tested on GST-Sp100 WT (wild type), GST-Sp100K297R and GST-p53 (250 ng each; 196 nM for Sp100 and 166 nM for p53).

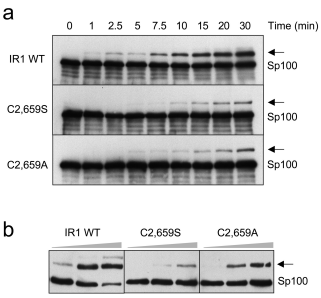

Figure 5 Cysteine mutants of IR1 have residual ligase activity. **(a)** *In vitro* SUMOylation of GST-Sp100 in the presence of wild-type (WT) or mutant IR1 in a time-dependent manner. Analysis was done by immunoblotting with antibodies against GST. **(b)** *In vitro* SUMOylation of GST-Sp100 in the presence of WT or mutant IR1 in a concentration-dependent manner (IR1 at 158 nM, 790 nM, or 1581 nM).

mutant (K297R) that lacked its major SUMOylation site. Notably, both fragments failed to stimulate modification of Sp100 K297R, demonstrating that their activity still depended on the presence of a genuine SUMO acceptor site. In conclusion, the 79-residue IR1+M fragment retains specificity for genuine SUMO acceptor sites, but lacks the target specificity observed for larger RanBP2 fragments. One interpretation of these finding is that IR2 and flanking regions in the larger fragments restrict modification to specific targets.

RanBP2 is not a HECT-related E3 ligase

Our previous analysis of the BP2ΔFG fragment included mutagenesis of two conserved cysteines[18]. The mutated fragment was catalytically active, but still contained 5 cysteines. Here we have mapped minimal ligase activity to the 52-residue fragment IR1, which contains a single cysteine. This allowed us to confirm whether RanBP2 requires a cysteine for its function. We generated two distinct mutants (C2659S and C2659A) and tested them in a time course for Sp100 modification (**Fig. 5a**). Both mutants were reduced in activity, but clearly stimulated Sp100 SUMOylation. Reduced activity could be partially compensated for by increased amounts of the mutants (**Fig. 5b**). Consequently, the single cysteine in IR1 is important but not absolutely essential for function, and RanBP2 is not a HECT-related E3 ligase.

Mapping of Ubc9 interaction sites in IR1

To further dissect RanBP2s E3 ligase activity, we wanted to identify single residues required for function of the minimal fragment IR1. As IR1 is markedly similar to IR2, we focused on residues identical in both fragments. Of those, we converted 15 to alanine or serine in single point mutants (indicated by dots in **Fig. 6a**). Each mutant was purified as a GST-fusion protein, cleaved with thrombin, and tested for SUMO E3 activity on Sp100. In the first set of experiments, IR1 fragments were used at 20 ng (158 nM) per reaction (**Fig. 6b**). Whereas some mutants including P2640A, K2645A, K2652A and P2655A behaved like WT IR1, others showed severely reduced or apparently abolished E3 activity. To discriminate between mutants with reduced activity and those that where completely inactive, we next assayed for concentration dependence. Increasing the concentration of T2656A almost fully restored its function, and increasing the concentration of the mutants P2654A, C2659S, D2676A and F2677A partially restored their function (**Fig. 6c**). A probable interpretation is that these mutants have reduced affinity for – or a greater k_{off} from - Ubc9. In contrast, the mutants L2651A, L2653A, F2657A and F2658A remained catalytically inactive irrespective of the amount used. These mutants may be completely unable to interact with Ubc9, or they may bind

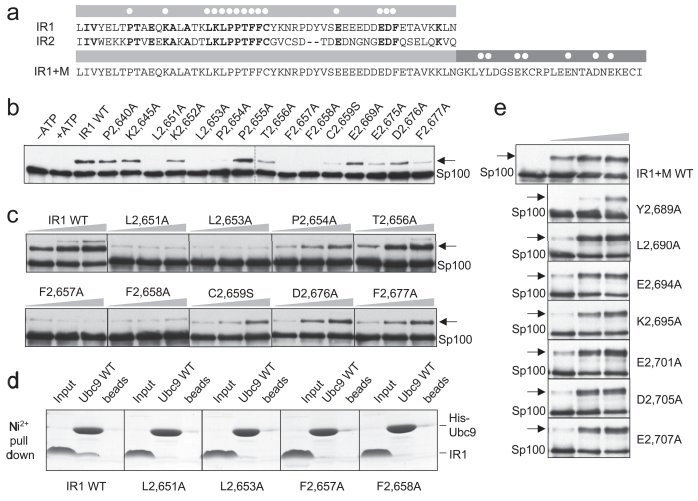

Figure 6 RanBP2 mutants defective for activity and Ubc9 binding. **(a)** Amino acid comparison of IR1, IR2 and IR1+M. Identical amino acids between IR1 and IR2 are in bold. Fifteen mutants in IR1 and seven in the IR1+M fragment were generated by individually changing indicated amino acids (dots) to alanine or serine (Cys2659). **(b)** GST-Sp100 SUMOylation in the absence or presence of 20 ng (158 nM) wild-type (WT) or mutant IR. Samples were analyzed by immunoblotting with antibodies against GST. **(c)** Sp100 SUMOylation was tested in the presence of three different concentrations of the indicated IR1 mutants (at 158 nM, 790 nM, and 1.58 μM). **(d)** His-Ubc9 immobilized on Ni^{2+} beads was incubated for 1 h with an approximately three-fold molar excess of WT or mutant IR1, and analyzed for binding by SDS-PAGE. Empty beads served as a control. Shown are total input and total bound fractions. **(e)** Sp100 SUMOylation was tested in the presence of three different concentrations of the indicated IR1+M mutants (10 nM, 80 nM and 640 nM).

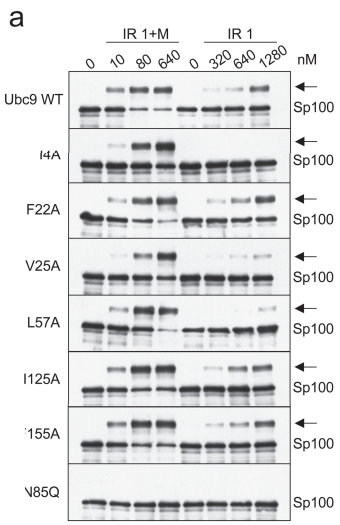

Figure 7 Ubc9 mutants defective in RanBP2-dependent modification. **(a)** Indicated wild-type (WT) and mutant Ubc9 proteins (56 nM) were tested on Sp100 modification in the presence of three concentrations of IR1+M and IR1. For this analysis, mutants were generated in His-Ubc9, and the tag was removed by thrombin. **(b)** Ubc9 structure[37] with mutations and the active cysteine indicated. Mutant residues with clear effects on IR1-dependent Sp100 SUMOylation are indicated in black.

Ubc9 but be unable to contribute to subsequent catalytic events. We tested this in pull-down experiments under low stringency conditions (**Fig. 6d**): wild-type IR1 binding was detected, but none of the four mutants bound Ubc9. Notably, circular dichroism (CD) spectra of wild-type and mutant IR1 fragments are very similar and reflect a largely unstructured conformation (data not shown, but see below). We next introduced several mutations in the M part of the IR1+M fragment, and analyzed the mutants for activity as we did for IR1. Of seven randomly selected mutants in hydrophobic and hydrophilic residues, 6 had no detectable effect. One mutant, Y2689A, showed a substantial reduction in activity towards Sp100 at low concentrations, but was functional at elevated levels (**Fig. 6e**). In summary, of 22 single point mutants analyzed, 9 were defective in function. Defects in five of these mutants could be compensated for by using increased concentrations, and four hydrophobic mutants were inactive at all concentrations tested. These inactive mutants also did not bind to Ubc9 in pull-down assays.

Ubc9 mutants in RanBP2-dependent SUMOylation

Notably, most of the critical residues that we identified in IR1 and M are bulky hydrophobic or aromatic residues. This suggested that RanBP2-Ubc9 interaction is mediated at least in part by hydrophobic interactions. We therefore generated six Ubc9 mutants in which surface-exposed hydrophobic residues were changed to alanine, and tested them for Sp100 modification in the presence of three different concentrations of IR1+M or IR1, respectively (**Fig. 7a,b**). Although all six stimulated Sp100 SUMOylation in the presence of IR1+M, mutants I4A, V25A and L57A were clearly reduced in activity. This effect was even more pronounced with the less efficient E3 ligase fragment IR1 (**Fig. 7a**). Notably, these mutants behaved exactly like wild-type Ubc9 in E3 ligase-independent SUMOylation of Sp100 and RanGAP1 (data not shown). Taken together, these findings implicate the Ubc9 residues Ile4, Val25 and Leu57 in RanBP2-Ubc9 interaction.

One additional mutant (N85Q) was included in this analysis, because Asn85 in Ubc9 and the corresponding asparagine in ubiquitin E2 conjugating enzymes has recently been described as essential for E2 and E2-RING E3 mediated isopeptide bond formation. Notably, mutating Ubc9 asparagine 85 to glutamine did not impair thioester bond formation[29] (see also **Supplementary Fig. 1** online), and HECT E3-dependent isopeptide bond formation was not affected in the ubiquitin E2 mutant UbcH5A N77Q[29]. From our finding that RanBP2 does not function as a HECT-type E3 ligase, we expected that it may require Asn85 for catalysis. Indeed, similar to the RING-type E3 ligase PIASxα in

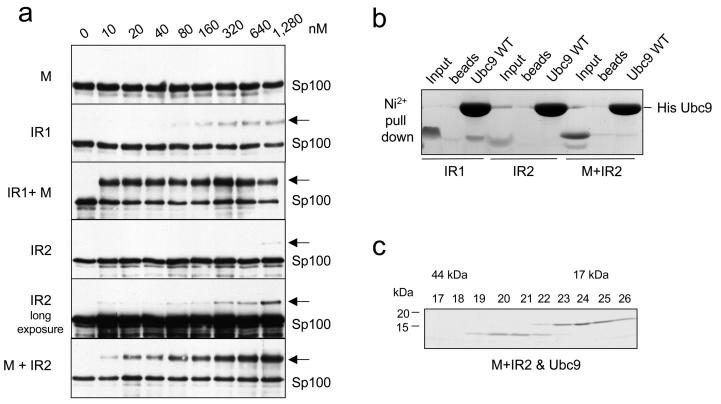

Figure 8 IR1+M and M+IR2 are both highly active in Sp100 SUMOylation. **(a)** IR2 and M+IR2 catalyze Sp100 SUMOylation. Indicated fragments were tested on GST-Sp100 modification as described in **Figure 3a**. A longer exposure of IR2-dependent reactions is also included to reveal its weak but detectable activity. **(b)** IR2 and M+IR2 do not stably interact with Ubc9. His-Ubc9 immobilized on Ni^{2+} beads was incubated for 1 h with IR1, IR2 and M+IR2, and analyzed for binding by SDS-PAGE. Empty beads served as a control. Shown are total input and total bound fractions. **(c)** M+IR2 does not co-elute with Ubc9: 50 μg of Ubc9 (14 μM) and M+IR2 (48 μM) was incubated for 1 h on ice and applied to gel filtration on Superdex 75. Fractions were analyzed by SDS-PAGE.

Stat1 SUMOylation[29], the RanBP2 fragments IR1 or IR1+M could not overcome the defect conferred by the N85Q mutation in Ubc9 in Sp100 SUMOylation (**Fig. 7a** and **Supplementary Fig. 1** online). Notably, N85Q is not completely inactive for isopeptide bond formation. Although Ubc9 N85Q was inactive in Sp100 modification even at high concentrations, it was still functional in RanGAP1 SUMOylation, albeit with reduced efficiency (**Supplementary Fig. 1** online).

Two overlapping catalytic domains in BP2ΔFG

Until this point we had focused on the IR1 fragment. Its similarity with IR2 suggested that IR2 might be catalytically active as well. However, the M fragment, which strongly stimulates IR1 activity in the context of the IR1+M fragment, exists only once in RanBP2, and is C-terminal of IR1 but N-terminal of IR2. We therefore tested both IR2 and the M+IR2 fragment for E3 ligase

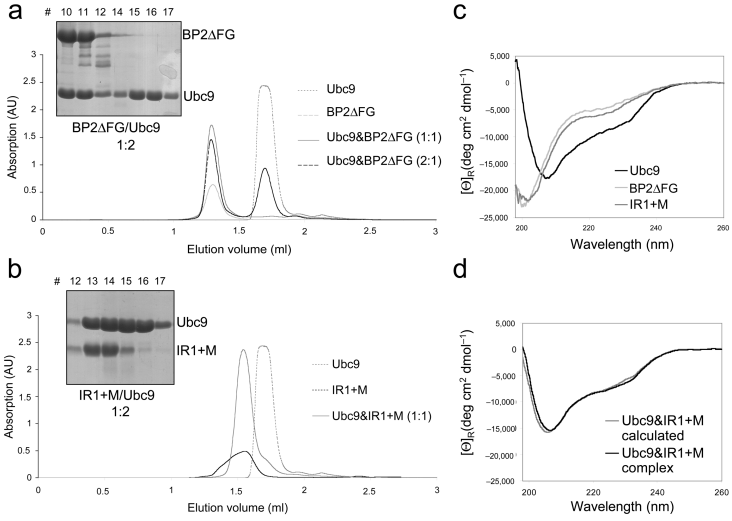

Figure 9 The largely unstructured BP2ΔFG binds Ubc9 in a 1:1 stoichiometry. **(a)** Size-exclusion chromatography of Ubc9, BP2ΔFG and both proteins at a 1:1 and 2:1 molar ratio shows that Ubc9 and BP2ΔFG form a 1:1 complex. Gel filtration traces are shown for all four column runs, peak fractions of the 2:1 analysis are also shown on SDS PAGE stained with Coomassie blue. **(b)** Experiments were done as in **a**; for Ubc9 and IR1+M, the 2:1 gel filtration trace was removed from the figure for clarity, but behaves analogously to that in **a**. **(c)** CD spectra of Ubc9, BP2ΔFG and IR1+M (5.5 μM each) demonstrate that both BP2ΔFG and IR1+M are largely unstructured proteins whereas Ubc9 shows characteristic hallmarks of a structured protein. The average of eight scans is reported and values are given in terms of ellipticity units per mole of peptide residue (Θ_R). **(d)** The measured spectrum of the IR1+M-Ubc9 complex shows very little difference to the theoretical noninteracting sum of the individual spectra. For formation of complex between IR1+M and Ubc9, both components were mixed (5.5 μM each) and incubated for 30 min at room temperature before measurement.

activity. Indeed, IR2 showed some activity at very high concentrations, albeit at much reduced levels compared to IR1. Notably, M+IR2 worked much more efficiently than IR2 alone. The 28-residue M fragment exerted no effect by itself (**Fig. 8a**), or when added to the isolated IR1 and IR2 fragments (data not shown). Taken together, these findings demonstrate that the single M domain has the potential to enhance catalysis of both the N-terminal IR1 and the C-terminal IR2 fragment. However, IR1 and IR2, and likewise IR1+M and M+IR2, clearly differed substantially in activity (at least a ten-fold higher concentration is required for comparable activity). We therefore also addressed whether IR2 and M+IR2 would interact with Ubc9 in pull-down assays. Neither IR2 nor M+IR2 bound to Ubc9 at detectable levels, even under low stringency conditions that allowed detection of IR1 binding (**Fig. 8b**). In addition, M+IR2 did not coelute with Ubc9 in gel filtration (compare **Fig. 2a** with **Fig. 8c**). It is therefore conceivable that their reduced activity compared to IR1 and IR1+M, respectively, is due at least in part to a reduced ability to stably interact with Ubc9. It remains to be determined whether this is due to a lower affinity or higher k_{off}.

BP2ΔFG binds Ubc9 with 1:1 stoichiometry

The finding that both IR1 and IR2 are active raised the question of whether the larger fragment, BP2ΔFG, binds to one or more Ubc9 molecules simultaneously. To address this, Ubc9 and BP2ΔFG were mixed at molar ratios of 1:1 and 2:1, and chromatographed on an analytical gel filtration column (**Fig. 9a**). To ensure saturated binding, very high molar concentrations (100 μM) were used. As revealed by absorption spectra and SDS-PAGE (shown for the 2:1 sample) only a 1:1 complex was formed. Similar results were obtained with the IR1+M fragment (**Fig. 9b**). During size-exclusion chromatography, BP2ΔFG and IR1+M eluted at positions of high apparent molecular mass when compared with globular molecular mass standards (**Fig. 9** and data not shown). To determine the absolute molecular mass of individual components and of Ubc9 complexes, we used multiangle laser light scattering (MALLS), a technique that is independent of the shape of molecules[30]. BP2ΔFG, IR1+M, Ubc9, and the complexes eluted with molecular masses comparable to the calculated values for monomeric species and 1:1 complexes, respectively (**Supplementary Fig. 2** online; for example, calculated and determined molecular masses for Ubc9-BP2ΔFG were 51 and 55 kDa, respectively; for Ubc9-IR1+M, they were 29 and 31 kDa, respectively).

BP2ΔFG is largely unstructured

The aberrant mobility and asymmetric peak of elution observed for BP2ΔFG and IR1+M in size-exclusion chromatography indicate an extended conformation. Notably, addition of Ubc9 (19 kDa) to IR1+M (10 kDa) did not shift the elution volume, but improved the peak to a more symmetrical and compact shape (**Fig. 9b**). The elution volume of the complex conforms to a globular protein of 30 kDa (corresponding to the mass of Ubc9-IR1+M), indicating that IR1+M changes from an extended to a more globular conformation upon Ubc9 binding. To explore this further, we used CD spectroscopy (**Fig. 9c**). In contrast to Ubc9, whose CD spectrum shows the expected hallmarks of secondary structure elements, the spectra of BP2ΔFG and IR1+M show very little α-helical or β-sheet content and are consistent with largely unstructured proteins. Upon incubation of IR1+M with Ubc9 for 30 min at room temperature, conditions that lead to quantitative complex formation, the CD spectrum of the complex was measured. It showed very little difference from the theoretical noninteracting sum of the individual spectra (**Fig. 9d**). Taken together, our findings suggest that BP2ΔFG and IR1+M are largely unfolded proteins that gain a more compact conformation upon binding to Ubc9. Interaction does not, however, lead to a substantial gain of secondary structure elements or to substantial changes in Ubc9 structure.

Discussion

Modular structure of RanBP2s catalytic domain

Here we dissected RanBP2's E3 ligase domain to gain insights into its mechanism (for a summary of data see **Supplementary Fig. 3** online). We found that RanBP2 has two minimal ~50-residue catalytically active fragments, IR1 and IR2. They are 40 % identical to each other, and are highly conserved between different vertebrate species (**Supplementary Fig. 4** online). To be efficient, they have to be used at very high concentrations (~1 μM). IR1 and IR2 are separated by the M domain (25 residues in human RanBP2). This

domain markedly enhances the catalytic activity when fused to the C terminus of IR1 or the N terminus of IR2, but is itself inactive. Consistent with the idea that M functions at least in part by increasing the affinity to Ubc9, IR1+M and M+IR2 work at substantially lower concentrations than IR1 or IR2 alone. Despite their structural and functional similarities, IR1 and IR2 (IR1+M and M+IR2) clearly differ in activity and Ubc9 binding. An important question for future studies is therefore whether both fragments function independent of each other in the context of full-length RanBP2, for example, on different targets, or whether the less active IR2 fragment serves, for example, an autoregulatory or inhibitory function (IR1+M is more active and less specific than IR1+2).

RanBP2 wrapping around Ubc9

One simple model, folding around Ubc9, may explain how IR1+M and M+IR2 could be functional equivalents (**Fig. 10**). Based on the observation that the catalytic domain of RanBP2 is largely unstructured, the fragment IR1+2 can be aligned in two distinct ways, such that the M and IR domains in IR1+M and M+IR2 can be positioned identically on Ubc9 (assuming an extended sheet structure, 36 residues would be

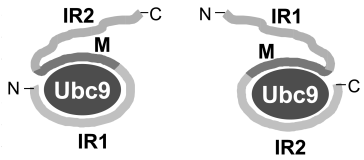

Figure 10 Model for RanBP2-Ubc9 interaction.

sufficient to fully wrap around Ubc9 at its largest diameter). In either conformation, one of the IR fragments would not engage in binding. Our model is consistent with the mobility of IR1+M with and without Ubc9 in molecular size-exclusion chromatography (extended shape for IR1+M, globular shape of the complex). It fits with the Ubc9 mutagenesis, which implicates far removed-residues on Ubc9's surface in IR1 binding (i.e. Ile4, Val25 and Leu57), and it offers an explanation for our finding that BP2ΔFG shows strong target preferences (Sp100 is modified efficiently, p53 is not), whereas the equally active IR1+M fragment does not (modification of p53 and Sp100). In our model, IR1+M would be fully involved in Ubc9 binding, whereas BP2ΔFG would have a substantial proportion of unengaged unstructured areas that might interfere with certain target binding.

Function without direct target interaction

The SUMO E2 enzyme Ubc9 recognizes many targets directly via a defined consensus sequence. This is, however, not enough for efficient modification. RanGAP1, the only known target efficiently modified in the absence of E3 ligases, has a second binding site for Ubc9[31]. Most other targets seem to lack a second interaction site, which may explain why stable interactions with Ubc9 have not been found (interactions were mainly observed in two-hybrid assays). These proteins are SUMOylated efficiently only in the presence of E3 ligases. Two distinct mechanisms could account for their stimulatory activity: first, they may serve as bridging factors, and by doing so stabilize the interaction between target and SUMO-charged Ubc9. Alternatively, they may function by accelerating the SUMO transfer time between Ubc9 and its targets such that even transient encounters are sufficient for modification. This could, for example, be achieved by allosterically changing Ubc9's catalytic cleft. Notably, these mechanisms are not mutually exclusive, and both have been discussed for ubiquitin RING-finger E3 ligases[7]. An allosteric effect has, for example, been proposed for Apc11, the 84-residue RING-finger component of the APC complex[32–34].

Although the bridging mechanism probably applies to PIAS E3 ligases, it is not compatible with our findings on RanBP2's catalytic domain. There is no evidence for target interactions, and shorter RanBP2 fragments stimulate SUMOylation of more targets than larger fragments. We therefore favor the idea that RanBP2 functions by accelerating SUMO transfer, possibly by an allosteric mechanism. Alternatively, RanBP2 may function by helping to position thioester-bound SUMO for efficient transfer. Although we have not addressed this experimentally, it is at least conceivable that RanBP2 binding influences non-covalent and/or covalent Ubc9-SUMO interactions.

A notable possibility for an allosteric mechanism has been raised by recent work by Pickart and coworkers: they demonstrated that a strictly conserved asparagine present in E2 enzymes is essential, for example, for Ubc9- and PIAS-dependent catalysis of Stat1 SUMOylation, and proposed that it serves to stabilize the

oxyanion intermediate formed during lysine attack. This would, however, require a change in asparagine side chain orientation relative to the conformation seen in existing E2 structures[29]. Here we extended their findings to demonstrate that RanBP2-dependent SUMOylation of Sp100 also requires Asn85. One hypothesis is therefore that RanBP2 (and perhaps PIAS E3s) accelerates catalysis by influencing the orientation of this asparagine side chain in Ubc9. A subtle change in side chain orientation of Asn85 would not be detectable in CD spectra, and high-resolution structural analysis will be required to resolve this issue. Notably, although Asn85 is also essential for E3-independent modification of Stat1[29] and Sp100, Ubc9 N85Q can SUMOylate RanGAP1 (**Supplementary Fig. 1** online). In contrast to Stat1 and Sp100, RanGAP1 stably interacts with Ubc9. It could therefore be modified even if isopeptide bond formation in N85Q Ubc9 were to be slowed down substantially.

In conclusion, the catalytic core domain of RanBP2 is distinct from that of any other known E3 ligase. It is an extended protein that interacts with Ubc9 via largely hydrophobic interactions. Stimulation of target SUMOylation seems to be catalyzed via enhancing Ubc9's activity rather than by target recruitment. Whether this indeed involves allosteric changes in Ubc9 awaits future structural analysis.

Material and methods

Plasmids. Plasmids for GST-BP2ΔFG (residues 2553–2838), Ubc9, SUMO1, Aos1, Uba2[18], GST-IR1+2 (residues 2633–2761), GST-IR1 (residues 2633–2685), GST-IR2 (residues 2711–2761), GST-M (residues 2683–2711)[25], GST-Sp100[35], GST-p53 and GST-HDAC4[19], His-Lef-1[28], GST-PIAS1[36] and wild-type His-Ubc9[31] have been described. GST-M-L (residues 2661–2735) and GST-M+IR2 (residues 2683–2761) constructs were generated by PCR and ligation into the BamHI and EcoRI sites of pGEX-2T. Single point mutants were generated by site-directed mutagenesis. GST-IR1+M (residues 2633– 2711) was derived from GST-IR1+2 by introduction of a stop codon in position 2712.

Expression and purification of recombinant proteins. Purification of SUMO1, Aos1, Uba2, and Ubc9 was done as described[18]. GST-BP2 constructs, GST-p53, GST-HDAC4, His-Lef-1 and His-Ubc9 were expressed in *E. coli* BL21 gold (Stratagene); GST-PIAS1 and GST-Sp100 were expressed in the *E.coli* strain Rosetta (Novagen). His- and GST-fusion proteins were purified according to standard protocols. Tags in GST- BP2ΔFG were removed by Factor Xa cleavage (Novagen) and subsequent gel filtration; GST-tags in other GST-BP2 constructs, GST-PIAS1, and His-Ubc9 were removed by cleavage with biotinylated thrombin according to manufacture's instructions (Novagen).

Antibodies. Rabbit anti-GST antibody was provided by L. Hengst (Max-Planck Institute for Biochemistry); mouse monoclonal anti-SUMO1 was obtained from Zymed Laboratories; rabbit anti-His was obtained from Santa Cruz Biotechnology; rabbit anti-RanBP2 had been described[18]. Secondary antibodies were obtained from Jackson Laboratories.

***In vitro* SUMOylation.** *In vitro* SUMOylation was done as described[18]. Unless stated otherwise, 250 ng of substrate (196 nM GST-Sp100, 127 nM GST-HDAC4, 278 nM His-Lef1 or 166 nM GST-p53), ATP, 10 ng Ubc9 (28 nM), 150 ng E1 (68 nM), 500 ng SUMO1 (2.2 μM) and 20 ng BP2 fragments (31 nM BP2ΔFG, 67 nM IR1+2, 109 nM IR1+M, 158 nM IR1, 115 nM M-L) or ~1 μg PIAS1 were incubated in a total volume of 20 μl transport buffer supplemented with protease inhibitors, 1 mM DTT, 0.05% (v/v) Tween and 0.2 mg ml^{-1} ovalbumin Grade VI (SIGMA) at 30°C for 30 minutes. Reactions were stopped by addition of 2x Laemmli buffer.

Ubc9 interaction studies. For gel filtration, 50 μg of indicated recombinant proteins were incubated on ice for 1 hour before application to gel filtration on Superdex 75 (Pharmacia). Fractions of 250 μl were collected and analyzed by 5–20% (w/v) SDS-PAGE. For Ni^{2+} pull-down assay, His-Ubc9 was bound to ProBound resin (Invitrogen) and incubated with indicated proteins in 200 μl transport buffer supplemented with protease inhibitors, 1mM DTT, 0.05% (v/v) Tween and 0.2 mg ml^{-1} ovalbumin at 4°C for 1 hour. After washing, bound and input samples were analyzed by SDS-PAGE. For the GST pull-down assay, 100 μl of bacterial lysate containing recombinant Ubc9 (0.5 μg μl^{-1}) was mixed with 50 μl beads containing 5 μg GST-IR1+2, GST-IR1+M and GST-IR1 in 1ml of PBS plus 0.1% (v/v) TritonX100 for 30 min at room temperature. After washing three times with PBS plus 0.1% (v/v) TritonX100, proteins associated with the beads were analyzed by 5–20% (w/v) SDS-PAGE.

Analytical gel filtration. Samples (50 μl) were applied to an analytical Superdex 200 PC 3.2/30 gel filtration column (Pharmacia) on the SMART system (Pharmacia) at room temperature. Individual proteins had concentrations of 278 μM Ubc9, 157 μM BP2ΔFG, or 526 μM IR1+M. For formation of complex, proteins were mixed at a molar ratio of 1:1 (100 μM each of BP2ΔFG and Ubc9; 182 μM each of IR1+M and Ubc9) or 1:2 (74 μM BP2ΔFG and 148 μM Ubc9; 109 μM IR1+M and 218 μM Ubc9), and incubated on ice for one hour before loading.

Multiangle laser light scattering (MALLS). Proteins were applied to an analytical Superdex 200 HR 10/30 gel filtration column (Pharmacia) coupled to a miniDAWN light scattering detector (Wyatt Technology) and a Dn-1000 differential refractive index detector (WGE Dr. Bures) at 4°C. Data analysis was carried out with the program Astra

using a d*n* dc^{-1} value of 0.185[30]. Protein concentrations were comparable to those used for the analytical gel filtration experiments, in the mixtures of BP2ΔFG-Ubc9 and IR1+M-Ubc9 a small excess of Ubc9 (~10 %) was used.

Circular dichroism. CD spectra were recorded on a Jasco J-715 spectropolarimeter provided with a PFD-350S temperature controller and a thermostatic cell compartment. For signal averaging and processing the Spectra Manager software J-700 was used. The spectra were registered at 30°C in the range 195-260 nm with a scanning speed of 50 nm min^{-1} in 1x PBS, pH 7.5, and 1mM DTT. Protein concentration (determined by UV) was 5.5 μM.

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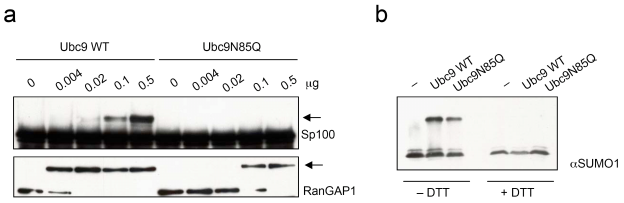
Supplementary Figure 1 Comparison of Ubc9 WT and Ubc9 N85Q for Sp100-RanGAP1 SUMOylation and thioester bond formation. **(a)** Indicated amounts of Ubc9 WT or N85Q mutant were applied to *in vitro* SUMOylation reactions (500 ng SUMO1, 150 ng E1 and ATP) of 250 ng GST-Sp100 (top) or 1.5 μg RanGAP1 (bottom). Samples were analyzed by immunoblotting with anti-GST or anti-RanGAP1 antibodies, respectively. **(b)** Ubc9 WT and N85Q mutant were compared for thioester bond formation (200 ng SUMO1, 100 ng Ubc9, 200 ng E1 and ATP). Analysis was done by SDS-PAGE in the absence or presence of DTT and immunoblotting with anti-SUMO1.



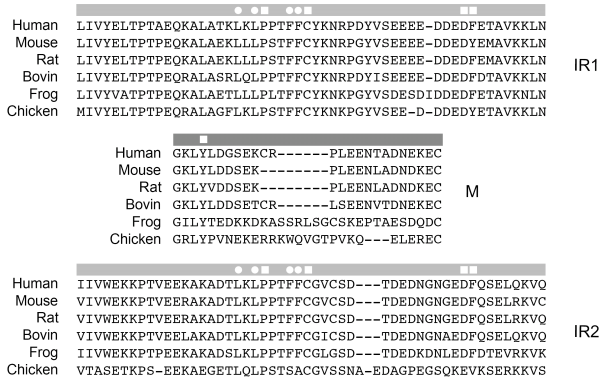
Supplementary figure 2 Calculated versus measured masses of light scattering analysis. BP2ΔFG, IR1+M, Ubc9 and the complexes eluted with molecular masses comparable to the calculated values for monomeric species and 1:1 complexes, respectively.

Protein	Calculated molecular weight	MALLS molecular weight
Ubc9	19 kDa	23 kDa
BP2ΔFG	32 kDa	34 kDa
IR1+M	10 kDa	14 kDa
Ubc9 & BP2ΔFG (1:1)	51 kDa	55 kDa
Ubc9 & IR1+M (1:1)	29 kDa	31 kDa

Supplementary Figure 3 Results of Ubc9 binding, 'auto-modification', ligase activity and specificity for the different RanBP2 fragments analyzed in this study. N.d.: not detectable, blanks: not tested. SUMO1 chain formation was clearly shown for the RanBP-2ΔFG fragment. In case of the smaller fragments it was not distinguished between chain formation and SUMOylation on different Lysines.

		Ubc9 binding	chains/SUMOylation	activity	specificity
BP2ΔFG (286 aa)	IR1 M IR2	√	++++	++++	++++
IR1+2 (129 aa)	IR1 M IR2	√	++	++++	+++
IR1 (53 aa)	IR1	√	(–/+)	++	++
IR2 (51 aa)	IR2	n.d.		+	
M-L (75 aa)	M	√	–	–	–
M (29 aa)	M	–		–	–
IR1+M (79 aa)	IR1 M	√	+	++++	++
M+IR2 (79 aa)	M IR2	n.d.		+++	

Supplementary Figure 4 Residues required for RanBP2's ligase activity are highly conserved. Sequence comparison between IR1 and IR2 from human (Homo sapiens gi|1009337), mouse (Mus musculus gi|19882199), rat (Rattus norvegicus gi|31156169, gi|29922782), bovine (Bos taurus gi|8039801), frog (Xenopus leavis gi|17499975, gi|17470945, gi|24088384, gi|17504982, gi|13486416) and chicken (Gallus gallus gi|25536955, gi|25339577, gi|27593034, gi|25509215). Sequences were obtained by translated BLAST search using the IR1+2 protein sequence as a query. The sequences for rat, frog and chicken were assembled from different EST-clones. White circles and squares highlight four essential and five important residues, respectively, that were identified in IR1 and M mutagenesis.



Chapter 3

SUMO modification of the ubiquitin-conjugating enzyme E2-25K

Andrea Pichler[1,3,*,#], Puck Knipscheer[2,*], Edith Oberhofer[3], Willem J. van Dijk[2], Roman Körner[3], Jesper Velgaard Olsen[4], Stefan Jentsch[3], Frauke Melchior[1,3] & Titia K. Sixma[2,#]

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[1]University of Göttingen, Department of Biochemistry I, Humboldt Allee 23, 37073 Göttingen, Germany
[2]The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[3]Max-Planck Institute for Biochemistry, Am Klopferspitz 18, D-82152 Martinsried, Germany
[4]University of Southern Denmark, Department of Biochemistry and Molecular Biology, Campusvej 55, Odense M, Denmark
[*]These authors contributed equally to the work
[#]communicating authors (pichler@biochem.mpg.de; t.sixma@nki.nl)

Posttranslational modification with small ubiquitin-related modifier (SUMO) alters functions of many proteins, but the molecular mechanisms and consequences of this modification are still poorly defined. During a screen for novel SUMO1 targets, we identified the ubiquitin-conjugating enzyme E2-25K (Hip2). SUMO attachment severely impairs E2-25K ubiquitin thioester and unanchored ubiquitin chain formation *in vitro*. Crystal structures of E2-25K(1–155) and of the E2-25K(1–155)–SUMO conjugate (E2-25K*SUMO) indicate that SUMO attachment interferes with E1 interaction through its location on the N-terminal helix. The SUMO acceptor site in E2-25K, Lys14, does not conform to the consensus site found in most SUMO targets (ψKXE), and functions only in the context of an α-helix. In contrast, adjacent SUMO consensus sites are only modified when in unstructured peptides. The demonstration that secondary structure elements are part of SUMO attachment signals could contribute to a better prediction of SUMO targets.

Introduction

Covalent attachment of ubiquitin and SUMO to proteins plays a major role in regulating cellular functions. Both modifications depend on a similar enzymatic cascade. Ubiquitination involves a single E1 ubiquitin-activating enzyme (Uba1), one of several E2 ubiquitin-conjugating enzymes, and one of many distinct E3 ubiquitin ligases. The latter are responsible for target selection (reviewed in[1,2]). Conjugation with SUMO requires the E1 heterodimer Aos1–Uba2 and the single E2 enzyme Ubc9, which contributes to target selection, and is often facilitated by E3 ligases[3,4]. Whereas ubiquitination can promote protein degradation, SUMOylation largely serves to regulate protein interactions. The list of known SUMO targets has grown substantially in recent years, but the mechanisms of target selection and the molecular consequences of modification are still poorly understood. Obtaining structural insights has been complicated by the fact that very few SUMO targets can be modified efficiently and at large quantities *in vitro*. In fact, only one SUMO conjugate has been structurally analyzed so far[5], and this is RanGAP1, the most efficient target known to date. Here we describe the identification, biochemical and structural analysis of a novel SUMO target, the ubiquitin-conjugating enzyme E2-25K.

Results

Identification of E2-25K as a novel SUMO target

To identify novel SUMO targets from HeLa cells, we made use of the observation that addition of GST–SUMO1 and ATP to cell extracts results in the appearance of GST–SUMO1 conjugates. Upon enrichment of modified proteins on GST columns and separation on SDS-PAGE, we applied mass spectrometry to identify putative targets. A protein band migrating at 65 kDa contained

both GST–SUMO1 and the 25-kDa ubiquitin-conjugating enzyme E2-25K, suggesting that the latter was covalently modified (**Fig. 1a**). E2-25K belongs to class II of ubiquitin-conjugating enzymes[6], and carries a C-terminal UBA domain in addition to a catalytic core. Its biological role is not yet understood, but different studies point to roles in Alzheimer disease[7], as a regulator of huntingtin[8], and in antigen processing and representation[9]. *In vivo* targets and E3 ligases for E2-25K-dependent ubiquitination are not known, but biochemical studies have revealed E2-25K's ability to catalyze the synthesis of unanchored Lys48-linked polyubiquitin chains[6].

To verify that E2-25K is a target for SUMO modification, we first tested its modification *in vitro*. E2-25K was efficiently SUMOylated even at low concentrations of Aos1–Uba2 and Ubc9 (68 nM and 28 nM, respectively) in the absence of E3 ligases (**Fig. 1b**). This places E2-25K amongst the best *in vitro* substrates for Ubc9-dependent SUMO modification known thus far.

Modification of endogenous E2-25K *in vivo* could also be demonstrated, but only after enrichment of SUMOylated proteins using a His-SUMO1-expressing cell line[10] and pull-down assays on Ni^{2+} beads (**Fig. 1c**). We could not detect SUMOylated E2-25K by direct immunoblotting of cell lysates with or without SUMO overexpression (data not shown). Although we cannot formally rule out the possibility that E2-25K is modified upon His-SUMO1 overexpression in the stable cell line, very low levels of steady state modification have been observed for many SUMO targets[11-13].

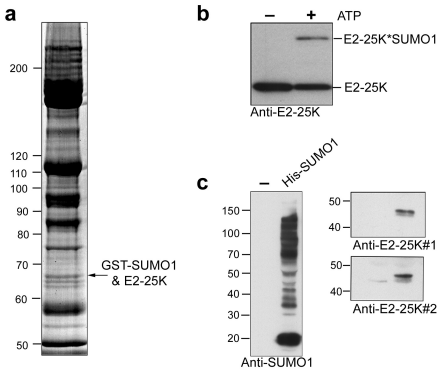

Figure 1 E2-25K is SUMOylated *in vitro* and *in vivo*. (**a**) Identification of E2-25K as a SUMO1 substrate: proteins that stably associated with GST–SUMO1 upon incubation in HeLa S3 cytosol were separated on SDS-PAGE. Mass spectrometry identified peptides for GST, SUMO1 and E2-25K in the indicated band. Size given in kDa. (**b**) E2-25K is SUMOylated *in vitro*: 500 ng E2-25K, 150 ng Aos1–Uba2, 10 ng Ubc9, and 1.5 µg SUMO1 in 20 µl reaction volume were incubated with or without ATP for 30 min at 30°C. Analysis was done by immunoblotting. (**c**) E2-25K is SUMOylated *in vivo*. Ni^{2+} pull-down under denaturing conditions from wild-type HeLa cells (left lanes) or HeLa cells stably expressing His-SUMO1 (right lanes). Immunoblotting was with two different anti-E2-25K and anti-SUMO1 antibodies. Size given in kDa.

SUMO inhibits ubiquitin - E2-25K thioester formation

To gain insight into the consequences of E2-25K SUMOylation, we generated modified and unmodified E2-25K and tested both for unanchored ubiquitin chain formation. SUMOylated E2-25K is severely reduced in activity (**Fig. 2a**). Two different steps leading to chain formation could be affected by SUMO attachment: transfer of ubiquitin from the E1 enzyme to E2-25K (thioester bond formation), or transfer of ubiquitin from the loaded E2 to an ubiquitin acceptor (isopeptide bond formation). SUMO attachment strongly impairs ubiquitin thioester bond formation (densitometric analysis of several experiments indicates a 5-12 fold inhibition)(**Fig. 2b**). To test whether it confers an additional effect on the isopeptide bond formation step, we compared the formation of di-ubiquitin by similar amounts of ubiquitin-charged unmodified and modified E2-25K (**Fig. 2c**). Here, SUMO has at best a minimal effect (up to 1.5-fold reduction of the transfer rate by densitometric analysis). To investigate SUMO's effect on the thioester formation step in more detail, we tested the effect of SUMO on E2-25K in an E1 concentration- (**Fig. 2d**) and time-dependent (**Fig. 2e**) manner. Here, we included E2-25K(1–155) lacking the C-terminal UBA domain, as we obtained high-quality crystals for structure solution only for the truncated protein (see below).

Direct comparison of full-length and truncated E2-25K was complicated by the observation that E2-25K and E2-25K(1–155) differ substantially in their requirement for E1 enzyme (compare top and bottom panel of **Fig. 2d**). As the truncated protein requires at least five fold less E1 for the same amount of thioester, this suggests an inhibitory function for the UBA domain. Notably, SUMO inhibited thioester formation for both full length (up to 12-fold inhibition) and truncated (up to 5-fold inhibition) E2-25K.

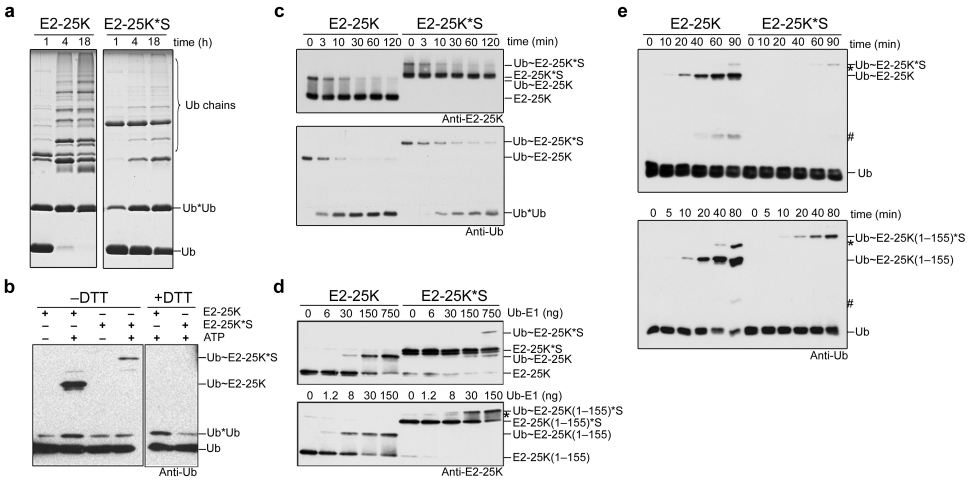

Figure 2 SUMOylation of E2-25K inhibits ubiquitin thioester formation. (**a**) Ubiquitin (Ub) chain formation. SUMO-modified (*S) or unmodified E2-25K (1.5 μg; arrows), 8 μg ubiquitin and 100 ng ubiquitin-E1 were incubated for indicated times with an ATP regenerating system at 37°C. Analysis was done by Coomassie blue staining. (**b**) Ubiquitin thioester formation. SUMO-modified or unmodified E2-25K (1 μg), 2 μg ubiquitin, 130 ng ubiquitin-E1, and ATP were incubated for 1 hour at 30°C. Analysis was done by immunoblotting. Left, nonreducing conditions allow detection of thioester; Right, reducing conditions. (**c**) Ubiquitin transfer. Equal amounts of E2-25K and E2-25K*SUMO ubiquitin thioesters were generated by incubating 10 μg E2-25K, 10 μg ubiquitin K48R, and 1.25 μg ubiquitin-E1 with ATP at 37 °C for 12 and 70 min, respectively. Ubiquitin-E1 was inhibited by EDTA, and wild-type ubiquitin was added to allow di-ubiquitin formation. Immunoblotting with anti-E2-25K (top) or anti-ubiquitin (bottom) followed. (**d**) Ubiquitin thioester formation of full-length and truncated E2-25K. 1 μg of SUMOylated or unmodified full-length (top) or truncated E2-25K(1–155) (bottom) was incubated with ATP, 1 μg ubiquitin and indicated concentrations of ubiquitin-E1 for 30 minutes at 30°C. Analysis under nonreducing conditions was by immunoblotting. Asterisk, ATP- independent unspecific band. (**e**) Experiment was done as in **d** but in a time course using 6 ng E1 for E2-25K(1–155) and 100 ng E1 for full-length E2-25. Asterisk, isopeptide linked ubiquitin to E2-25K thioester; #, di-ubiquitin.

A crystal structure of the E2-25K*SUMO conjugate

SUMO may inhibit E2-25K thioester formation by interfering with the E1-E2 interaction, by masking of the catalytic cysteine, or by inducing a structural change in E2-25K. We addressed this question by solving and comparing the crystal structures of E2-25K(1–155) and the E2-25K(1–155)*SUMO conjugate at 1.8 Å and 2.3 Å, respectively, using *in vitro* purified and modified protein (**Fig. 3** and **Table 1**). The truncated protein is active in unanchored ubiquitin chain formation in contrast to a slightly shorter fragment[14], that lacks main chain interactions and may not have folded correctly. The structure of unmodified E2-25K(1–155) shows the canonical E2-core fold (r.m.s. deviation of 1.0 Å for 147 of 155 Cα atoms) when compared to its yeast homolog Ubc1[15]. In agreement with mass spectrometry analysis the structure of modified E2-25K(1–155) (**Fig. 3a**) clearly reveals attachment of the C terminus of SUMO to Lys14 (**Fig. 3b)**. SUMO binding buries ~250 Å^2 of the accessible surface area of E2-25K. In a recently solved NMR structure of the RanGAP*SUMO complex[5], no interactions were observed between SUMO and the target other than the isopeptide bond. The E2-25K interface with SUMO, however, involves three hydrogen bonds and several hydrophobic interactions, primarily between exposed side chains of the E2-25K N-terminal helix and the backbone of the kinked SUMO C terminus (**Fig. 3c**). A kinked C terminus is also seen in structures of tetra-ubiquitin[16,17] and in a structure-based model for the ubiquitin thioester with yeast Ubc1[15], but kinking is absent in structures of complexes of deconjugating enzymes and E1[18-21].

Superposition of modified and unmodified E2-25K(1–155) gives an r.m.s. deviation of 0.70 Å (using all Cα atoms), demonstrating that SUMO does not cause substantial conformational changes as a modifier in its substrate. The only substantial difference in secondary structure elements is in the N-terminal helix, which is somewhat bent outward when SUMO is linked. Some changes are also seen in the loops between

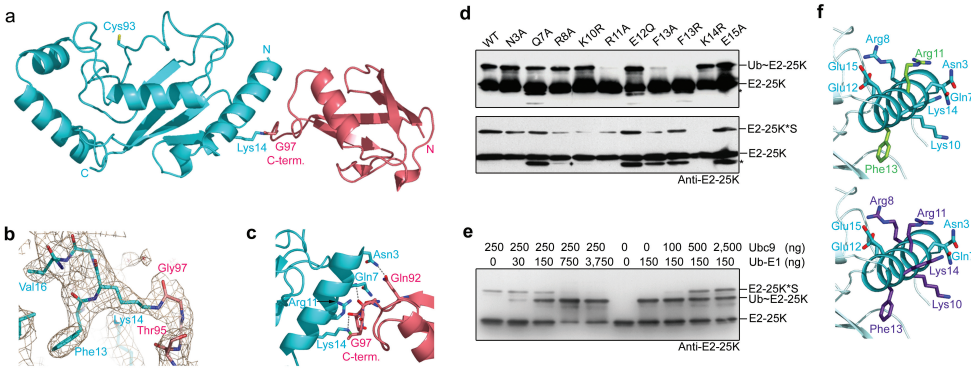

Figure 3 Crystal structure of SUMO-modified E2-25K. (**a**) The E2-25K*SUMO structure showing the covalent linkage between E2-25K (cyan) Lys14 and the C terminus of SUMO (pink). The E2-25K catalytic cysteine is yellow. (**b**) Refined electron density map ($2mF_o$- DF_c) at the linker region between E2-25K (cyan) and SUMO (pink). (**c**) The interface between E2-25K and SUMO is small but defined. Hydrogen bonds and interface side chains are shown. (**d**) Mutation analysis of the N-terminal α-helix. Top, thioester bond formation. Wild-type (WT) E2-25K or mutants (500 ng), 150 ng ubiquitin E1, 1 µg ubiquitin, and ATP were incubated for 30 min at 30°C. Analysis was done under nonreducing conditions. Bottom, SUMOylation. E2-25K wild-type or mutants (250 ng), 150 ng SUMO E1, 500 ng SUMO1, 250 ng Ubc9 and ATP were incubated 30 min at 30°C. Asteriks indicates a faster-migrating form that has also been noticed by others[38]. (**e**) Competition between ubiquitin thioester formation and SUMOylation: 500 ng E2-25K, 150 ng SUMO E1, 1 µg SUMO1, 1 µg ubiquitin, ATP and indicated amounts of ubiquitin E1 and Ubc9 were incubated 30 min at 30°C. (**f**) Position of the residues individually mutated in E2-25K. Top, residues that affect thioester formation (green). Bottom, residues affecting SUMOylation (purple). For both, residues not affecting thioester formation or SUMOylation are cyan.

the secondary structure elements, but notably, the loop containing the active site cysteine shows no substantial shifts upon SUMO modification. SUMO itself also does not undergo substantial structural changes, as its secondary structure is very similar to that of its unconjugated yeast homologue SMT3 and to that of ubiquitin[16].

Ubc9 and ubiquitin E1 compete for E2-25K access

As our structural data clearly reveal that SUMO conjugation does not result in major structural changes or masking of the catalytic cysteine, reduced thioester formation could best be explained by steric interference of SUMO with the E2-25K–E1 interaction. This interpretation is consistent with studies implicating the N terminus of conjugating enzymes in E1 interaction[21-23]. To gain further evidence for this model, we mutated nine residues in the N-terminal helix of E2-25K and tested them for ubiquitin thioester formation (**Fig. 3d**, top). Mutations in two residues, Arg11 and Phe13, caused strongly reduced thioester formation, indicating E1 interactions at these sites. Of these, Arg11 is buried upon SUMO attachment (**Fig. 3b,f**), in agreement with the idea that SUMO inhibits E1 interaction by steric hindrance *in vitro*.

Notably, SUMOylation of E2-25K also involves Arg11 and Phe13 (**Fig. 3d**, bottom). These results suggest that the ubiquitin E1 and Ubc9 directly compete for an overlapping surface on E2-25K (**Fig. 3f**). To address this experimentally, we allowed for simultaneous ubiquitin thioester formation and SUMOylation (**Fig. 3e**). In the first set of experiments we kept Ubc9 constant and increased ubiquitin E1 concentrations; in the second set we kept E1 constant and increased Ubc9 concentrations. These experiments revealed clear competition between both reactions, with a strong preference for E1-mediated thioester formation. This indicates that E2-25K can be SUMOylated only in uncharged form and at low ubiquitin E1 concentrations.

A helical SUMO acceptor site

As shown above, the acceptor lysine for SUMO conjugation in E2-25K is Lys14. This was rather surprising because Lys14 is not part of a consensus motif found in most SUMO targets (ψKXE, where ψ is a hydrophobic residue and X is any residue). Ubc9 requires both the bulky hydrophobic and the acidic side chains of the conventional consensus motif to recognize and modify its targets[24-26]. Notably, acidic residues are not required for modification of the E2-25K

acceptor site; instead, the basic residues Arg8, Lys10, Arg11 and the aromatic hydrophobic residue Phe13 seem to be involved (**Fig. 3d,f**). SUMOylation sites of mutated proteins were analyzed by mass spectrometry. Whereas most mutants involve Lys14 as major modification site, the weakly modified mutant F13A is SUMOylated at Lys10 (data not shown).

Not only the primary sequence, but also the secondary structure of the E2-25K acceptor site differs substantially from that of previously characterized SUMO targets. Whereas Lys14 is part of an α-helix, most of the known SUMO target sites fall either in unstructured N or C termini or in loop regions[12,25]. We therefore wondered whether secondary structure in conjunction with a specific primary sequence defines a SUMO acceptor site. E2-25K was an ideal model substrate for this question, as it contains three consensus site lysine residues (Lys10, Lys18 and Lys28) in close proximity to Lys14 (**Fig. 4a,b** and **Supplementary Fig. 1** online). All four lysines are exposed to the solvent in the E2-25K structure, and are found in α-helices or β-strands. We first analyzed single point mutants in each of these lysine residues for their modification *in vitro* (**Fig. 4**). Notably, only the K14R mutant was incompetent for modification, indicating that Lys10, Lys18 and Lys28 are not modified in the context of properly folded E2-25K. We then used WT and mutant peptides comprising the N-terminal helix of E2-25K (residues 2–19) to analyze the competence of Lys10 and Lys14 for modification in the absence of a helical structure (**Fig. 4c**). Circular dichroism was used to confirm the lack of helical structure (data not shown). In this case the consensus site residue Lys10 was strongly preferred over the nonconsensus residue Lys14. In summary, we showed that the ψKE motif surrounding Lys10 is recognized only as part of an extended structure, whereas the helical motif surrounding Lys14 is recognized only in the context of an α-helix, and requires basic rather than acidic residues for recognition.

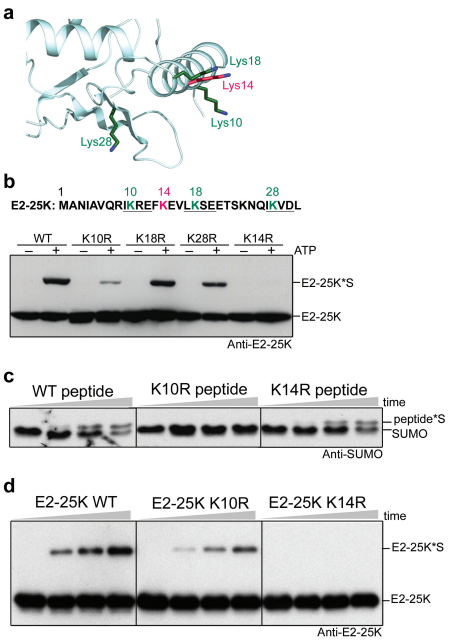

Figure 4 SUMO target sites are defined by their structural context. (**a**) Position of four lysine residues in the N terminus of E2-25K. (**b**) In the folded protein, Lys14 is the preferred substrate. Wild-type (WT) or indicated E2-25K mutant (500 ng), 1 µg SUMO1, 300 ng Aos1– Uba2, 500 ng Ubc9 and ATP were incubated for 30 min at 30°C. (**c**) In an unfolded peptide, Lys10 is preferred. Indicated peptide (20 µg), 1.5 µg SUMO-E1, 340 ng Ubc9, 15 µg SUMO, and ATP were incubated for 0–5 hours. (**d**) In folded E2-25K protein, Lys14 is preferred. Wild-type or indicated E2-25K mutant (7.5 µg), 12 µg SUMO-E1, 425 ng Ubc9, 20 µg SUMO, and ATP were incubated for 0–2 hours at 37 °C.

Discussion

Here we described the ubiquitin-conjugating enzyme E2-25K as a novel target for SUMOylation. While it is efficiently modified *in vitro* by Ubc9 and the SUMO E1 enzyme, we observed very low levels of modification *in vivo* that we could only detect upon enrichment from a cell line that stably overexpresses His-SUMO1. Because SUMO-modifying enzymes should have access to E2-25K (distributed throughout the cell), this may be due to a constant turnover by SUMO isopeptidases, or due to strict regulation of the modification. *In vitro*, SUMO interferes with E2-25K ubiquitin thioester formation. The physiological relevance of E2-25K SUMOylation cannot easily be addressed, because no E2-25K-dependent *in vivo* E3 ligases or substrates are available. At least two different scenarios are compatible with the low steady state levels of E2-25K SUMOylation: first, SUMOylation of E2-25K may act as an inhibitor of E2-25K-dependent ubiquitination, and may be spatially or temporally regulated. Alternatively, E2-25K may go through constant cycles of modification and demodification, which may contribute to assembly and disassembly of

E1-E2-E3. In this scenario, SUMOylation of E2-25K may even act as a positive regulator of E2-25K dependent ubiquitination. Such a model is supported by the observation that ubiquitin E2 N termini are involved not only in E1 but also in E3 interaction[27]. A related model has been proposed for one subfamily of ubiquitin E3 ligases, members of which are regulated by neddylation, another ubiquitin-related modification[28,29].

SUMO modification can have substantial consequences for the binding properties of its targets, either allowing or prohibiting formation of complex with partners. Whether this is due to conformational changes in the acceptor protein or due to the addition or masking of binding interfaces is unclear in most cases. A recent NMR study by Macauley et al.[5] of the RanGAP1*SUMO1 complex indicates that SUMO1 is connected to RanGAP1 like 'beads on a string'. The crystal structure analysis of SUMO-modified and unmodified E2-25K again demonstrates the absence of conformational changes in the target. Together these studies lend credit to the idea that SUMO generally functions by masking and/or providing binding interfaces rather than by inducing conformational changes to its target.

Acceptor sites for SUMO frequently fit a ψKXE consensus motif. In RanGAP1, this motif is part of an extended loop structure that is recognized by Ubc9[25]. Some nonconsensus acceptor sites have been identified in other proteins, but the structural context of these is largely unknown. An exception is PCNA, which is SUMOylated on the nonconsensus residue Lys164, which is also part of a loop structure[12]. In E2-25K both the primary sequence surrounding the acceptor site, Lys14, and the secondary structure element (an α-helix) are distinct. Although E2-25K has several consensus motifs, including one surrounding Lys10, these are not modified in the folded protein. Thus, the ψKXE motif surrounding Lys10 is recognized only as part of an extended structure, whereas the helical motif surrounding Lys14 is recognized only in the context of an α-helix, and requires a phenylalanine upstream of the lysine, and basic rather than acidic residues for recognition. These findings strongly underscore the importance of structural elements in lysine recognition for SUMOylation, which is critical for better prediction of SUMO targets. The identification and structural analysis of other proteins with helical SUMO acceptor sites and their interaction with Ubc9 will be important for precise definition of this novel motif in the future.

Table 1 Data collection and refinement statistics

	E2-25K(1–155)	E2-25K(1–155)*SUMO
Data collection		
Space group	$P2_12_12_1$	$P4_32_12$
Cell dimensions (Å)		
a, b, c	38.26, 68.15, 78.26	58.39, 58.39, 162.84
Resolution (Å)	50-1.8 (1.90-1.80)	84 50-2.3 (2.42-2.30)
R_{merge}	0.072 (0.548)	0.070 (0.511)
I / σI	17.8 (3.3)	30.6 (3.8)
Completeness (%)	100 (100)	99.9 (99.9)
Redundancy	7.1 (7.3)	13.4 (13.0)
Refinement		
Resolution (Å)	50-1.8	50-2.3
No. reflections	19,623	13,245
R_{work} / R_{free}	16.7/21.9	21.0/27.9
No. atoms		
Protein	1,278	1,869
Ligand/ion	4	0
Water	261	83
B-factors		
Protein	30.6	57.9
Ligand/ion	42.0	
Water	39.5	57.2
R.m.s deviations		
Bond lengths (Å)	0.017	0.018
Bond angles (°)	0.622	0.646

One crystal was used per data set. Values in parenthesis are for highest resolution shell.

Methods

Plasmids, enzymes and antibodies. GST–SUMO1 (1–97) and SUMO enzymes have been described[30,31]. GST–E2-25K was a gift from C. Pickart (Johns Hopkins University)[14]. GST–E2-25K(1–155) was cloned in pETM30 (European Molecular Biology Laboratory). Mutants were generated by site directed mutagenesis. For small-scale experiments, wild-type E2-25K and mutants were expressed and purified according to standard protocols, and the GST-tag was removed by cleavage with biotinylated thrombin (Novagen). Mammalian ubiquitin E1 was from Affinity or Boston Biochem. Ubiquitin was either obtained from Sigma or recloned from a vector kindly provided by N. Dantuma (Karolinska Institute) into the pETM30 vector; GST-tag was removed by TEV cleavage. Affinity-purified rabbit anti-E2-25K against full-length bovine protein was generated as described[30]. A second rabbit anti-E2-25K was provided by C. Pickart (Johns Hopkins University)[32]. Mouse monoclonal anti-SUMO (anti-GMP1), mouse monoclonal anti-ubiquitin, and secondary antibodies were obtained from Zymed, Santa Cruz, and Jackson Laboratories, respectively.

Identification of SUMOylated E2-25K in HeLa cell extracts. HeLa S3 cytosol (40 ml), 200 µg GST-SUMO1, 2 µg RanBP2ΔFG[31] and ATP were incubated for 1h at 37°C (subsequent analysis showed that RanBP2 had no effect on E2-25K SUMOylation *in vitro*, data not shown). Modified proteins were enriched on glutathione beads, washed with 50 mM Tris pH 8.0, 750 mM NaCl, 1% (v/v) NP40, eluted with 20 mM glutathione, and separated on a 7% (w/v) SDS-PAGE. Coomassie blue-stained protein bands were digested by trypsin (Promega, sequencing grade) as described[33] and desalted using homemade miniaturized reversed-phase columns[34].
MALDI-TOF mass spectra were acquired on a Reflex III instrument (Bruker Daltonik) in positive ion reflector mode. As a matrix, α-cyano-hydroxycinnamic acid was used. The E2-25K modification site was identified by online reverse-phase nanoscale liquid chromatography tandem mass spectrometry using an AB-MDS Sciex QSTAR pulsar quadrupole time-of-flight mass spectrometer.

Detection of endogenous E2-25K*SUMO1 by pull-down. A HeLa cell line expressing HisSUMO1 was provided by R. Hay (University of St. Andrews)[10]. Cells from eight 100 mm^2 flasks were lysed under denaturing condition using a standard protocol (Qiagen), and His-SUMO1-modified proteins were enriched on Ni^{2+}ProBond resin (Invitrogen).

***In vitro* SUMOylation and ubiquitination and thioester bond formation.** Small-scale *in vitro* SUMOylation assays were done as described[31]. E2-25K–ubiquitin thioester and chain formation assays were essentially done as described[31], using Uba1 and ubiquitin instead of Aos1–Uba2 and SUMO1. Chain formation involved incubation for up to 16 hours in the presence of an ATP regenerating system. Di-ubiquitin formation was carried out by initial thioester formation using Uba1, E2-25K and ubiquitin K48R. E1 activity was blocked by addition of 10 mM EDTA. Equal amounts of thioesters for SUMOylated and unmodified E2-25K were used for di-ubiquitin formation initiated by addition of excess of wild-type ubiquitin.

Expression and purification of E2-25K*SUMO1 conjugates for crystallization. GST–E2-25K(1–155) was expressed in *Escherichia coli* using IPTG induction overnight at 15°C. Purification included glutathione affinity chromatography and TEV cleavage of the GST–tag followed by size-exclusion chromatography (Superdex 75, Pharmacia). E2-25K was SUMO-modified on large scale by incubating 10 mg E2-25K(1–155) with 100 µg E1, 800 µg Ubc9 and 7.5 mg SUMO in the presence of 5 mM ATP and 5 mM MgCl2 for 4 hours at 37 °C. SUMO-modified E2-25K was purified by size-exclusion chromatography (Superdex 75) followed by anion-exchange chromatography (MonoQ).

Crystallization and data collection. Both SUMO-modified and unmodified E2-25K(1–155) were stored in 20 mM Tris pH 8.0, 100 mM NaCl, 5 mM β-mercaptoethanol and 0.1 mM PMSF at 5-10 mg ml^{-1}. E2-25K(1–155) crystals were grown at room temperature using hanging drops against 17% (w/v) PEG MME 5000, 70 mM $(NH_4)_2SO_4$, 100 mM Tris pH 8.5. E2-25K(1– 155)*SUMO crystals grew in sitting drops at room temperature against 17% (w/v) PEG 4000, 10% (v/v) glycerol, 200 mM $MgCl_2$ and 100 mM Tris pH 8.5. Cryoprotection was achieved with mother liquor supplemented with 20% (v/v) glycerol. Data were collected from a single crystal at 100 K on European Synchrotron Radiation Facility beam line ID14-2 (λ = 0.933) for E2-25K and ID14-4 (λ = 0.939) for E2-25K*SUMO. Data were processed with Mosflm and Scala[35].

Structure determination and refinement. A systematic procedure involving a subset of available E2 structures (PDB entries 1FXT, 1AYZ, 1AAK, 1QCQ, 1JAT, 1J7D and 1FZY) provided optimal phasing after molecular replacement[35], side chain mutation, automated side chain fitting in ARP/wARP[36] and rigid body refinement[35]. The best model (PDB entry 1FZY), which had an R_{factor} of 0.32 and R_{free} of 0.44, improved considerably compared with molecular replacement (R_{factor}=0.41, R_{free}=0.49), allowing almost complete ARP/wARP autobuilding of E2-25K in both crystal forms. SUMO was built manually using O[37] in the E2-25K*SUMO structure. Rebuilding and refinements were done with O[37] and Refmac[35]. Percentages of residues in most favored, additionally allowed, generously allowed and disallowed regions of the Ramachandran plot for E2-25K were 90.5, 8.8, 0.7 and 0.0, respectively. For E2-25K*SUMO they were 91.9, 7.4, 1.5 and 0.0, respectively. Crystallographic parameters are summarized in **Table 1**. All structure figures were generated using PyMOL (http://www.pymol.org).

Coordinates. Atomic coordinates and structure factors for E2-25K(1-155) and E2-25K(1-155)*SUMOM have been deposited in the Protein Data Bank (accession codes 2BEP and 2BF8, respectively).

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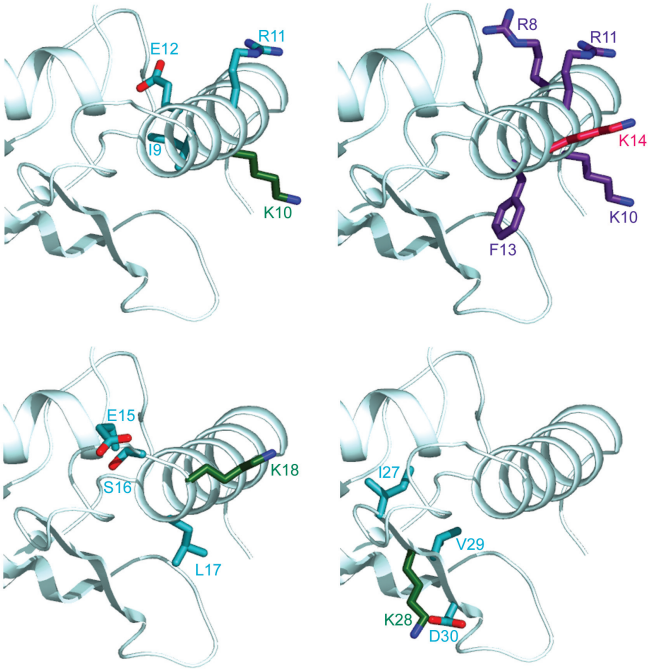

Supplementary Figure 1 Conventional SUMO sites in E2-25K structure (ball-and-stick representation), showing how the lysines (green) are exposed but the rest of the sequence motifs are buried in the folded protein. Top right panel shows Lys14 (purple), the actual site of the SUMO modification, with the amino acids that are involved in the sumoylation process according to mutation analysis (**Fig. 3d**)

Chapter 4

Ubc9 sumoylation regulates SUMO target discrimination

Puck Knipscheer[1], Willem J van Dijk[1], Alex Fish[1], Jesper V Olsen[2], Matthias Mann[2], Frauke Melchior[3],Titia K Sixma[1,#] and Andrea Pichler[3, 4, #]

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Puck Knipscheer[1], Willem J van Dijk[1], Alex Fish[1], Jesper V Olsen[2], Matthias Mann[2], Frauke Melchior[3],Titia K Sixma[1,#] and Andrea Pichler[3, 4, #]

[1]The Netherlands Cancer Institute and Center for Biomedical Genetics, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[2]Max-Planck Institute for Biochemistry, Am Klopferspitz 18, D-82152 Martinsried, Germany
[3]Georg August University of Göttingen, Humboldtallee 23, 37073 Göttingen, Germany
[4]Max F. Perutz Laboratories, Medical University Vienna, Dr. Bohrgasse 9, 1030 Vienna, Austria
#communicating authors (andrea.pichler@meduniwien.ac.at; t.sixma@nki.nl)

Posttranslational modification with *s*mall *u*biquitin related *mo*difier SUMO is a widespread mechanism for rapid and reversible changes in protein function. Considering the large number of known targets, the number of enzymes involved in modification seems surprisingly low: a single E1, a single E2, and a few distinct E3 ligases. Here, we show a novel mechanism of target discrimination: auto-sumoylation of the E2 conjugating enzyme Ubc9, while not altering its activity towards HDAC4 and E2-25K, impairs activity on RanGAP1 and strongly enhances activity on the transcriptional regulator Sp100. Enhancement is dependent on the presence of a SUMO interacting motif (SIM) in Sp100, in close proximity to its SUMO acceptor site. The crystal structure of SUMO modified Ubc9 demonstrates how the newly created binding interface can provide the gain in affinity otherwise provided by E3 ligases. This reveals a new concept for the central role of Ubc9 in target selection and modification.

Introduction

Posttranslational modification with SUMO is important for maintaining cellular integrity. Sumoylation regulates a large number of proteins involved in various cellular processes like transcriptional regulation (Girdwood et al., 2003; Seeler and Dejean, 2003; Yang et al., 2003), nucleo-cytoplasmatic transport (Pichler and Melchior, 2002), genome integrity (Muller et al., 2004), DNA repair (Hoege et al., 2002; Stelter and Ulrich, 2003) and cell cycle progression (Hay, 2005). This requires SUMO modification to be highly specific and tightly regulated. Isopeptide bond formation between SUMO and a specific lysine on its substrate depends on an energy-dependent enzymatic cascade analogous to the ubiquitination system. In an ATP-dependent reaction SUMO is activated by a single specific E1 activating enzyme, the heterodimer between Aos1 and Uba2 (also referred to as SAE1/SAE2) resulting in an E1-SUMO thioester linkage. SUMO is then transferred to the E2 conjugating enzyme Ubc9, again forming a thioester. The catalytic cleft of Ubc9 directly interacts with many substrates via their SUMO consensus motif (ΨKxE/D), but this interaction by itself is not sufficient for efficient SUMO transfer to the target lysine. Target modification therefore often depends on a third class of enzymes, the E3 ligases (Hay, 2005; Johnson, 2004; Pichler, 2004), which enhance SUMO transfer from the E2 to the substrate. So far, three different types of SUMO E3 ligases have been identified: the Siz/Pias SP-RING family (Johnson and Gupta, 2001; Kahyo et al., 2001; Kotaja et al., 2002; Sachdev et al., 2001; Schmidt and Muller, 2002), the nucleoporin RanBP2/Nup358 (Pichler et al., 2002; Pichler et al., 2004) and Pc2 (polycomb group protein 2) (Kagey et al., 2005; Kagey et al., 2003). While target recognition is mediated in part by the E2 Ubc9, E3 ligases contribute to substrate specificity and accelerate catalysis, either by stabilizing the interaction between a specific substrate and SUMO loaded E2 (SP-Ring type), or by positioning the loaded E2 for optimal transfer (RanBP2; (Reverter and Lima, 2005)). One exceptional target that does not require an E3 ligase for efficient modification is RanGAP1 (Mahajan et al., 1997; Matunis et al., 1996). Here, stabilization of the interaction

with SUMO loaded Ubc9 is mediated via a second binding interface between RanGAP1 and Ubc9 (Bernier-Villamor et al., 2002).

If one considers the large number of known SUMO targets that are modified in a temporally and spatially controlled fashion, the number of known SUMO E3 ligases seems rather low. One obvious possibility to explain this discrepancy is that many E3 ligases still await identification. This would be in line with the large number of E3 ligases known to exist in the ubiquitin field. Alternatively, regulation of sumoylation may take place largely at the level of individual target proteins. Both posttranslational modification and regulated localization can determine target accessibility to the conjugation machinery. For example, a number of proteins, including HSF-1, contain a phosphorylation-dependent sumoylation motif (PDSM) (Hietakangas et al., 2003; Hietakangas et al., 2006). Finally, regulation of the enzymes themselves could serve to determine specific target recognition. One example is the Siz1 dependent mitosis specific sumoylation of septins at the yeast bud neck (Johnson and Gupta, 2001). While Siz1 resides in the nucleus during interphase it translocates to the bud neck during mitosis. Interestingly, SUMO itself seems involved in regulating its enzymes, as it was recently described that sumoylation of the SUMO E3 ligase PIASy enhances Tcf-4 sumoylation (Ihara et al., 2005).

Modulation of localisation and activity may not be restricted to E3 ligases, but could also include Ubc9, as this enzyme directly contributes to target recognition. While nothing is known about regulated Ubc9 localisation and/or phosphorylation, posttranslational modification of Ubc9 with SUMO has frequently been observed in *in vitro* sumoylation assays ((Bencsath et al., 2002); this study). More importantly, several screens for new SUMO targets from yeast to men revealed the existence of sumoylated Ubc9 in cells (Hannich et al., 2005; Wohlschlegel et al., 2004; Zhao et al., 2004; Zhou et al., 2004); Of note, two of these studies expressed tagged SUMO from its endogenous promotor, which excludes artificial Ubc9 sumoylation due to overexpression. Finally, a recent study on mice reported tissue specific levels of sumoylated Ubc9, pointing to an important role of this modification in Ubc9 regulation (Nacerddine et al., 2005).

We previously reported that the ubiquitin E2 conjugating enzyme E2-25K is sumoylated at a non-conventional SUMO acceptor site in its N-terminus. Modification masked the E1 interaction site and thereby resulted in E2-25K inhibition (Pichler et al., 2005). Based on these findings, we

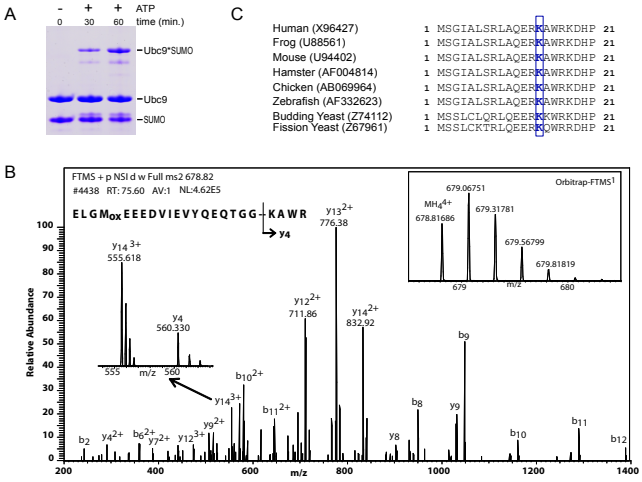

Figure 1. Ubc9 is SUMO1 modified at lysine 14. (A) In vitro sumoylation of Ubc9: 4 μg Ubc9, 500 ng Aos1-Uba2 and 3 μg SUMO1 were incubated at 37 °C in a 10 μl reaction volume without and with ATP for the indicated time periods. Samples were separated on an SDS-PAGE and stained with Coomassie. (B) Identification of sumoylated lysine-14 in Ubc9 by orbitrap tandem mass spectrometric analysis. Tryptic digestion of the sumoylated protein results in a 19mer-peptide remnant from the C-terminus of SUMO-1 (ELGMEEEDVIEVYQEQTGG-) conjugated to the targeted lysine. This leads to a mass shift of 2169.9 Da (when methionine is oxidized) and a missed tryptic cleavage at the modified lysine residue. The figure shows the fragmentation pattern of the quadruply-charged tryptic peptide (^{14}-KAWR^{17}) derived from Ubc9 that contain the sumoylated K14. The y series of ions (C-terminus containing fragments) that are produced due to fragmentation are labeled, as well as those from the b ion series (N-terminus containing fragments). The inset shows the intact peptide ion selected for sequencing. (C) Sequence alignment of the N-terminal helix of Ubc9 from various species. The SUMO attachment site, marked in blue, is highly conserved from yeast to men.

initially suspected that Ubc9 sumoylation would serve a similar negative regulatory mechanism.

Here we report the surprising finding that Ubc9 sumoylation, while taking place at a similar non-consensus site in the N-terminal helix of Ubc9, instead serves as a modulator of target selection. While modification of some targets is not affected by Ubc9 sumoylation, Ubc9 modified with SUMO is strongly impaired for RanGAP1 modification. Most strikingly, sumoylation significantly increases Ubc9 efficiency in Sp100 modification, to an extent that can otherwise only be achieved in the presence of E3 ligases. The crystal structure of SUMO*Ubc9 demonstrates no structural change in Ubc9, but reveals a newly created binding interface involving Ubc9 and SUMO. In combination with our finding that a SUMO interaction motif (SIM) in Sp100 is required for the enhanced modification, these data demonstrate that Ubc9 sumoylation serves to recruit Ubc9 to a subclass of SUMO targets.

Results and discussion

SUMO is conjugated to lysine 14 in Ubc9

To gain insight in the role of Ubc9 sumoylation, we first aimed to identify the SUMO acceptor site. Based on our previous experience with E2-25K sumoylation, where sumoylation did not take place on a consensus site motif, we decided to employ mass spectrometry to identify the Ubc9 sumoylation site. For this, we first performed an in vitro sumoylation assay by incubating the SUMO E1 (Aos1-Uba2), Ubc9 and SUMO1 in the presence of ATP (Figure 1A). Although relatively high protein concentrations are required to conjugate SUMO to Ubc9 compared to other substrates, we can clearly see the formation of Ubc9*SUMO in time. We excised the Ubc9*SUMO band from the gel and after in gel reduction, alkylation and tryptic digestion the sample was applied to orbitrap tandem mass spectrometric analysis (Olsen et al., 2004). The expected remnant 19mer-peptide from the SUMO1 C-terminus was conjugated to lysine 14 in Ubc9 leading to a mass shift of 2169,9 Da and disappearance of the peptide 14-KAWR-17 from Ubc9 (Figure 1B). No other modified peptides were found indicating that lysine 14 was the only SUMO attachment site.

Despite the presence of a consensus site for SUMO attachment at position 65 (FKDD), our data clearly demonstrate that the non-consensus site surrounding lysine 14 serves as the SUMO acceptor site on Ubc9. This site is located on the N-terminal α-helix of Ubc9, and it is structurally equivalent to the E2-25K sumoylation site. Interestingly, the motif surrounding lysine 14 is distinct from that in E2-25K (see below), but conserved in Ubc9 suggesting a general importance for this modification (Figure 1C). The relatively inefficient modification of Ubc9 in vitro is in contrast with the fact that Ubc9 was found sumoylated in many in vivo target screens (see above), which indicates that Ubc9 sumoylation in vivo is most likely regulated by an E3 ligase.

Ubc9 sumoylation does not impair SUMO thioester formation

The N-terminus of E2 conjugating enzymes is of major importance for E1 interaction and thioester formation (Bencsath et al., 2002; Huang et al., 2005; Tatham et al., 2003). Consistent with this, we previously found that E2-25K sumoylation interferes with ubiquitin E1 interaction resulting in an impaired ability to form ubiquitin thioesters (Pichler et al., 2005). As Ubc9 is modified at a structurally equivalent lysine on the N-terminal α-helix, we tested whether SUMO modification of Ubc9 also affected E1 interaction and thioester formation. For this, we purified SUMO modified and unmodified forms of Ubc9 from an *in vitro* sumoylation reaction and compared their E1 interaction in a native gel mobility assay. While

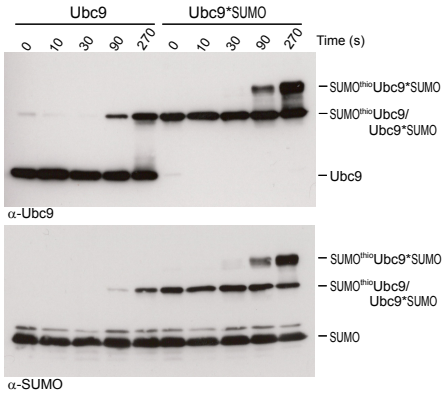

Figure 2. Ubc9 sumoylation does not affect its ability to form thioester bonds. 25 nM of either Ubc9 or Ubc9*SUMO were incubated with 70 nM Aos1-Uba2, 4.4 μM SUMO1 and ATP **at 30**°C. Reactions were stopped at indicated time points with a non-reducing buffer and separated on a 5-20% SDS gel. Analysis by immunoblotting was either with α-Ubc9 raised in goat (upper panel) or with α-SUMO1 (lower panel).

SUMO modified E2-25K is strongly reduced in E1 (Uba1) interaction compared to free E2-25K, SUMO modification of Ubc9 only has a mild effect on E1 (Aos1-Ub2) interaction (Supplementary Figure 1). Furthermore, we show that the Ubc9K14R mutant is not affected in E1 interaction in contrast to the charge-changing K14E mutation (Bencsath et al., 2002). Next, we studied the effect of Ubc9 sumoylation on thioester formation. Equimolar amounts of modified and unmodified Ubc9 were incubated with the SUMO E1, SUMO1 and ATP under conditions allowing thioester formation but not isopeptide bond formation on Ubc9, and followed the reaction over time. As shown in Figure 2, sumoylation of Ubc9 does not affect SUMO thioester formation. The mild effect of Ubc9 sumoylation on E1 interaction does not impair Ubc9*SUMO thioester formation in our assay conditions, which is in contrast to sumoylation of E2-25K. This indicates a functionally different role for SUMO modification on the SUMO E2 Ubc9, compared to the ubiquitin E2, E2-25K.

Sumoylation of Ubc9 causes a shift in target specificity

Since Ubc9 sumoylation did not affect SUMO thioester formation, we went on to test putative consequences on target modification. For this, we selected four different well-characterized SUMO targets, RanGAP1, Sp100, HDAC4 and E2-25K that vary in acceptor sites and requirements for E3 ligases and examined SUMO modification in a multiple turnover assay. First, we tested RanGAP1, a target that is efficiently sumoylated in the absence of an E3 ligase. Recombinant RanGAP1, Aos1-Uba2, SUMO1 and ATP were incubated for 30 min with increasing concentrations of Ubc9 or Ubc9*SUMO, and modification was analysed by immunoblotting with α-RanGAP1 (Figure 3A, top panel). To rule out that Ubc9 itself is modified

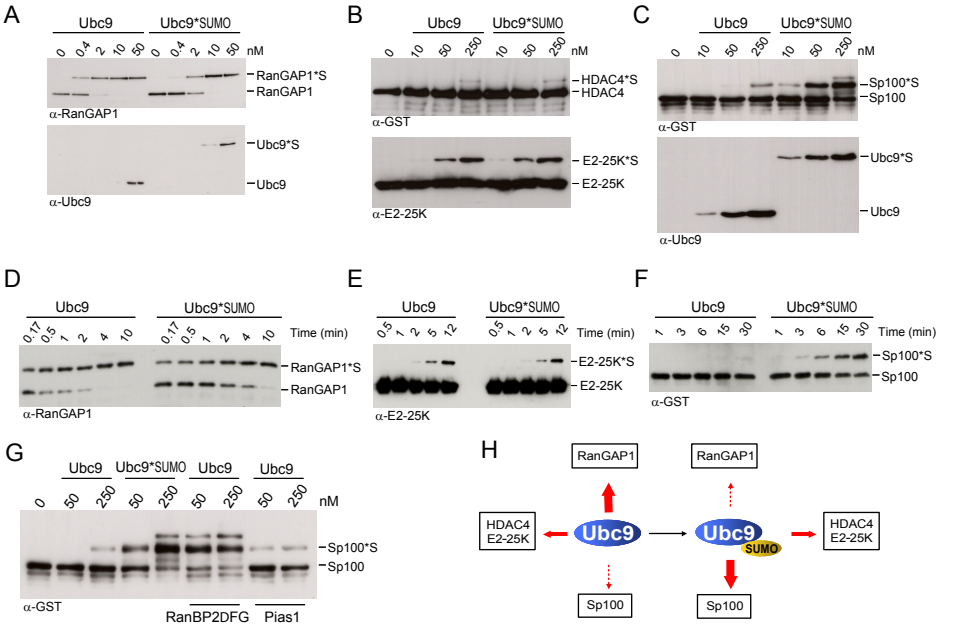

Figure 3. Sumoylation of Ubc9 causes target discrimination. (A) 2.2 μM RanGAP1 was incubated with 4.4 μM SUMO1, 70 nM Aos1-Uba2, ATP and indicated concentrations of either Ubc9 or Ubc9*SUMO at 30 °C for 30 minutes. Samples were separated on a 6% and 12% SDS-PAGE and analysed by immunoblotting with α-RanGAP1 and α-Ubc9, respectively. (B) In vitro sumoylation assays were performed as in (A) but with 127 nM GST-HDAC4 and 2.2 μM E2-25K. Separation was on 7% or 12.5% SDS gels and immunoblotting with α-GST and α-E2-25K, respectively. (C) Sumoylation as in (A) but with 200 nM GST-Sp100. Samples were separated on 7% and 12.5% SDS-PAGE and western blotting was performed with α-GST or α-Ubc9. (D) Single turnover transfer of SUMO from thioester to RanGAP1 was followed by adding preformed thioesters to 12.5 nM RanGAP1 at 4 °C for the indicated time points. (E) As in (D) but at 30 °C and with 595 nM E2-25K. (F) As in (E) but with 225 nM GST-Sp100. (G) Sumoylation of RanGAP1 in the presence of E3s, assay as in (C) but supplemented as indicated with either 8 nM RanBP2ΔFG or 170 nM PIAS1. (H) Model of the switch in target specificity via Ubc9 sumoylation.

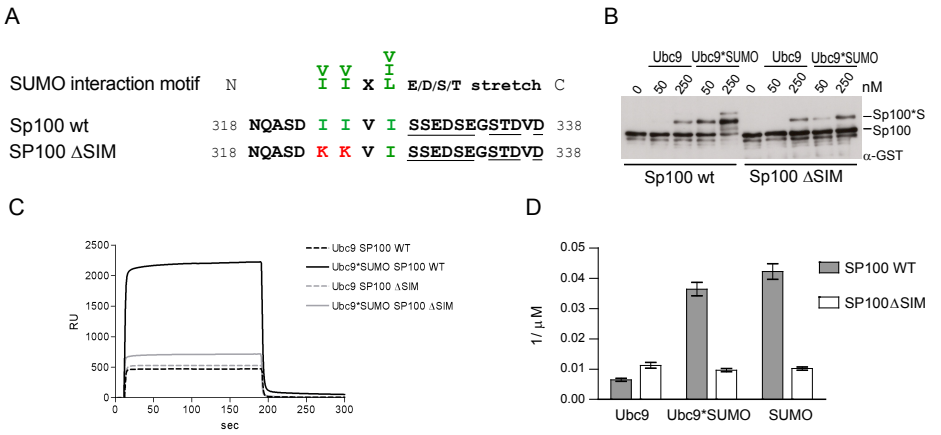

Figure 4. Enhanced sumoylation of Sp100 with Ubc9*SUMO depends on SUMO interacting motif (SIM) in Sp100. (A) SIM consensus motif aligned with the Sp100 wt and Sp100ΔSIM mutant sequences. The SIM is marked in green, mutations in SP100 are indicated in red, and acidic residues or potential phosphorylation sites are underlined. (B) In vitro sumoylation assay performed as in Figure 3C using each 200 nM GST-Sp100 wt or GST-Sp100ΔSIM, 4.4 μM SUMO1, 70 nM Aos1-Uba2, ATP and indicated amounts of Ubc9 or Ubc9*SUMO. Detection was with α-GST. (C) Apparent association constants for Ubc9, Ubc9*SUMO and SUMO towards Sp100 and Sp100ΔSIM as determined by surface plasmon resonance.

during the reaction, the same samples were also tested in an immunoblot with α-Ubc9 antibodies (Figure 3 A, lower panel). While RanGAP1 could be quantitatively modified with either form of Ubc9, there was a significant and reproducible difference in efficiency: five-fold higher concentrations of modified Ubc9 compared to unmodified Ubc9 were needed to obtain similar levels of RanGAP1 sumoylation. As Ubc9 is the rate-limiting factor in this experimental set up, this suggests a five-fold reduction in modification rate.

Surprisingly, when we then turned to HDAC4 and E2-25K, this negative effect of SUMO modification on Ubc9 function could not be generalized. As shown in Figure 3B, both HDAC4 (top panel) and E2-25K (bottom panel) were modified with equal efficiency by Ubc9 and Ubc9*SUMO. Of note, E2-25K and HDAC4 are rather inefficient targets for E3 independent modification. Therefore, much higher concentrations of Ubc9 were needed, compared to RanGAP1 sumoylation shown in Figure 3A.

In parallel, we tested another well-known SUMO substrate, Sp100 (Figure 3C). Again, we were in for a surprise: in this case Ubc9*SUMO had increased activity compared to Ubc9. In fact, Sp100 modification reached levels usually observed only in the presence of an E3 ligase. We therefore went on to compare directly Ubc9*SUMO with the combinations Ubc9 / RanBP2ΔFG and Ubc9 / PIAS1 (Figure 3D). Indeed, at 250 nM Ubc9*SUMO was as efficient in Sp100 sumoylation as RanBP2ΔFG, and clearly better than the E3 ligase PIAS1.

We have shown that SUMO modification of Ubc9 can either decrease, increase, or not affect target modification under multiple turnover conditions. The fact that thioester formation is not affected by Ubc9 modification suggests that the obtained changes in target modification take place at the final conjugation step, the transfer from the SUMO-Ubc9 thioester to the target. To prove this hypothesis we tested RanGAP1, E2-25K and Sp100 modification with modified and unmodified Ubc9 under single turnover conditions (Figure 3E-G). We preformed Ubc9 or Ubc9*SUMO thioesters and stopped the reaction with EDTA to inhibit the E1 enzyme and thus Ubc9 reloading. The reaction mixture was subsequently incubated with a target and the transfer of SUMO to the target was measured over time. Consistent with the multiple turnover reactions, SUMO modified Ubc9 results in an approximately 5 fold reduction in RanGAP1 modification, E2-25K modification is not affected and Sp100 modification is strongly enhanced compared to the reactions with unmodified Ubc9. Taken together, these findings suggest that Ubc9 sumoylation does not alter Ubc9's catalytic activity, but positively or negatively interferes with the SUMO transfer to selected substrates, and thereby modulates the target choice (Fig 3H).

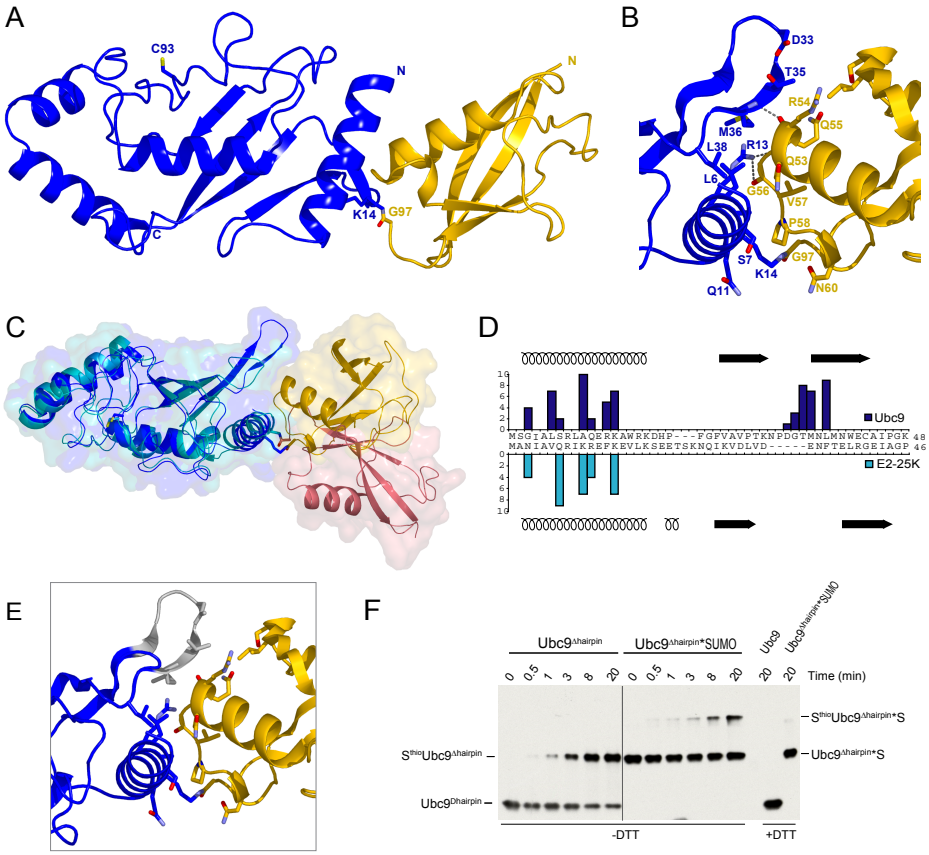

Figure 5. Crystal structure of Ubc9*SUMO. (A) The Ubc9*SUMO structure demonstrates the covalent linkage between Ubc9 (blue) Lys14 and the C-terminus of SUMO (yellow). The catalytic cysteine of Ubc9 is shown in yellow. (B) The interface between Ubc9 and SUMO with hydrogen bonds shown in gray dotted lines and interface side chains shown as sticks. (C) Superposition of Ubc9 and E2-25K, both conjugated to SUMO, in cartoon representation with surface area in the background. Ubc9*SUMO is blue (Ubc9) and yellow (SUMO), E2-25K*SUMO is cyan (E2-25K) and pink (SUMO). (D) Percentage of surface area buried per residue as calculated by PISA (Krissinel and Henrick, 2005) in the interface of E2 and SUMO, plotted against the sequence of Ubc9 compared to E2-25K. Ubc9 residues are shown in blue, E2-25K residues shown in cyan. Secondary structure is depicted on top and bottom. (E) Structural model of the Ubc9$^{\Delta hairpin}$ mutant. Deleted β-hairpin is shown in gray, Ubc9 without the hairpin shown in blue, SUMO shown in yellow. (F) Thioester formation assay for Ubc9$^{\Delta hairpin}$ (670 nM) versus Ubc9$^{\Delta hairpin}$*SUMO (670 nM) with 260 nM Aos1-Uba2, 6.7 μM SUMO. Incubation was at 30°C and reactions were stopped at indicated time points with a non-reducing buffer and separated on a 15% SDS gel. Analysis by immunoblotting was with α-Ubc9 raised in goat.

A SIM recruits Ubc9*SUMO to Sp100

Next, we set out to elucidate the mechanism behind the enhancement of Sp100 modification by Ubc9*SUMO. One possibility is that SUMO attachment adds a new binding interface to Ubc9, allowing it to better interact with Sp100, similar to its role in RanGAP1 - RanBP2 interaction at nuclear pore complexes (Mahajan et al., 1997; Matunis et al., 1998; Reverter and Lima, 2005). Several groups mapped a motif required for non-covalent SUMO interaction, the so-called SUMO interaction motif or SIM (also referred to as SUMO binding motif or SBM) (Hannich et al., 2005; Hecker et al., 2006; Minty et al., 2000; Song et al., 2004; Song et al., 2005). The minimal motif consists of a short hydrophobic stretch (V/I)(V/I)X(V/I/L), which can bind SUMO in both orientations and is flanked in many cases by additional acidic residues or potential phosphorylation sites (Figure 4A)(Hecker et al., 2006; Song et al., 2005). Structural studies of SUMO bound to a SIM have revealed that the hydrophobic residues add a β-strand to a β-sheet of SUMO (Reverter and Lima, 2005; Song et al., 2005). Interestingly,

this binding motif was identified in SUMO targets but also in enzymes of the SUMO cascade like the SUMO E1 and several SUMO E3s (Hecker et al., 2006; Minty et al., 2000; Reverter and Lima, 2005; Song et al., 2004).

Sp100 interaction with SUMO was recently demonstrated by a yeast two hybrid assay (Hecker et al., 2006), suggesting the presence of a SIM. Therefore, we analysed the Sp100 protein sequence for such a motif and indeed found a hydrophobic stretch at position 323 to 326 followed by an acidic region (Figure 4A). We mutated the first two hydrophobic amino acids of the SIM motif in Sp100 to lysines (I323K, I324K), mutations earlier shown to effectively disrupt SUMO interaction of such motifs (Song et al., 2004). We tested this Sp100 SIM mutant (Sp100ΔSIM) for sumoylation with unmodified and modified Ubc9 (Figure 4B). In the presence of unmodified Ubc9, both wt and mutant Sp100 were sumoylated at similar low levels, indicating that mutagenesis did not affect Sp100 folding. However, only Sp100 wt was efficiently modified by sumoylated Ubc9 in contrast to the Sp100 SIM mutant. Mutation of the SIM in Sp100 clearly reverses the enhancing effect of Ubc9*SUMO compared to Ubc9 alone. To prove that this is indeed due to binding of the SIM to SUMO on Ubc9 we performed surface plasmon resonance (SPR) experiments in a Biacore flow system. GST-Sp100 wt and GST-Sp100ΔSIM were bound to the chip via amine coupling and their interaction with Ubc9, Ubc9*SUMO as well as with free SUMO was measured. The shape of the response curve is indicating either a relatively low binding affinity, or a high off rate (Fig 4C). By measuring response curves at various concentrations we determined the affinity for free SUMO and modified and unmodified Ubc9 for Sp100 and Sp100ΔSIM (Figure 4D). The affinity of Ubc9 for the target Sp100 (Kd=155 ± 11 μM) presumably reflects the affinity of the E2 for the SUMO target site ΨKxE/D. The presence of SUMO in Ubc9*SUMO increases the affinity for Sp100 five-fold (Kd=27.5 ± 1.7). This increase is caused by the presence of the SIM on Sp100 since mutation of the SIM reduces the affinity of Ubc9*SUMO for Sp100 (Kd = 104 ± 6) to levels comparable to unmodified Ubc9. The increase in affinity for sumoylated Ubc9 is due to an additional interface that is solely formed by the SUMO moiety since the affinity of Sp100 for free SUMO (Kd=23.7 ± 1.4) is very similar to Ubc9*SUMO and is reduced to the same extent by mutation of the SIM (Kd=99 ± 5). However, the SIM mutations in Sp100 do not completely abolish the interaction between SUMO and the SIM since there is still some affinity of the Sp100 SIM mutant for free SUMO. This also explains the fact that the Sp100 SIM mutant has residual activity with SUMO modified Ubc9 compared to unmodified Ubc9 in Sp100 modification assay (Fig 4B).

These results show that the affinity of Ubc9*SUMO for Sp100 is not higher than the added affinities for Ubc9 and SUMO and we conclude that there is no cooperativity between the Ubc9 and SUMO binding sites on Sp100. Therefore, Ubc9 sumoylation introduces an additional binding interface for selected targets resulting in enhanced target modification.

Crystal structure of Ubc9*SUMO

To study the effect of covalent SUMO modification on the Ubc9 conformation we solved the crystal structure of Ubc9 modified with SUMO1 (Table 1). We used a deletion mutant of SUMO1 lacking the flexible N-terminal 20 amino acids (SUMOΔN20) for crystallization. As shown in Figure 5A the structure of the covalent complex (Ubc9*SUMO) confirmed complex is very similar to non-linked SUMO, except for the C-terminal tail which connects SUMO to Ubc9. The r.m.s. deviation with other SUMO crystal structures is between 0.66 and 0.85 Å for the SUMO core domain (70 Cα atoms) (Lois and Lima, 2005; Mossessova and Lima, 2000; Pichler et al., 2005; Reverter and Lima, 2004; Reverter and Lima, 2005). The interface between Ubc9 and SUMO is small but defined and buries 345 Å^2 of solvent-accessible surface area on Ubc9 and 353 Å^2 on SUMO. It comprises 4 direct and several water-mediated hydrogen bonds. On the Ubc9 side, the residues interacting with SUMO are situated on the N-terminal helix and on, or close to, the β-hairpin between the first and the second β-strand (Figure 5B). For SUMO, besides the C-terminus, residues 53-58 of the loop between α-helix 1 and β-strand 3 play the most important role in the interaction with Ubc9, contributing 3 hydrogen bonds to the interface (Figure 5B).

Most important in the interface is the covalent bond between SUMO and Ubc9. Since SUMO linkage to Ubc9 takes place on the equivalent lysine compared to E2-25K and both target sites do not conform to classical consensus sites

Table 1: Data Collection and Refinement Statistics

	Ubc9*SUMO
Data collection	
Space group	$P2_12_12_1$
Cell dimensions: a, b, c (Å)	27.5, 66.6, 122.6
Resolution (Å)	60-2.2 (2.32-2.21)
R_{sym}	14.4 (43.9)
I/σI	5.2 (1.8)
Completeness (%)	97.3 (81.5)
Redundancy	12.4 (10.0)
Refinement	
Resolution (Å)	60-2.2
Number reflections	117850
R_{work}/R_{free} (%)	18.3 / 25.8
Number of atoms	
Protein	1895
Water	150
Ion	3
Rms Deviations	
Bond lengths (Å)	0.015
Bond angles (°)	1.601

Highest resolution shell is shown in parenthesis.

and are situated on a helix, we compared the sequence context around the target sites. For the E2-25K helical acceptor site, the following residues have been shown to be most important for sumoylation: Arg8, Lys10, Arg11 and Phe13 (Pichler et al., 2005). Of these residues, only Arg8 is conserved in Ubc9. Clearly, the E2s have different needs for the molecular environment of their modification sites and further investigation is required to show whether there are general requirements for sumoylation of helical acceptor sites.

Although SUMO is attached to the equivalent site in both Ubc9 and E2-25K, it is obvious from the structure that the orientation of SUMO relative to the E2s is substantially different in Ubc9*SUMO compared to the E2-25K*SUMO complex (Pichler et al., 2005). Upon superposition of the two E2s there is only minimal overlap between the conjugated SUMO molecules (Figure 5C). A major difference between E2-25K and Ubc9 is a β-hairpin which protrudes from the Ubc9 core domain into the solvent (Figure 5B and 5D). This hairpin consists of a 5 amino acid insertion (31-35) that is conserved in Ubc9 orthologs but not found in other E2 conjugating enzymes. It interacts with SUMO in addition to the interaction with the N-terminal helix of Ubc9 (Figure 5D). This extra interaction with the Ubc9-specific β-hairpin could explain the different positioning of covalently bound SUMO on Ubc9 compared to E2-25K, and be the origin of the functional difference between these E2s upon sumoylation. To test this hypothesis we created a mutant of Ubc9 that lacks this β-hairpin ($Ubc9^{\Delta hairpin}$), in which we replace amino acid 30 to 36 with two glycines to retain the hairpin character (Figure 5D and 5E). If this mutant can no longer stabilize the position of SUMO, it should behave like E2-25K*SUMO and $Ubc9^{\Delta hairpin}$*SUMO should show reduced E1 interaction and thioester formation. Indeed, as can be seen in Figure 5F, thioester formation is inhibited in this SUMO modified $Ubc9^{\Delta hairpin}$ and to a similar extent as observed for SUMO modified E2-25K (Pichler et al., 2005). Therefore, we conclude that the positioning of SUMO with respect to the E2 is important for the functional role of this modification.

Does the position of SUMO attached to Ubc9 give insight how modification affects Ubc9 target choice? From our structural data we can not explain the inhibitory effect on RanGAP1 sumoylation: Comparison of the Ubc9*SUMO structure with the Ubc9-RanGAP1 complex (Bernier-Villamor et al., 2002) (Supplementary Figure 1) shows that RanGAP1 and SUMO are located on different sites of Ubc9 and therefore steric hindrance between the target and SUMO is unlikely. However, from the structure we cannot exclude that the SUMO conjugated to Ubc9 has an effect on the positioning of the thioester. Since the transfer of SUMO from the thioester to the target is known to be extremely efficient for RanGAP1 specifically, thioester positioning could become rate limiting for RanGAP1 and not for the other targets. In contrast to RanGAP1, for Sp100, the structure does offer an explanation for the change in modification with SUMO modified Ubc9.

The newly created binding interface

In addition to the binding interface of Ubc9 with the consensus site of Sp100, SUMO modification of Ubc9 creates an additional, high affinity binding interface for Sp100. The newly created combinatorial binding interface is shown in Figure 6 and shows the positioning of the catalytic cleft and the SIM interaction site on the surface of Ubc9*SUMO. This demonstrates that the SIM has to be positioned at a defined distance to the SUMO attachment site in the substrate. The mechanism of creating a second Ubc9 binding interface is analogous to the additional Ubc9 interaction that is found on RanGAP1 or created

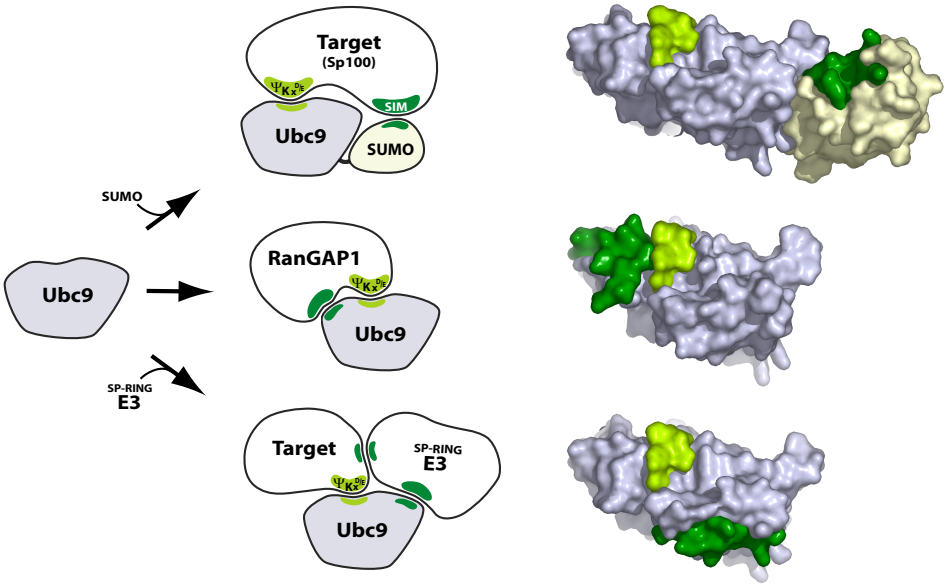

Figure 6. Model of Ubc9 target discrimination via gain in affinities. Left panel shows a cartoon of Ubc9-target interactions, mediated either through SUMO, target or an E3 ligase . Right panel demonstrates the distinct surfaces in Ubc9 involved in these interactions. The catalytic cleft in Ubc9 interacts with its targets via the SUMO consensus motif (light green) which usually results in inefficient in vitro sumoylation. Stabilization of this interaction enhances modification achieved by gain in affinity via introducing additional binding interfaces (dark green). Sumoylation of Ubc9 increases interaction of targets with a SIM in a defined distance as we demonstrated for Sp100 in this study (top panel). RanGAP1 itself contains two binding interfaces (Bernier-Villamor et al., 2002)(middle panel) and is an unusually efficient SUMO substrate. Similarly the SP-RING E3 ligases can stabilize the interaction between the target and Ubc9 resulting in enhanced modification (bottom panel).

by the SP-RING E3 ligases which also stabilise the interaction between loaded E2 and the target (Figure 6). Notably, each of these mechanisms involve a different surface on Ubc9 in addition to the catalytic cleft but all function by enhancing E2-target interaction (Fig 6).

In conclusion, this study has shown that sumoylation of the SUMO conjugating enzyme Ubc9 regulates target discrimination. Modification of some substrates is inhibited, others are unaffected, and selected substrates are enhanced. Enhancement is due to the generation of an additional binding interface that recognizes targets containing a SIM correctly oriented with respect to the SUMO acceptor site. The function of Ubc9 modificaton with SUMO is therefore comparable to the function of RING E3 ligases. Together, these findings emphasize the unique role of Ubc9 in target discrimination, and add one building block to how target specific sumoylation can be accomplished with a limited number of enzymes.

Experimental procedures

Plasmids and antibodies

SUMO(ΔN20), Ubc9, RanBP2ΔFG, Aos1-Uba2, RanGAP1 (Pichler et al., 2002; Pichler et al., 2005), GST-Sp100 (Seeler et al., 2001), GST-HDAC4 (Kirsh et al., 2002), GST-Pias1 (Sapetschnig et al., 2002) and GST-E2-25K (Haldeman et al., 1997) were described before. GST-Sp100 mutants were generated by site-directed mutagenesis. Mouse α-SUMO1 was obtained from Zymed, mouse α-Ubc9 used for pull downs was from BD Biosciences, goat α-Ubc9, rabbit α-GST, goat α- RanGAP1 and rabbit α-E2-25K were described before (Pichler et al., 2002; Pichler et al., 2005). Secondary antibodies were from Jackson Laboratories, Biorad or Biosource.

Protein expression and purification

Purification of SUMO1, Aos1-Uba2, GST-Sp100, GST-HDAC4, E2-25K, RanGAP1 RanBP2ΔFG and Pias1 was performed as described (Pichler et al., 2002; Pichler et al., 2005; Pichler et al., 2004). Ubc9 and Ubc9$^{\Delta hairpin}$ were expressed in BL21(DE3) cells using IPTG induction overnight at 15 °C. Purification was performed on a POROS S column in 20 mM Bis-Tris pH 6.5, 50 mM NaCl, 0.1 mM PMSF, 1 mM DTT, and eluted with a salt gradient between 0.05 to 1 M NaCl. Ubc9 containing fractions were

concentrated, pooled and further purified by gel filtration on a Superdex 75 column in 20 mM Tris pH 8.0, 100 mM NaCl, 0.1 mM PMSF, 1 mM DTT.

Multiple and single turnover in vitro sumoylation and thioester bond formation

In vitro modification and thioester bond formation assays for Ubc9 and E2-25K were performed as described in Pichler et al 2002. Thioester formation assays for the Ubc9$^{\Delta hairpin}$ mutant were performed in buffer containing 20 mM HEPES pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 0.05% Tween 20, 0.2 mM ovalbumin and 0.1 mM DTT with 260 nM Aos1-Uba2, 6.7 µM SUMO and 670 nM Ubc9$^{\Delta hairpin}$ or Ubc9$^{\Delta hairpin}$*SUMO. Reactions were started by addition of 5 mM ATP and time points were immunoblotted and analysed with α-Ubc9.

Single turnover assays were performed by generating the E2-SUMO thioester at 30°C in buffer containing 20 mM HEPES pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 0.05% Tween 20, 0.2 mM ovalbumin and 1 mM DTT. Reaction mix contained 0.9 µM Aos1-Uba2, 7.2 µM SUMO1, 3.7 µM Ubc9 or Ubc9*SUMO and 5 mM ATP and was incubated for 4 minutes. The reaction mixture containing the E2-SUMO-thioester was then diluted in the same buffer supplemented with 10 mM EDTA (dilution buffer) to inactivate the E1. For GST-Sp100 and E2-25K thioesters were diluted 15 times after which 225 nM GST-Sp100 or 595 nM E2-25K was added and time point were taken during the incubation at 30°C. Since the RanGAP1 reaction is extremely fast thioesters needed to be diluted 250 times with dilution buffer to be able to follow the reaction upon addition of 12.5 nM RanGAP1 at 4°C. Samples were run on 15 or 8% SDS gels and bands were visualized by immunoblotting against α-GST, α-E2-25K or α-RanGAP1. A control blot was analysed with α-Ubc9 (suppl data).

Surface plasmon resonance

Surface plasmon resonance spectroscopy (SPR) was performed at 25°C on a BIAcore T100. Roughly 8000 response units of GST, GST-SP100WT or GST-SP100ΔSIM were immobilized on different flow cells of a CM5 sensor chip (Biacore) using amino coupling. Different concentrations of Ubc9, Ubc9*SUMO or SUMO in running buffer (25 mM HEPES pH 7.3, 100 mM NaCl, 0.5 mM EDTA and 0.05% Tween-20) were injected across the chip at 30 ml/min. Saturation binding values were measured for each protein, using the flow cell with GST as reference, and plotted as a function of concentration. The apparent dissociation constants (Kd) and association constants (Ka) for the interactions were determined by fitting the data according to a steady state affinity model using the Prism 4.03 software (GraphPad Software, Inc.).

Ubc9*SUMO purification and crystallization

Ubc9 and Ubc9$^{\Delta hairpin}$ were modified with SUMO1 (or SUMOΔN20) in large scale by incubating 15 mg Ubc9, 1 mg Aos1-Uba2, 11 mg SUMO1, and 5 mM ATP in 3 ml of 20 mM Tris 8.0, 100 mM NaCl, 0.1 mM DTT and 5 mM $MgCl_2$ for 4 to 6 hours at 37 °C. Ubc9*SUMO and Ubc9 were separated by applying the reaction mixture to a MonoQ column. The Ubc9 and Ubc9*SUMO containing peaks were pooled separately and both were next applied to a MonoS column as a final purification step. After concentration proteins were stored at -80 °C in a buffer containing 20 mM Tris 8.0, 100 mM NaCl, 1 mM DTT and 0,1 mM PMSF. Ubc9*SUMO (4.2 mg/ml) was crystallized at room temperature using hanging drops against 24% (w/v) PEG3350, 200 mM Sodium Formate and 100 mM Bis-Tris Propane. Cryoprotection was achieved by increasing the concentration of PEG3350 in the mother liquor to 40% (w/v).

Mass spectrometry.

The protein band containing sumoylated Ubc9 was excised from an SDS-PAGE gel and subjected to in-gel reduction, alkylation, trypsin digestion and subsequent sample desalting and concentration, as previously described [Olsen 2004]. The resulting peptide mixture was analyzed by nano-HPLC-MS/MS using an Agilent 1100 nanoflow system connected to a hybrid linear ion trap orbitrap (LTQ-Orbitrap) mass spectrometer (Thermo Electron, Germany), equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark), essentially as described (Olsen et al., 2005).

Data collection and structure determination

Data were collected from a single crystal at 100K using a Cu-Kα source, helios optics (Bruker) and a MAR345 X-ray detector. Processing of the data was performed using MOSFLM and SCALA (Collaborative Computational Project 4, 1994). The structure was solved by molecular replacement with the program Molrep (Collaborative Computational Project 4, 1994) using both Ubc9 (Tong et al., 1997) and SUMO (Pichler et al., 2005). Rebuilding was done with Coot (Emsley and Cowtan, 2004) and ARP/wARP (Perrakis et al., 1999) and refinement was done with REFMAC (Collaborative Computational Project 4, 1994). Percentages of residues in the most favoured, additionally allowed, generously allowed and disallowed regions of the Ramachandran plot were 92.5, 6.5, 1.0 and 0.0 respectively. The model includes 150 water molecules and one formate molecule from the crystallization buffer. Crystallographic parameters are summarized in Table 1. Buried solvent-accessible surfaces areas between the molecules were calculated with the program PISA (Krissinel and Henrick, 2005). Besides the described Ubc9-SUMO interface around K14 there were two other interfaces found by the program, one between two Ubc9 molecules and another between Ubc9 and another SUMO molecule in the crystal lattice (manuscript in preparation). Multiangle laser light scattering and gel filtration experiments showed only monomers, indicating that these interactions are not present in solution for Ubc9*SUMO.

All structure figures were generated using Pymol (http://www.pymol.org). Atomic coordinates and structure factors have been deposited to the Protein Data Bank with accession code xxxx.

Gel shift assays

Interaction of SUMO E1 with Ubc9 and Ubc9*SUMO was determined by a native gel mobility shift assay. Ubc9 or Ubc9*SUMO were incubated with increasing amounts of E1 for 15 minutes at room temperature. Bound and unbound forms were separated on a 4.5 % polyacrylamide

gel (acrylamide:bis, 37.5:1) in a Tris-Borate buffer pH 8.0, containing 2% glycerol. Loading and running buffer were also buffered with Tris-Borate pH 8.0. Gels were soaked in a 0.1% SDS solution prior to the blotting procedure. Analysis was done using α-Ubc9. E2-25K and E2-25K*SUMO gel shifts were performed identical except that the gel contained 8 % polyacrylamide gel (acrylamide:bis, 29:1) in a Tris buffer pH 8.3 and a Tris-glycine running buffer was used. Analysis was done using α-E2-25K.

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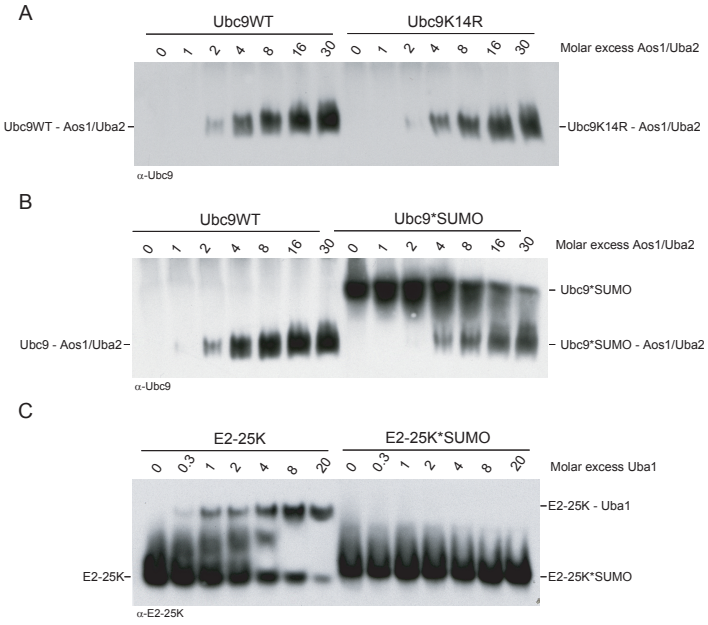
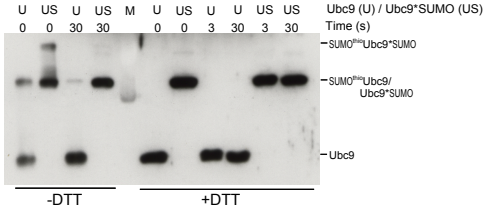
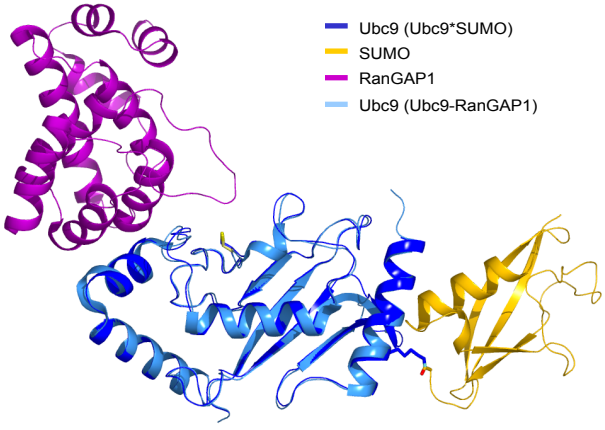


Supplementary figure 1: SUMO modification of Ubc9 has only minor effect on E1 interaction. (A) Ubc9 or Ubc9*SUMO (400 nM) were incubated with increasing amounts of SUMO E1 and complex formation was visualized by immunoblotting on a native gel using an α-Ubc9 antibody. Free Ubc9 is positively charged under these conditions and therefore does not enter the gel while Ubc9 in complex with E1 does. (B) Same as in (A) but comparing Ubc9WT with Ubc9*SUMO. (C) Same as in (A) but comparing ubiquitin E1 interaction with E2-25K and E2-25K*SUMO using an α-E2-25K antibody.



Supplementary figure 2: Control blot for single turnover reactions. Preformed thioesters were added to 225 nM Sp100 at t=0. At the start of the reaction about half of the Ubc9 or Ubc9*SUMO is loaded with SUMO, after 30 minutes most of the loaded complexes are gone and no self-conjugated Ubc9 or Ubc9*SUMO has been formed.



Supplementary figure 3: RanGAP1 interacts with Ubc9 on the opposite site compared to the covalently linked SUMO. Superposition of Ubc9 from the Ubc9-RanGAP1$^{420-589}$ structure (Bernier-Villamor et al. 2002) and the Ubc9*SUMO structure. Catalytic cysteine of Ubc9 is shown in yellow.

Chapter 5

Non-covalent interaction between Ubc9 and SUMO promotes SUMO chain formation

Puck Knipscheer[1], Willem J van Dijk[1], Jesper V Olsen[2], Matthias Mann[2] and Titia K Sixma[1,#]

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Puck Knipscheer[1], Willem J van Dijk[1], Jesper V Olsen[2], Matthias Mann[2] and Titia K Sixma[1,#]

[1]The Netherlands Cancer Institute and Center for Biomedical Genetics, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[2]Max-Planck Institute for Biochemistry, Am Klopferspitz 18, D-82152 Martinsried, Germany
[#]communicating author: t.sixma@nki.nl

The ubiquitin related modifier SUMO regulates a wide range of cellular processes by post-translational modification with one, or a chain of SUMO molecules. Sumoylation is achieved by the sequential action of several enzymes in which the E2, Ubc9, transfers SUMO from the E1 to the target mostly with the help of an E3 enzyme. In this process Ubc9 forms a thioester bond with SUMO, but also interacts with SUMO non-covalently. Here we show that this non-covalent interaction promotes the formation of short SUMO chains on targets such as Sp100 and HDAC4. We present a crystal structure of the non-covalent Ubc9-SUMO1 complex, showing that SUMO is located far from the E2 active site and resembles the non-covalent interaction site for ubiquitin on UbcH5c and Mms2. Structural comparison suggests a model for poly-sumoylation involving a mechanism analogous to Mms2-Ubc13 mediated ubiquitin chain formation.

Introduction

SUMO is a ubiquitin-related post-translational modifier that plays an important role in many cellular pathways including transcriptional regulation, intracellular transport, DNA repair and replication (Pichler and Melchior, 2002; Hoege *et al.*, 2002; Yang *et al.*, 2003; Stelter and Ulrich, 2003; Seeler and Dejean, 2003; Girdwood *et al.*, 2003; Muller *et al.*, 2004). Sumoylation of substrates generally functions by modulating their interaction properties with other proteins. Although SUMO has been detected mostly as single molecule modification, recent reports show that formation of SUMO chains is also observed for SUMO1 in vitro (Pichler *et al.*, 2002; Pedrioli *et al.*, 2006; Yang *et al.*, 2006) and for SUMO2/3 in vitro and in vivo (Tatham *et al.*, 2001; Fu *et al.*, 2005).

The process of SUMO modification is chemically similar to that of ubiquitin conjugation and the enzymes involved are mostly homologous. This involves the ligation of the C-terminus of the modifier to a lysine residue in the substrate, mediated by a highly regulated three-step cascade. For SUMO this requires Aos1-Uba2 as the E1 or activating enzyme, Ubc9 as the E2 or conjugating enzyme and in most cases an E3 ligase such as PIAS, Pc2 or RanBP2 (Melchior, 2000; Johnson, 2004). In the first step a thioester bond is formed between the modifier and the catalytic cysteine of the E1 enzyme in an ATP-dependent reaction. This thioester bond is subsequently transferred to the catalytic cysteine of the E2 enzyme, and in the last step the modifier is ligated to the ε-amino group of a lysine on the substrate with or without the help of an E3 ligase. In contrast to ubiquitin conjugation the E2 enzyme in sumoylation plays an active role in target recognition by interacting with a ΨKxE/D consensus site sequence present on most, but not all targets (Sampson *et al.*, 2001).

There are four vertebrate SUMO isoforms with partially overlapping target specificity. SUMO2 and SUMO3 differ only by three N-terminal residues and they share 45% sequence identity with SUMO1. The recently identified SUMO4 is more similar to SUMO2/3 (87%) than to SUMO1 (41%). Most of the SUMO1 in cells is found in conjugates whereas there is a large pool of free cellular SUMO2/3 (Saitoh and Hinchey, 2000; Tatham *et al.*, 2001; Ayaydin and Dasso,

2004). Apparently, only SUMO2/3 form chains on substrates in vivo, whereas SUMO1 chains have only been shown in vitro (Pichler *et al.*, 2002; Pedrioli *et al.*, 2006; Yang *et al.*, 2006). The SUMO2/3 chains are linked through lysine 11, located in a traditional SUMO consensus motif in the flexible N-terminus of SUMO2/3 and seem to play a role in PML localization (Fu *et al.*, 2005). The single yeast SUMO homologue, Smt3, also forms chains in vivo, which are important for the regulation and assembly of the synaptonemal complex during meiosis (Bylebyl *et al.*, 2003; Cheng *et al.*, 2006).

Transient interaction is an important feature of the sumoylation process. Ubc9, the E2, takes a central place by interacting with the E1, SUMO, the E3 and the target at various stages. Structural studies have revealed the nature of interaction of Ubc9 with a target (Bernier-Villamor *et al.*, 2002) and with an E3 enzyme (Reverter and Lima, 2005). Mutational analysis has indicated the interface between Ubc9 and the SUMO E1 to be mainly through its N-terminal helix and the loop between the first and the second β-strand (Bencsath *et al.*, 2002). This would suggest a similar interaction as was recently shown for another ubiquitin-like molecule, Nedd8, with its E1 APPBP1-UBA3 (Huang *et al.*, 2005).

Ubc9 interacts with SUMO both in the thioester intermediate, a complex which has been structurally characterized for several ubiquitin E2s with ubiquitin (Hamilton *et al*. 2001; McKenna *et al.*, 2003b), as well as in a non-covalent manner. This non-covalent Ubc9-SUMO interaction involves the N-terminal helix of Ubc9, as well as the loop between this helix and the first β-strand, a surface that is also partially used for E1 interaction (Liu *et al.*, 1999; Bencsath *et al.*, 2002; Tatham *et al.*, 2003). As a consequence, SUMO and the E1 can compete directly for interaction with the E2 (Bencsath *et al.*, 2002). The role of the non-covalent interaction between SUMO and Ubc9 is unclear and functional studies have been complicated by this shared interaction site. This non-covalent binding between E2 and modifier is not unique for SUMO, as ubiquitin can also interact non-covalently with some of its E2 enzymes. The details of this interaction were recently shown for ubiquitin bound to UbcH5c (Brzovic *et al.*, 2006) and to the E2 variant enzyme Mms2 (Brzovic *et al.*, 2006; Lewis *et al.*, 2006). The E2-variant Mms2 is thought to position ubiquitin for creation of lysine 63 linked chains of ubiquitin by the E2 Ubc13. The non-covalent interface of UbcH5c is also important for chain formation, but this is thought to follow a different mechanism.

Here we show the crystal structure of a non-covalent complex between SUMO1 and Ubc9. SUMO1 interacts through its β-sheet with a secutive stretch in Ubc9 connecting the first helix and strand. This site is located distant from the active site cysteine and resembles the Mms2 and UbcH5c ubiquitin non-covalent interfaces. We show that both SUMO1 and SUMO2 similarly interact with Ubc9. The high resolution structure enabled us to identify Ubc9 and SUMO mutants that specifically inhibit the interaction between the two proteins. These mutants were used to show that interference with this non-covalent interaction does not affect SUMO thioester formation but strongly reduces SUMO2 chain formation on several targets. A model is presented in which the non-covalent interaction between SUMO and Ubc9 mediates SUMO chain formation involving a mechanism similar to K63-linked ubiquitin chain formation by the Mms2-Ubc13 heterodimer.

Results

Crystal structure of non-covalent Ubc9-SUMO complex

To get insight in the functional importance of the non-covalent interaction between Ubc9 and SUMO we solved the crystal structure of this complex using human Ubc9 and SUMO1 lacking the flexible N-terminal 20 amino acids (SUMOΔN20) (Table I). Ubc9-SUMO crystals were grown by mixing the two components in a hanging drop crystallization set-up. The quality of the crystals allowed high resolution data collection after which the structure was solved by molecular replacement using the structures of Ubc9 (Tong *et al.*, 1997) and SUMO1 (Pichler *et al.*, 2005) as search models. The crystal structure shows that non-covalent interaction of SUMO1 with Ubc9 occurs on the backside of Ubc9 with respect to the active site cysteine (Figure 1A).

There are no large conformational changes in either Ubc9 or SUMO upon complex formation with r.m.s. deviations of 0.79 Å for SUMO (using the core 77 Cα atoms) and 0.60 Å for Ubc9

(using all Cα atoms) compared to previous crystal structures. The interface between the two proteins buries 727 Å² of solvent-accessible surface area on Ubc9 and 642 Å² on SUMO. This interface is relatively hydrophilic, with 5 salt bridges, 8 direct hydrogen bonds and another 12 hydrogen bonds mediated through defined water molecules, but there are also many van der Waals interactions. On the Ubc9 side, all the residues involved in the interaction are situated in one continuous stretch of sequence at the end of the N-terminal helix, the first β-strand and the intervening loop (Figure 1B and 1E). This compact region of Ubc9 interacts with 3 of the 5 β-strands in SUMOs β-sheet. On SUMO most contacts are with β-strand 5, but also β-strand 1 and 3 and the loops connecting these strands are involved in the interface. (Figure 1B and 1F). Details of the interactions are presented in Figure 1C and Supplementary Figure 1.

We compared our Ubc9-SUMO structure with the non-covalent complexes of ubiquitin with the E2 enzyme UbcH5c and the E2-variant Mms2 both determined by NMR (Brzovic *et al.*, 2006; Lewis *et al.*, 2006). Although UbcH5c and Ubc9 are only 36% identical they both show the E2 specific α/β-fold and superimpose with a r.m.s. deviation of 2.5 Å using 136 Cα atoms. The E2-variant Mms2 is only 15% identical to Ubc9 and adopts a E2-like fold lacking the C-terminal helix. It superimposes on Ubc9 with a r.m.s. deviation of 1.9 Å using 115 Cα atoms. Secondary structures of ubiquitin and SUMO bound to the E2(-like) proteins are also highly similar even though their sequence is only 18% conserved (r.m.s. deviation 1.4 Å using 75 or 72 Cα atoms for ubiquitin bound to UbcH5c and Mms2 respectively). Roughly, the interaction sites of ubiquitin on UbcH5c and SUMO1 on Ubc9 are conserved, both ubiquitin and SUMO1 interact with the backside of the E2, at least 20 Å away from the active site cysteine, and both use their β-sheet for this interaction. Also, the solvent-accesible surface area buried in the complexes is comparable, for Mms2-Ub this is 641 Å² and 650 Å² respectively, and for UbcH5c-Ub it is 567 Å² and 556 Å². Comparison with the Mms2-ubiquitin structure shows that the interaction of ubiquitin with the E2 variant is more similar to the UbcH5c-ubiquitin interaction than to that between SUMO and Ubc9 (figure 1E).

If we superpose only the E2s it becomes clear that the relative orientations of the modifiers are slightly different (Figure 1D, 1E). SUMO1 is rotated 28.4 degrees towards the N-terminal helix of Ubc9 compared to ubiquitin on UbcH5c. The tilting of the ubiquitin in the Mms2 structure is 27.7° compared to the SUMO, but only 14.4° if we compare it with ubiquitin interacting with UbcH5c (figure 1E). Although crystal contacts could be involved, we did see the same orientation for SUMO in a second crystal form (data not shown). The difference in the position of the modifiers results in a change of interaction surfaces on the E2s, where SUMO interacts with Ubc9 mainly N-terminally, the ubiquitin interaction surface on UbcH5c and Mms2 is shifted somewhat towards the C-terminus (figure 1E).

SUMO1 and SUMO2 interact with Ubc9 with similar affinities

Both SUMO1 and SUMO2 bind non-covalently to Ubc9 (Tatham *et al.*, 2003) and even though they are only 44% identical, the residues in the interface with Ubc9 are relatively well conserved (9 identical, 4 homologous, 3 different) (Figure 1F). Of the three non-conserved residues, Gly 81 (Glu 77 in SUMO2) only makes main chain contacts, and the other two, changing Ile 27 into an alanine (Ala 23 in SUMO2) and Val 87 into a threonine (Thr 83 in SUMO2) can be accommodated in the interface without problems. Therefore we deduce that the interaction mode of Ubc9 with SUMO1 and 2 are likely to be very similar, in agreement with NMR studies of this interface (Liu *et al.*, 1999; Tatham *et al.*, 2003).

In order to determine the affinity of the interaction between Ubc9 and SUMO1 and 2 we used isothermal calorimetric analysis. In isothermal calorimetry (ITC) the absorbance or release of energy of mixing two components that interact with each other can be measured as heat changes. These changes in heat can be used to determine the binding constant and thermodynamic parameters of the reaction. A dissociation constant of 250 +/- 70 nM has been reported for the Ubc9-SUMO1 interaction using ITC (Tatham *et al.*, 2003). In our hands the interaction between Ubc9 and SUMO1 was stronger, but with a Kd of 82 +/- 23 nM still in the same order of magnitude (Figure 2A). The heat exchange or enthalpy contribution to the binding is relatively small (maximally 6 kcal/mol under these conditions) but high enough to calculate the Kd accurately. The only obvious differences in Kd measurement between ours and Tatham et al. are a small pH difference (pH=8.0 versus 7.5 respectively) and the presence of an

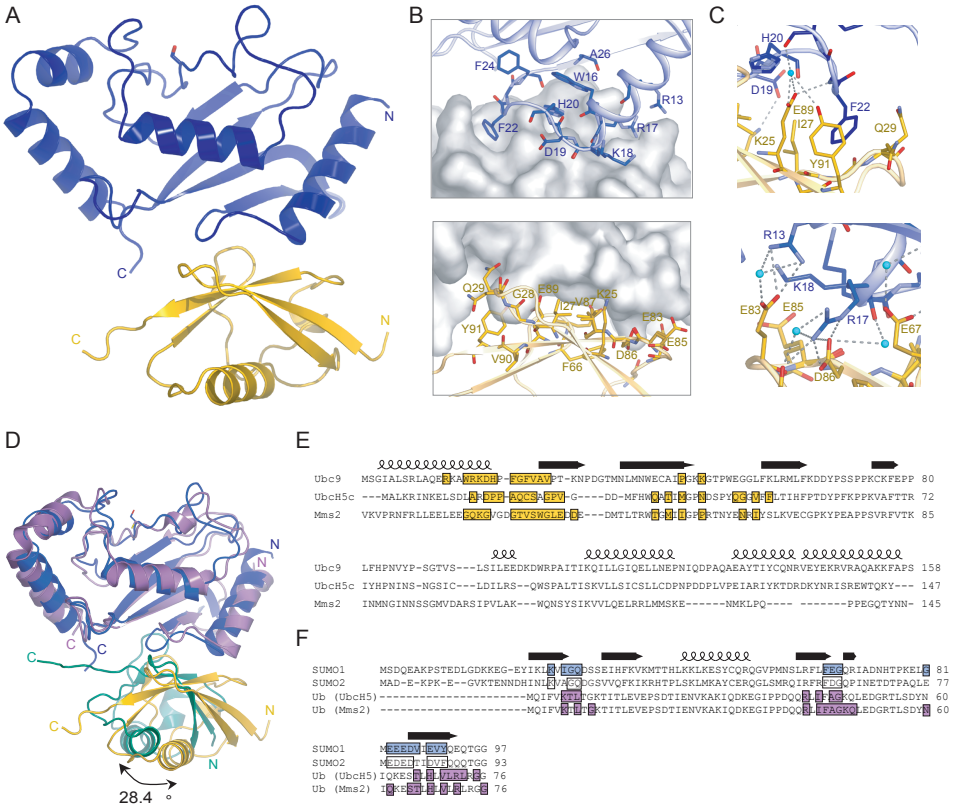

Figure 1 Structure of non-covalent Ubc9-SUMO1 complex.
(**A**) Cartoon representation of the Ubc9-SUMO1 crystal structure, Ubc9 in blue and SUMO1 in yellow. The catalytic residue is shown in sticks. (**B**) Details of the interaction site. Residues of Ubc9 (upper panel) and SUMO1 (lower panel) involved in the interaction shown in sticks, counterpart shown as surface representation. (**C**) Close-up of Ubc9-SUMO1 interaction. (**D**) Superposition of the UbcH5c-Ubiquitin complex (purple and green respectively) and the Ubc9-SUMO1 complex. Only UbcH5c and Ubc9 were used for the superposition, the angle between ubiquitin and SUMO is indicated. (**E**) Sequence alignment of Ubc9, UbcH5c and Mms2 showing secondary structure elements of Ubc9. Residues that loose at least 20% of their solvent accessible surface area upon complex formation with SUMO/ubiquitin are shown on a yellow background. (**F**) Sequence alignment of SUMO1, SUMO2 and ubiquitin with secondary structure elements of SUMO1 on top. Residues of SUMO1 involved in Ubc9 interaction (determined as in (E)) are shown on a blue background and identical residues in SUMO2 are framed. Residues of ubiquitin involved in UbcH5c or Mms2 interaction have a purple background.

N-terminal His-tag on Ubc9 in their experiments. Both of these factors could contribute to the 3-fold difference in Kd.

When we performed identical ITC measurements replacing SUMO1 for SUMO2, we were not able to measure any reproducible heat exchange during the measurement. Nevertheless, if we collected the sample from the flow cell after the experiment and run it on an analytical gel filtration column we observe complete complex formation between SUMO2 and Ubc9 (data not shown). Since the heat exchange of the reaction for SUMO1 binding was very small it seems likely that for SUMO2 the enthalpic contribution is even smaller and the reaction is mostly entropically driven, so it can not be measured by ITC.

For a direct comparison of the affinities of Ubc9 with SUMO1 and SUMO2 we therefore used an analytical gel filtration shift experiment. First, we tested the method using high protein concentrations by mixing pure samples of Ubc9 (50 μM) and an excess of SUMO1 or SUMO2 (100 μM) in 25 μl, incubation at 4 °C for 10 minutes prior to running it on a Superdex 75 gel filtration column. Both for SUMO1 (Figure 2B, upper panel) and for SUMO2 (Figure 2B, lower panel) all of the Ubc9 was shifted to the Ubc9-SUMO complex peak while the excess of SUMO eluted in a peak

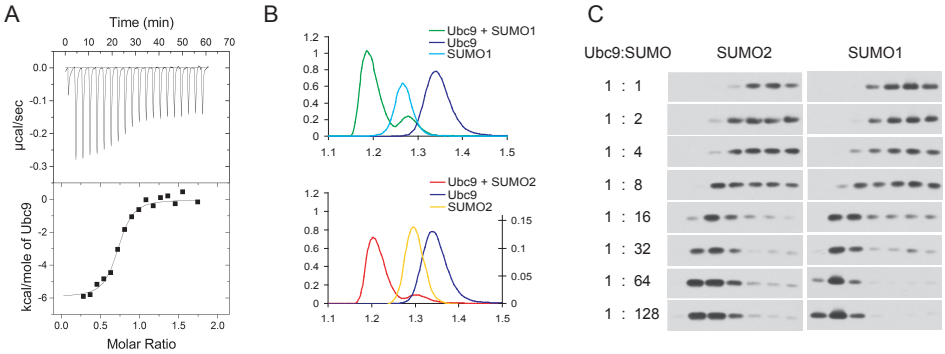

Figure 2 SUMO1 and SUMO2 have similar affinity for Ubc9.
(**A**) Isothermal calorimetry data for non-covalent interaction between Ubc9 and SUMO1. Raw (upper panel) and processed (lower panel) data for 7 µM SUMO titrated with 12 µl injections of 70 µM Ubc9. Processed data points were fitted to a model describing a single set of binding sites. Thermodynamic parameters for the interaction are $\Delta H = -5.96 \pm 0.2$ kcal/mol and $-T\Delta S = -3.87$ kcal/mol (**B**) Chromatograms of analytical gel filtration runs for Ubc9 with SUMO1 (upper panel) and Ubc9 with SUMO2 (lower panel). Runs of single proteins contained 60 µM Ubc9 or 300 µM SUMO1/2, complex runs contained 50 µM Ubc9 and 100 µM SUMO1/2. (**C**) Gel-filtration based shift assays visualized by western blot analysis using anti-Ubc9. For SUMO2 (left panel) as well as for SUMO1 (right panel) several gel-filtration runs were performed with a constant Ubc9 and increasing SUMO concentrations (molar ratio is depicted on the left).

overlapping with the free SUMO peak. To compare affinities we used lower concentrations of Ubc9 (390 nM) with varying SUMO concentrations, followed by gel filtration chromatography and western blotting of the fractions using an anti-Ubc9 antibody. This allowed visualization of the free Ubc9 peak shifting to the SUMO bound peak upon increased SUMO concentrations in the samples (Figure 2C). Both SUMO1 and SUMO2 are able to shift the Ubc9 peak under these conditions and the peak shift occurs at similar SUMO1 and SUMO2 concentrations indicating similar affinities of Ubc9 for SUMO1 and SUMO2.

Ubc9H20D and SUMO1E67R inhibit non-covalent interaction

Although several groups have reported the non-covalent interaction between Ubc9 and SUMO, the function of this interaction has been subject of speculation. Based on mutant analysis it has been proposed that the interaction is needed for SUMO thioester formation (Tatham *et al.*, 2003). However, since the binding sites for SUMO and the E1 on Ubc9 partially overlap it was difficult to create interface mutants that do not affect E1 interaction, and consequently, thioester formation. Now, based on our high-resolution structure, we

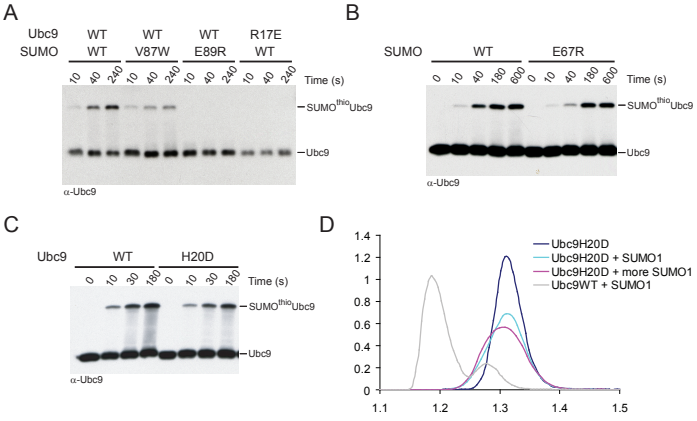

Figure 3 Mutants that interrupt non-covalent Ubc9-SUMO binding but not thioester formation
(**A**) Thioester formation followed in time for Ubc9 and SUMO1 wild type and mutants. Concentrations were: 100 nM E1, 900 nM Ubc9 and 3 µM SUMO1. (**B**) Thioester formation assay comparing SUMOWT with SUMOE67R. Concentrations were: 200 nM E1, 1.4 µM Ubc9 and 15 µM SUMO1. (**C**) Thioester formation assay as in (B) comparing Ubc9WT with Ubc9H20D. (**D**) Non-covalent binding studied using analytical gel-filtration for Ubc9H20D with SUMO1. Curve indicated as 'Ubc9 + SUMO1' was 44 µM Ubc9H20D and 108 µM SUMO1, 'Ubc9 + more SUMO1' was 27 µM Ubc9H20D and 136 µM SUMO1. Free Ubc9H20D and the complex between Ubc9WT and SUMO are indicated for clarity.

Table II Summary of SUMO mutant data

SUMO	WT	E67R	G68Y	V87W	E89R
Ubc9 binding	++	-	+/-	+/-	-
E1 thioester	++	+	+/-	+	-
Ubc9 thioester	++	+	-	+/-	-

Table III Summary of Ubc9 mutant data

Ubc9	WT	R17E	G23R	V25W	V25R	H20D
SUMO binding	++	-	++	++	++	-
E1 interaction	++	-	++	++	++	+/-
Ubc9 thioester	++	-	nd	nd	nd	++

searched for Ubc9 or SUMO1 mutants that only abolish the non-covalent interaction between Ubc9 and SUMO.

For SUMO1 we generated the following mutants: E67R, G68Y, V87W and E89R. These mutants were tested for their ability to interact with Ubc9 non-covalently, as well as for their activity in E1 and Ubc9 thioester formation. As summarized in Table II, all of these mutants showed decreased non-covalent interaction with Ubc9 and SUMOE67R as well as SUMOE89R were completely unable to interact with Ubc9 in the analytical gel filtration assay (Supplementary Figure 2 and data not shown). SUMO E89R however was also impaired in both E1 and Ubc9 thioester formation and was therefore not a good candidate to study the function of the Ubc9-SUMO non-covalent interaction (Figure 3A and Supplementary Figure 2). The SUMO E67R mutant only has a minor defect in E1 and Ubc9 thioester formation and would, from these mutants, be the best candidate to study the role of non-covalent Ubc9-SUMO interaction (Figure 3B and Supplementary Figure 2).

For Ubc9 we tested mutations in four residues, R17E, H20D, G23R and V25W and V25R (Table III). These mutants were tested for SUMO1 interaction and, in addition, for their ability to interact non-covalently with the E1 as well as their activity in Ubc9~SUMO thioester formation (Figure 3A, Supplementary Figure 2, data not shown). Valine 25 is equivalent to serine 22 in UbcH5c, mutating this residue inhibited non-covalent interaction with ubiquitin (Brzovic *et al.*, 2006). In Ubc9 however, mutation of this residue did not affect the non-covalent binding of SUMO probably due to the fact that it is less well buried in the Ubc9-SUMO interface (Supplementary Figure 2). Also, the G23R mutation did not abolish Ubc9-SUMO non-covalent interaction. In contrast, the E17R and H20D mutants do disturb the interface and are strongly inhibited in Ubc9-SUMO interaction (Figure 3D, Supplementary Figure 2). However, Ubc9E17R was also deficient in E1 interaction and strongly reduced in Ubc9~SUMO thioester formation and was therefore excluded from further studies. Ubc9H20D was the only Ubc9 mutant that abolished the non-covalent interaction with SUMO (Figure 3D) without affecting thioester formation (Figure 3C) even though it does show a reduction in E1 interaction (Supplementary Figure 2C). This mutation is therefore suited for further analysis of the function of non-covalent interaction between Ubc9 and SUMO.

Non-covalent Ubc9-SUMO interaction promotes SUMO chain formation

In both Mms2 and in UbcH5c the non-covalent interaction with ubiquitin is involved in ubiquitin chain formation (VanDemark *et al.*, 2001; Brzovic *et al.*, 2006). Therefore, we first tested

Table I Data Collection and Refinement Statistics

	Ubc9-SUMO
Data collection	
Space group	$P2_1$
a, b, c (Å)	49.5, 35.0, 72.9
α, β, γ (°)	90.0, 93.4, 90.0
Resolution (Å)	50-1.4 (1.48-1.40)
R_{sym} (%)	6.0 (22.7)
I/σI	6.3 (1.6)
Completeness (%)	99.2 (99.3)
Redundancy	3.5 (3.1)
Refinement	
Resolution (Å)	50-1.4
Number reflections	49557
R_{work}/R_{free}	14.0 (17.7)
Number of atoms	
Protein	2130
Ligand/ion	1 Na^+
Water	408
B-factors	
Protein	12.5
Ligand/ion	24.6
Water	26.8
Rms Deviations	
Bond lengths (Å)	0.014
Bond angles (°)	1.554

Highest resolution shell is shown in parenthesis.

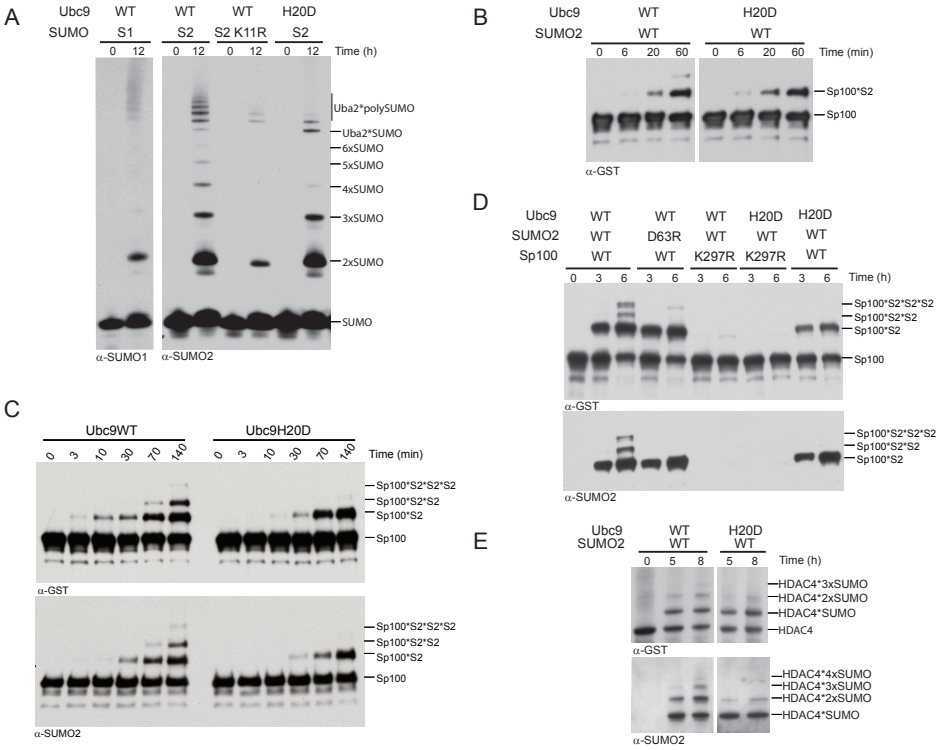

Figure 4 Non-covalent Ubc9-SUMO interaction promotes SUMO chain formation.
(**A**) Free SUMO chain formation for SUMO1, SUMO2 and SUMO2K11R with Ubc9WT and Ubc9H20D. Formation of SUMO chains is followed in time using SUMO1 or SUMO2 antibodies as indicated. Concentrations were: 100 nM E1, 400 nM Ubc9 and 20 μM SUMO. **(B)** Sumoylation of Sp100 with SUMO2 comparing Ubc9WT with Ubc9H20D. Concentrations were: 830 nM GST-Sp100, 175 nM E1, 400 nM Ubc9 and 20 μM SUMO2 and detection was with anti-GST. **(C)** SUMO chain formation on SP100 with SUMO2, comparing Ubc9WT with Ubc9H20D. Concentrations are identical to (B), formation of GST-Sp100*SUMO2 conjugates is followed in time using either a GST antibody (upper panel) or a SUMO2 antibody (lower panel). **(D)** SUMO chain formation on Sp100 comparing several mutant proteins. Concentrations were: 1.3 μM GST-Sp100, 10 nM E1, 300 nM Ubc9 and 10 μM SUMO and detection was with anti-GST or anti-SUMO2. (**D**) Assay as in (C) but using GST-HDAC4 as a target.

the Ubc9 mutant that interferes with Ubc9-SUMO non-covalent interaction, for free SUMO chain formation. Wild type and mutant Ubc9 were incubated with SUMO, E1 and ATP at 37 °C and SUMO chain formation was followed in time (Figure 4A). Both SUMO1 and the SUMO2 K11R mutant were included as controls and, as expected, they hardly form SUMO chains as has been shown before (Tatham *et al.*, 2001). SUMO2WT readily forms chains with Ubc9WT, however, the Ubc9H20D mutant is less productive in chain formation (Figure 4A). Even though the difference is not as pronounced as compared to SUMO1, it is very reproducible. Also, there is a striking difference in formation of higher order Uba2 conjugates occurring as a side effect of the reaction. Of note, Uba2 has been picked up as a target for sumoylation (Zhao *et al.*, 2004; Hannich *et al.*, 2005), and we have confirmed the presence of SUMO modified Uba2 by mass spectrometry (data not shown).

Next, we tested substrate sumoylation using the transcriptional regulator Sp100 as a target. If we stop the reaction after one hour we observe mostly mono-sumoylation of Sp100 and, importantly, this is equally efficient for Ubc9WT compared to Ubc9H20D (Figure 4B). However, at the latest time point in the Ubc9WT reaction a higher band also appears that could potentially correspond to Sp100*2xSUMO. To investigate whether this indicates SUMO chain formation we performed a similar experiment but extended the incubation time. At later time points we now observe several higher bands using Ubc9WT that do not appear if

we use Ubc9H20D, shown on western blots with either GST or SUMO2 antibodies (Figure 4C). Even though this SUMO2 antibody appears to cross react strongly with GST-Sp100, we do see an enhancement of the higher molecular weight bands using this antibody.

In order to check whether the higher molecular weight bands are a result of SUMO2 chain formation, or mono-sumoylation on multiple sites in Sp100, we analyzed the Sp100*SUMO2 Sp100*2xSUMO2 and Sp100*3xSUMO2 samples by mass spectrometry. In all samples one major modification site in Sp100 was found, this was K297, the lysine that has previously been identified as the SUMO modification site (Sternsdorf *et al.*, 1997). Also, one minor site was found to be modified (K387) for 5% or less compared to the major site. This indicates that most of the 'poly'-sumoylation is due to chain formation of SUMO2. In fact, the mass spectrometric analysis primarily identified K11, and to a small extent K5, as the acceptor lysines on SUMO2 itself. To verify that Sp100 is primarily modified on the in vivo relevant lysine (K297) and to confirm chain formation at lower enzyme concentrations, we performed a similar sumoylation reaction using less enzyme, comparing Sp100WT with the Sp100 K297R mutant. Under these conditions we can still show SUMO2 chain formation on Sp100WT while Sp100K297R is hardly modified (Figure 4D). Additionally, in a similar assay, the SUMO2 K11R mutant is also reduced in SUMO2 chain formation (Supplementary Figure 3). These results demonstrate that SUMO chains are formed on Sp100 via the consensus site lysine 297, and that SUMO2 uses primarily lysine 11 to make these chains.

To examine the importance of the non-covalent interaction between Ubc9 and SUMO2 for the formation of these chains we tested the Ubc9H20D mutant in the same Sp100 chain formation assay. Figure 4D shows that this mutant is impaired in SUMO2 chain formation on Sp100. To verify this result we also tested the SUMO2D63R mutant, which is the equivalent of the SUMO1E67R mutation. It inhibits SUMO2-Ubc9 non-covalent interaction while it is still competent in Ubc9 thioester formation (data not shown). This SUMO mutant also inhibits poly-sumoylation of Sp100 (Figure 4D). These data strongly suggest that non-covalent interaction between Ubc9 and SUMO2 promotes poly-sumoylation of Sp100.

To investigate whether the importance of the non-covalent binding site for poly-sumoylation is a target specific effect we tested SUMO2 chain formation on another well-known SUMO target, histone deacetylase 4 (HDAC4). Also with this target we see a profound inhibition of SUMO chain formation upon disruption of the non-covalent interaction site between SUMO and Ubc9 (Figure 4E).

To gain insight in the mechanism of SUMO chain formation and the functional role of the non-covalent Ubc9-SUMO interaction in this process we compared our structure with the structure of the Mms2-Ubc13 heterodimer (Eddins *et al.*, 2006). In this complex, the E2 variant (Mms2) can bind an acceptor ubiquitin non-covalently and form a heterodimer with a functional E2 (Ubc13) that is activated with ubiquitin. Modeling as well as structural studies have shown that in this complex the K63 of the acceptor ubiquitin is in close proximity to the activated cysteine of Ubc13, providing a mechanism for K63-linked ubiquitin chain formation (VanDemark *et al.*, 2001; McKenna *et al.*, 2003b; Lewis *et al.*, 2006). The recently published crystal structure of the Ubc13~Ub intermediate in complex with Mms2 also shows this quaternary complex as it has a ubiquitin molecule from a neighboring complex bound to the acceptor ubiquitin site on Mms2 (Figure 5A)(Eddins *et al.*, 2006).

A complex like this could also be formed between two identical E2s, which would require the E2 to interact with itself. Although Ubc9 is not a homodimer in solution, self-interaction has been shown in yeast two hybrid systems (Hateboer *et al.*, 1996; Kovalenko *et al.*, 1996). If we superpose the Ubc9-SUMO complex on Mms2 bound to ubiquitin from the Mms2-Ubc13~Ub structure, a free Ubc9 on Ubc13, and SUMO on the thioester ubiquitin (Eddins *et al.*, 2006), we can create a model that resembles the Mms2/Ubc13/ubiquitin complex (Figure 5B). In this model we can see that, although SUMO does not have a lysine at the equivalent position to K63 in ubiquitin, the N-terminus of the non-covalently bound SUMO is close to the active site of the adjacent Ubc9. For SUMO2 there are 14 residues of N-terminal tail that are not present in the structure, and the K11 and K5 are respectively 4 and 10 residues upstream from the last residue in the structure and could therefore both easily reach the active site of the donor Ubc9. Figure 5 shows the comparison of the Mms2-Ubc13-2x ubiquitin structure (Figure

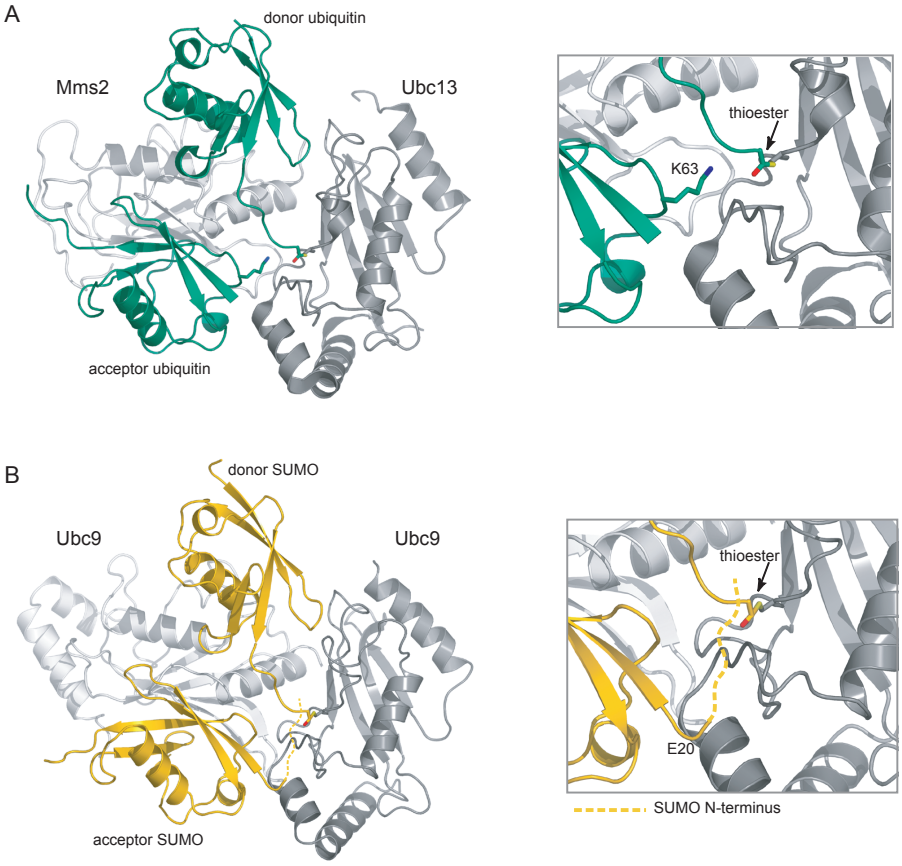

Figure 5 A model for SUMO chain formation.
(**A**) K63 ubiquitin chain formation by the Mms2-Ubc13 heterodimer (Eddins *et al.*, 2006)(PDB-code: 2GMI). Close-up shows Mms2 K63 in sticks and the Ubc13 thioester active site. (**B**) Structural model for SUMO chain formation. The non-covalent Ubc9-SUMO1 complex was superposed on Mms-Ub and another Ubc9 molecule was superposed on Ubc13 (see (A)). Thioester SUMO was modeled by superposition on ubiquitin replacing the C-terminal tail with the one from ubiquitin. Close-up shows acceptor SUMO N-terminus and donor SUMO-Ubc9 thioester active site.

5A) with a similar model comprising 2x Ubc9-2x SUMO (Figure 5B). This attractive model provides a possible explanation why chain formation takes place on the N-terminal tail of SUMO2 and why the non-covalent interaction between Ubc9 and SUMO promotes this chain formation.

Discussion

We have presented here the crystal structure of the non-covalent complex between Ubc9 and SUMO1. The resemblance of this structure with the non-covalent complex between ubiquitin and UbcH5c as well as with the E2 variant Mms2 and ubiquitin underlines the conservation of this interaction between homologous pathways. The UEV (ubiquitin conjugating enzyme variant) domain of Tsg101 (and VPS23) also interacts non-covalently with ubiquitin but this interaction is different as it primarily uses a β-tongue motif formed by the extended first and second beta-strands and the loop between them (Sundquist *et al.*, 2004).

NMR studies have been performed on the non-covalent interface between Ubc9 and SUMO1, 2 and 3 (Liu *et al.*, 1999; Tatham *et al.*, 2003), all indicating that the interaction site on Ubc9 is primarily formed by the N-terminal region of Ubc9. We have now shown the high resolution details

of this interaction and found that SUMO interacts with the N-terminal helix, the first β-strand and the intervening loop of Ubc9. Recently, a model for the interaction between Ubc9 and SUMO3 was created by docking approaches in combination with NMR interaction data (Ding *et al.*, 2005). In this model, the equivalent residues of SUMO3, compared to SUMO1 in our structure, interact with Ubc9. Since SUMO2 and 3 are completely identical in the interacting residues, and since we have shown that SUMO1 and SUMO2 interact with Ubc9 with similar affinities we can conclude that all SUMO isoforms interact with Ubc9 using the same interaction site and with similar affinities. With a Kd of ~80 nM, the interaction between SUMO and Ubc9 is relatively strong compared to the interaction between ubiquitin and UbcH5c with a Kd of ~300 μM (Brzovic et al 2006), and between ubiquitin and Mms2 with a Kd of ~100 μM (McKenna *et al.*, 2003a). Even though the buried surface areas between E2 and modifier are comparable in all three structures, there are more hydrogen bonds and salt bridges in the Ubc9-SUMO interface which might account for the difference in affinity.

Two mutations, one in Ubc9 (H20D) and one in SUMO (E67R in SUMO1 or D63R in SUMO2), were identified that individually interfere with Ubc9-SUMO non-covalent interaction. These mutants enabled us to show that the interaction between Ubc9 and SUMO promotes SUMO chain formation on both Sp100 and HDAC4. There are several possible mechanisms that can explain the role of this interaction in chain formation. The simplest one would be that one Ubc9 molecule interacts with two SUMO molecules and the non-covalently bound SUMO acts as a direct acceptor for the thioester bound SUMO (donor). This mechanism is not likely since the distance from the N-terminus of the acceptor SUMO to the active cysteine is too long to bridge the distance to the acceptor lysines K5 or K11, even if the N-terminal residues adopt an extended conformation.

Another possibility is that chain formation occurs by a mechanism similar to what has been proposed for UbcH5c (Brzovic *et al.*, 2006). There, the non-covalent interaction between ubiquitin and UbcH5c is promoting large assemblies of activated UbcH5c~ubiquitin which are required for processive BRCA1-directed ubiquitination. However, in our hands the Ubc9~SUMO thioester does not form these large assemblies (data not shown) and even though we can not completely rule out that assembly formation may occur with a very low affinity we do not favor this mechanism. Our favored hypothesis, however, is that the poly-sumoylation that we observe involves a mechanism that follows the well-established model for ubiquitin chain formation as seen in Mms2-Ubc13 dependent K63 poly-ubiquitination. We have shown here that the non-covalent site on Ubc9 closely resembles the non-covalent binding site of ubiquitin on Mms2 and that a model involving two Ubc9 molecules and two SUMO molecules superimposes well on the Mms2-Ubc13-ubiquitin structures. In this complex the N-terminus of SUMO would be exposed to covalent modification with a donor SUMO molecule, leading to chain formation. This model would explain why the non-covalent interface between Ubc9 and SUMO is needed for SUMO chain formation and why the lysines in the N-terminal tail are used as linking sites.

The chain formation that has been observed for SUMO2 (Tatham *et al.*, 2001; Cheng *et al.*, 2006) is less processive than that which has been seen for ubiquitin. This lower processivity is most likely an intrinsic feature of Ubc9 and not due to the in vitro reaction conditions since poly-ubiquitin chains are easily formed in vitro, whereas the SUMO chains remain shorter. However, SUMO chains have not been extensively studied in vivo and, since the yeast homologue of SUMO, Smt3, seems to be able to form long chains in vivo (Bylebyl *et al.*, 2003), it would not be unlikely that additional factors are required for processive SUMO chain formation. Nevertheless, the actual mechanism for chain formation seems once again surprisingly similar between the ubiquitin and SUMO pathways.

Materials and methods

Plasmids and antibodies

Ubc9, SUMO1(ΔN20), Aos1-Uba2 (Pichler *et al.*, 2005), GST-Sp100 (Seeler *et al.*, 2001) and GST-HDAC4 (Kirsh *et al.*, 2002) plasmid construction has been described before. Ubc9 and SUMO1 mutants were generated by site-directed mutagenesis. Mouse α-SUMO1 was obtained from Santa Cruz and Zymed, mouse α-SUMO2 from Zymed and goat α-GST from Amersham. Goat α-Ubc9 and goat α-SUMO2 were kindly provided by F. Melchior (Georg-August University, Göttingen). Secondary antibodies were from Biorad or Biosource.

Protein expression and purification

Purification of Ubc9, Aos1-Uba2, GST-Sp100, GST-HDAC4 was performed as described (Pichler *et al.*, 2002; Pichler *et al.*, 2005) and SUMO2 were expressed in BL21(DE3) cells using IPTG induction overnight at 15 °C. Purification was performed on an Superdex 75 column in 20 mM Tris pH 8.0, 100 mM NaCl, 0.1 mM PMSF, 1 mM DTT followed by an anion exchange (monoQ) column.

Analytical gel filtration

Protein-protein interaction studies by analytical gel filtration were performed on a Superdex 75 column on the SMART system (Pharmacia) in a buffer containing 20 mM Tris pH 8.0 and 100 mM NaCl. Concentrations as indicated in the figure legends were mixed and incubated for 10 minutes at 4 °C prior to loading (25-50 μl) on the column. Eluted fractions were run on SDS-PAGE and either analyzed by coomassie staining, or by western blot analysis using an antibody against Ubc9.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) experiments were performed with the VP-ITC Micro Calorimeter (MicroCal, Inc.) at 30°C. Stock solutions of Ubc9 and SUMO were prepared by dialysis of the purified proteins against a buffer containing 20 mM Tris pH 8.0 and 5 mM β-mercaptoethanol at 4°C and were degassed before use. The sample cell (1.4 ml) contained SUMO (5-10 μM) which was titrated with Ubc9 (50-100 μM) using 12 μl injections. The injections after saturation were used to determine the background signal. Corrected data were analyzed using software supplied by the ITC manufacturer to calculate the dissociation constant (K_d,). Parameters were obtained for a model describing one set of binding sites, using non-linear least-squares fitting.

Native gel shift assays

Interaction of SUMO E1 with Ubc9WT and mutants was determined by a native gel mobility shift assay. Ubc9 was incubated with increasing amounts of E1 for 15 minutes at room temperature. Bound and unbound forms were separated on a 4.5 % polyacrylamide gel (acrylamide: bis, 37.5:1) in a Tris-Borate buffer pH 8.0, containing 2% glycerol (or: on a 8 % polyacrylamide gel (acrylamide:bis, 29:1) in a Tris buffer pH 8.3 and a Tris-glycine running buffer). Loading and running buffer were also buffered with Tris-Borate pH 8.0. Bands were visualized with coomassie staining.

E1 thioester formation

E1 thioester formation was performed at 8 °C in the same buffer as the thioester formation. Aos1-Uba2 (3 μM) was mixed with SUMO (22 μM) and reactions were started by addition of 5 mM ATP. Samples from indicated time points were denatured in non-reducing loading buffer, run on SDS-PAGE and stained with Coomassie.

In vitro thioester formation and target sumoylation assays

Ubc9WT and mutant thioester formation assays were performed at 30 °C in buffer containing 20 mM Tris pH 8.0, 100 mM NaCl, 5 mM MgCl2 and 0.1 mM DTT. Reactions contained Aos1-Uba2, SUMO and Ubc9 in concentrations as indicated in the figure legends. Reactions were started by addition of 5 mM ATP and at the indicated time points samples were denatured in non-reducing loading buffer. Samples were run on SDS-PAGE, immunoblotted and analyzed with α-Ubc9.

Target sumoylation and free SUMO chain formation assays were performed at 37°C in buffer containing 20 mM HEPES pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 0.05% Tween 20, 0.2 mM ovalbumin and 1 mM DTT. Reaction mix contained Aos1-Uba2, SUMO1 or SUMO2, Ubc9 and either Sp100 or HDAC4 in case of target sumoylation, in concentrations as indicated in the figure legends. After start of the reaction by the addition of 5 mM ATP, samples were taken at the indicated time points and mixed with denaturing and reducing sample buffer. Samples were run on 8% SDS gels or 4-12% NuPage gels (Invitrogen) and bands were visualized by immunoblotting against α-GST, α-SUMO1 or α-SUMO2.

Mass spectrometry

The protein bands containing GST-SP100 conjugated with several SUMO2 molecules were excised from an SDS-PAGE gel and subjected to in-gel reduction, alkylation, trypsin digestion and subsequent sample desalting and concentration, as previously described (Olsen *et al.*, 2004). The resulting peptide mixture was analyzed by nano-HPLC-MS/MS using an Agilent 1100 nanoflow system connected to a hybrid linear ion trap orbitrap (LTQ-Orbitrap) mass spectrometer (Thermo Electron, Germany), equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark), essentially as described (Olsen *et al.*, 2005).

Crystallization, data collection and structure determination

Ubc9-SUMO(ΔN20) crystals were grown at 4 °C by mixing equimolar amounts of Ubc9WT or Ubc9C93S and SUMO(ΔN20) (450 μM each) in a hanging drop with a mother liquor consisting of 16.5 % (w/v) PEG3350, 100 mM BisTris pH 5.5 and 15 % (w/v) glycerol. The complex crystallized using either Ubc9WT or Ubc9C93S, as large plates with a maximum of 400 μM in their largest dimension. Cryoprotection was achieved by increasing the concentration of glycerol to 25 % (w/v). Data were collected on a single Ubc9C93S-SUMO1 crystal in a separate high and low resolution dataset at 100 K on European Synchrotron Radiation Facility beam line ID14-2 (λ=0.933). Data were processed with MOSFLM and SCALA (Collaborative Computational Project 4, 1994).

The structure was solved by molecular replacement with the program Molrep (Collaborative Computational Project 4, 1994) using both Ubc9 (Tong *et al.*, 1997, PDB code 1U9A) and SUMO (Pichler *et al.*, 2005, PDB code 2BF8). Rebuilding was done with ARP/wARP (Perrakis et al., 1999) and Coot (Emsley and Cowtan, 2004), and refinement was done with REFMAC (Collaborative Computational Project 4, 1994). Percentages of residues in the most favored, additionally allowed, generously allowed and disallowed regions of the Ramachandran plot were 93.5, 6.0, 0.5 and 0.0 % respectively. The model includes 408 water molecules and one sodium ion from the protein storage

buffer. Crystallographic parameters are summarized in Table I. Buried solvent-accessible surfaces areas between the molecules were calculated with the program Areaimol (Collaborative Computational Project 4, 1994) and contacts between Ubc9 and SUMO were analyzed with the program NCONT (Collaborative Computational Project 4, 1994).
All structure figures were generated using Pymol (http://www.pymol.org). Atomic coordinates and structure factors have been deposited to the Protein Data Bank with accession code xxxx.

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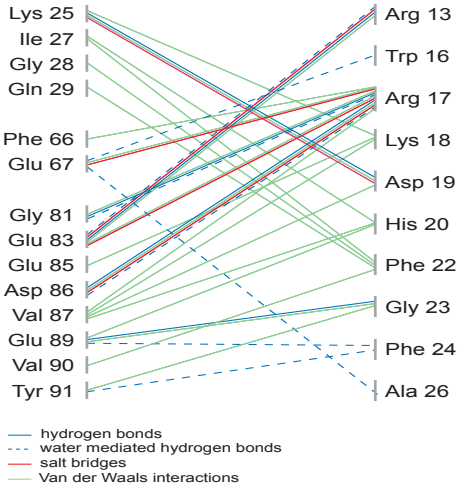
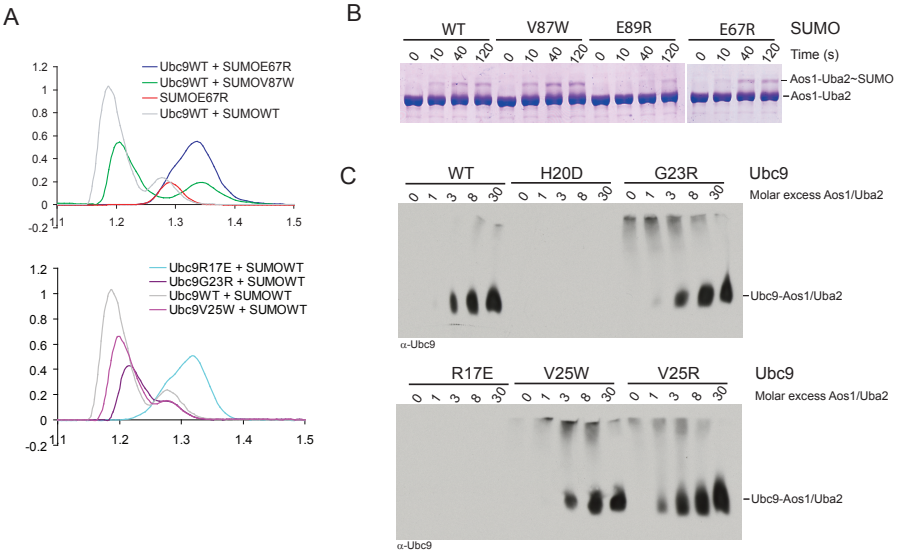
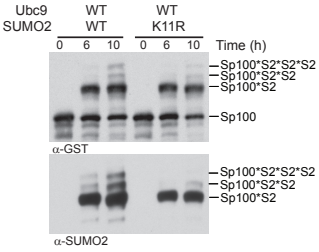
Supplementary Figure 1 Interactions between Ubc9 and SUMO
Diagram showing the details of non-covalent interactions in the Ubc9-SUMO structure. Hydrophobic interactions, salt bridges and hydrogen bonds between indicated residues are shown.



Supplementary Figure 2 Analysis of the SUMO and Ubc9 mutants
(**A**) Non-covalent interaction between Ubc9 and SUMO mutants. Chromatograms of analytical gel filtration runs for Ubc9WT with SUMO mutants (upper panel) and Ubc9 mutants with SUMOWT (lower panel). (**B**) E1 (Aos1-Uba2) SUMO thioester formation assay for various SUMO1 mutants. (**C**) Analysis of E1 interaction of Ubc9 variants. Ubc9 WT and mutants were incubated with increasing amounts of SUMO E1 and complex formation was visualized by immunoblotting on a native gel using an α-Ubc9 antibody. Free Ubc9 is positively charged under these conditions and therefore does not enter the gel while Ubc9 in complex with the E1 does. Of note, Ubc9H20D interaction with the E1 is reduced but not completely abolished since the complex becomes visible at a longer exposure times.



Supplementary Figure 3 SUMO2 K11R mutant is reduced in chain formation.
SUMO chain formation on Sp100 comparing SUMO2WT to SUMO2K11R. Concentrations were: 1.3 µM GST-Sp100, 10 nM E1, 200 nM Ubc9 and 20 µM SUMO and detection was with anti-GST or anti-SUMO2.



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Chapter 6

Discussion

Discussion

SUMO modification is a special type of post-translational modification important in many biological processes. Sumoylation of target proteins is a highly regulated process and requires three sequential steps involving an E1, E2 and mostly an E3 enzyme. To understand this multi-step reaction in detail we need to know the enzymes involved in each step, the order in which the consecutive steps are taking place, the start and end states of these steps and the chemical nature of each reaction including intermediate states. Moreover, for a full understanding of the function of SUMO modification in the cell, we also need to know how this process is regulated. In this thesis we have presented structural and biochemical data concerning the process of SUMO modification and its regulation at several steps of the enzymatic cascade.

In the process of sumoylation, E2 enzymes or SUMO conjugating enzymes, play a central role by accepting a SUMO molecule from the E1 and transferring it, often with the help of an E3 enzyme, to the target. The E2 is also partially involved in target recognition since it interact with the SUMO consensus site (ΨKxE/D) present on many targets. In chapter 4 and chapter 5 we describe how the SUMO protein itself regulates the function of the E2 enzyme, Ubc9, in two different ways. The first example of Ubc9 regulation by SUMO involves a non-covalent interaction (chapter 5). This interaction between SUMO and Ubc9 takes place at a site different from the active site and does not influence the basic activity of sumoylation. However, we show that it is important for the formation of SUMO chains possibly through a mechanism that is also used in K63-linked ubiquitin chain formation. In this case, the regulation of Ubc9 influences the type of SUMO signal generated by the sumoylation cascade. In chapter 4 we described how SUMO can regulate Ubc9 in a different manner by covalent modification (chapter 4). This sumoylation of Ubc9 takes place on a lysine situated on the N-terminal helix of Ubc9, which differs again in position from the active site and is also distinct from the non-covalent interaction with SUMO. Modification of Ubc9 with SUMO causes a shift in target specificity, some targets are enhanced in SUMO modification while others are inhibited in modification. This mechanism enables SUMO signaling to discriminate between targets by modification of a single target, its own E2. The enhancement of sumoylation of specific targets is dependent on the interaction of the target with SUMO on Ubc9, through a SUMO interaction motif (SIM). This SIM-mediated enhancement of sumoylation resembles the function that is usually achieved by an E3 enzyme, so E3 function can be provided by E3 enzymes but also by the SUMO modification of the E2 Ubc9.

E3 enzymes, or ligases, can also be considered as regulators of SUMO modification as they can enhance the sumoylation of specific targets. In many cases the details of how they perform their function is not known. We have studied one of these E3 enzymes, the nucleoporin RanBP2, and show that it functions by directly influencing the E2 enzyme without interacting with the target itself (chapter 2). This is different from another group of SUMO E3 ligases, the SP-RING family, that seem to function by bringing the E2 and the target together.

Once targets are modified by this highly regulated sumoylation cascade, the consequence of this modification is often not easy to determine. We have studied the biochemical effect of sumoylation for two targets. The first one is Ubc9 itself, where sumoylation leads to target discrimination, as has been explained above. We also identified the ubiquitin conjugating enzyme E2-25K as a target for SUMO modification. Here the effect of sumoylation is different since it inhibits the basic enzymatic activity of the E2. SUMO modified E2-25K shows a reduction in ubiquitin thioester formation, which is probably because SUMO interferes with E1 interaction. These two SUMO targets, Ubc9 and E2-25K, show how different the functional effect of sumoylation can be, even in homologous proteins. We identified the Ubc9 specific region that is responsible for this difference.

Although the function of sumoylation of these two targets differs, their target site is similar. In both Ubc9 and E2-25K SUMO modification takes place on a non-consensus site lysine located on the N-terminal helix of the protein. For E2-25K we have shown that it is only modified when the site is present on a helix and not when it is unstructured. Secondary structure may therefore be an additional determinant for SUMO site

specificity.
A different way in which the SUMO pathway can be regulated is by the formation of SUMO chains. Both for SUMO2 and SUMO3 chain formation has been observed *in vivo*. We have shown that the non-covalent interaction of SUMO with Ubc9 is important for chain formation. Therefore, the formation of SUMO chains is, at least partially, regulated by the E2 enzyme. However, it is likely that specific E3 enzymes are involved in chain formation *in vivo*.
We have described regulation of E2 and E3 enzymes in sumoylation, studied the consequence of sumoylation for several targets, and studied the mechanism of SUMO chain formation. However many questions are still to be answered before we fully understand the process of sumoylation and its regulation.

The mechanism of sumoylation
As for the process of SUMO modification, information that is lacking is mainly concerning the chemistry of the reactions, the reaction intermediates and the specificity determining factors. How does the E1 perform its successive sub-reactions, how does it transfer SUMO to the E2 and what determines the directionality of this transfer. How is SUMO transferred from the E2 to the target and how do the different E3s catalyze this reaction? To answer these and other questions in detail, structural and biochemical information is needed specifically on intermediate steps in the SUMO modification cascade. This is not an easy task since many of these intermediates are unstable and protein interactions are often transient. Smart tricks are needed to stabilize these intermediates and protein complexes and it remains to be seen whether this will be possible in the near future.

SUMO signaling
SUMO is a versatile modifier, it can have various effects on the modified protein like a change in localization, altered activity, enhanced stability, modulation of interaction with other proteins or a combination of these. We have studied the biochemical consequence of sumoylation of two E2 conjugating enzymes, E2-25K and Ubc9, but the function for many other SUMO targets is unknown. Also, the role of the SIM, and the various types of SUMO modification in SUMO signaling needs to be further investigated. The recently identified SIM is found in many proteins including several proteins involved in the sumoylation cascade. We have described how a SIM present on a SUMO target can result in the enhanced sumoylation of that target in the presence of SUMO modified Ubc9. These studies have all been performed *in vitro*, and currently we are testing this mechanism *in vivo* in collaboration with Frauke Melchior (University of Göttingen, Germany) and Andrea Pichler (Max F. Perutz Laboratories, Austria).
In contrast with sumoylation, where only one SIM has been identified so far, in ubiquitination a large variety of ubiquitin binding domains (UBDs) have been identified. The identification of other SUMO binding motifs may simply lag behind compared to the ubiquitin binding domains, but this could also reflect a difference in function between SUMO and ubiquitin. SUMO may have a more prominent function in, for example, blocking certain binding sites or changing the function of a target protein, in addition to its function as an interaction site modulator. Various examples of SUMO targets where sumoylation does not merely change the interaction surface but has an other functional effect, have been described in chapter 1.
SUMO signaling is not as simple as only mono-sumoylating with a single isoform. Four SUMO isoforms are present in mammalian cells and several studies have recently shown the importance of SUMO2 and SUMO3 chains. We have studied the mechanism of SUMO chain formation *in vitro* and showed that a the non-covalent interaction between SUMO and Ubc9 promotes the formation of chains. It would be of interest to test our mechanistic model *in vivo*, using the mutants that inhibit this non-covalent interaction.
With chain formation, the complexity of signaling in the SUMO pathway is expanding and is approaching the level as seen in ubiquitination. In the ubiquitin field the existence of so called 'mixed chains', chains combining linkages through several lysines, has been suggested which may also apply to the SUMO field. Obviously, there is possibility for several more layers of complexity but it remains to be seen whether they exist *in vivo*.

Regulation of sumoylation
We have presented two examples of how SUMO can regulate its own enzymatic cascade, by covalent and non-covalent interaction with Ubc9. It is unlikely that this is the only way the SUMO pathway is regulated. Already several additional

regulatory mechanisms are known that can affect overall sumoylation. Examples are the viral protein Gam1 which is able to inhibit E1 activity, or reactive oxygen species that induce the formation of cross-links between the SUMO E1 and E2 and thus inhibit sumoylation. Other regulatory mechanisms occur at the substrate level, examples of which are the temporally and spatially regulation for some targets and the fact that modification of some targets depends on prior phosphorylation. The cell may have other ways to control sumoylation and one could think of additional modifications of the enzymes in the SUMO pathway. This could be analogous to the modification of E3-ligases in ubiquitination, or, alternatively it would be possible that additional posttranslational modification of targets is required as a prerequisite for their sumoylation. Finally, much is still to be learnt about the role of isopeptidases in regulation. The specificity for most of these SUMO deconjugating enzymes is not clear yet but it is very likely that they are important factors in the regulation of SUMO signaling.

SUMO target identification

Since SUMO was discovered a decade ago, hundreds of substrates have been identified and this list is still growing. We have identified two new SUMO targets, Ubc9 and E2-25K, but many more are likely to exist. In order to understand the full biological impact of sumoylation, we need to identify the majority of SUMO targets and study the functional consequence of their sumoylation. *In vivo* detection and verification of SUMO targets however, is often difficult due to two reasons; one is that generally, at a given moment, only a small fraction of the total amount of a protein is sumoylated, and the second is that the SUMO isopeptidases are extremely active and remove SUMO from the target as soon as cells are broken for analysis. This last issue could potentially be solved with specific and efficient inhibitors of SUMO deconjugating enzymes, preferable working in intact cells, however, these are not yet available. The problem of low steady-state modification of proteins on the other hand, is intrinsic to sumoylation and can not be easily circumvented. New, highly sensitive, methods for identifying SUMO modified proteins from cells may accelerate the search for new SUMO targets. Mass spectrometry techniques may at some point be sensitive enough to analyze endogenous sumoylated proteins which would be of great value. An unbiased way of identifying new SUMO targets may also shed light on the specificity of sumoylation and the importance of the sequence surrounding the modified lysine.

Currently, many targets are identified by actively searching for the ΨKxE/D consensus sequence and, even though this has lead to the identification of many SUMO targets, there are also several examples where SUMO modification takes place on sites not conforming to this consensus site. Therefore, we can not exclude the possibility that many additional targets will be identified on alternative target sites. We have shown that in case of E2-25K and Ubc9 sumoylation takes place on a non-consensus site and, in both cases, on a site situated on an α-helix. In contrast to the traditional consensus sequence which is preferred in unstructured regions, helical sites may have a different preferred sequence. It would be useful to understand the requirements for such a helical SUMO consensus site. This is currently subject of studies in collaboration with Hans Langedijk (Pepscan Systems, The Netherlands).

The process of sumoylation is complex and subject to many levels of regulation. We have provided new insights into the function of an E3 enzyme, the way SUMO can regulate E2s, the biochemical consequence of sumoylation of the two targets, and the regulation of SUMO chain formation.

Summary

To ensure optimal control of cellular processes proteins need to be tightly regulated, which is mostly controlled at the level of synthesis, degradation or modification. Protein modification is a powerful tool, especially in signaling cascades, since it can quickly change the properties of a protein, for example by literally switching them on or off. The modifier can be a small functional group, like in phosphorylation, methylation or acetylation, but it can also be a protein.
The best known example of protein modification is ubiquitin, which regulates protein degradation as well as processes like DNA repair, signal transduction and endocytosis. Several ubiquitin like modifiers have been identified that share a common fold with ubiquitin but have different functional roles. The **S**mall **U**biquitin related **MO**difier SUMO is one of these ubiquitin-like molecules and functions in various cellular processes like intracellular transport, cell cycle regulation, DNA repair and regulation of transcription. Both ubiquitin and SUMO are ligated to their target proteins using a similar mechanism involving three regulated enzymatic steps that are catalyzed by an E1, E2 and an E3 enzyme.
Since many crucial cellular processes are regulated by SUMO modification, it is evident that the SUMO pathway can play a role in disease as well. In fact, a role for SUMO modification has been implicated in cancer, neurodegenerative diseases and viral infection. For a better understanding of the mechanism of sumoylation and its role in human diseases it is essential to study the proteins, and protein complexes, involved in great detail. The work in this thesis provides insight into the regulation of sumoylation by describing biochemical and structural aspects of SUMO modification; the catalytic mechanism of a SUMO E3 ligase, two distinct examples of the structure and function of SUMO modified targets, and insights into the mechanism of SUMO chain formation.

Although E1 and E2 enzymes are homologous between SUMO and ubiquitin conjugation this is not the case for all E3 enzymes. Ligases or E3 enzymes play an important role in sumoylation since they are able to enhance the modification of specific targets. One particular SUMO E3 ligase, the nucleoporin RanBP2, is unrelated to any other known E3 ligase. RanBP2 is an elongated protein that forms the cytoplasmic fibers of the nuclear pore complex (NPC) and its E3 ligase activity has been shown to reside in a domain towards the C-terminus of the protein. In **chapter 2** the mechanism of the E3 ligase RanBP2 has been studied biochemically and it was shown that RanBP2 does not work like HECT E3-ligases, which form an intermediate thioester with the modifier, and also not like the RING type E3 ligases, which act as adapters between target and E2. Instead, RanBP2 enhances sumoylation, at least in part, by altering the catalytic properties of Ubc9 without interacting with the target. Binding of RanBP2 to Ubc9 changes the properties of RanBP2 from being largely unstructured to a more compact conformation. A model where RanBP2 wraps around Ubc9 and accelerates SUMO transfer by an allosteric mechanism was proposed. That the principle of this mechanism was correct was later confirmed by others in the crystal structure of the catalytic fragment of RanBP2 in complex with Ubc9 and SUMO modified RanGAP1. This structure suggests that RanBP2 positions the Ubc9~SUMO thioester in an optimal conformation to accelerate SUMO transfer to the target.

Even though hundreds of SUMO targets have been identified, the biochemical consequence of SUMO modification is in many cases not known in detail. However, sumoylation generally leads to a change in protein interactions, activity, location or stability. In **chapter 3**, the identification of the ubiquitin conjugating enzyme E2-25K as a SUMO target has been described. We show that SUMO modification of E2-25K affects its enzymatic activity, inhibiting its ability to form ubiquitin thioesters as well as unanchored ubiquitin chains. Structure determination of the sumoylated E2-25K showed that the non-consensus target lysine is situated on the N-terminal helix creating a small but defined interface between E2-25K and the modifier. The target lysine of E2-25K is only recognized when present on a helix, suggesting that structural context is important for specific target site recognition that could lead to a better target site prediction. The inhibition of the enzymatic activity of E2-25K upon its sumoylation is most likely due to interference with the E1 interaction.

In **chapter 4** we show that E2-25K is not the only conjugating enzyme that is regulated by sumoylation, as we found that the SUMO conjugating enzyme, Ubc9, itself is subject to covalent modification with SUMO. The Ubc9*SUMO crystal structure shows that the position of SUMO with respect to Ubc9 is different compared to SUMO linked to E2-25K, even though sumoylation takes place on the equivalent lysine situated on the N-terminal helix. This difference is mediated by the interaction of SUMO with a β-hairpin of Ubc9, a feature unique for the SUMO E2 not present on any ubiquitin E2. As a result of this specific interaction and orientation of the modifier, sumoylation of Ubc9 functions completely different compared with E2-25K. SUMO ligated to Ubc9 does not inhibit its ability to form SUMO thioesters, but results in an altered substrate specificity; it inhibits the modification of certain targets and enhances the modification of others. The enhancement of Sp100 modification by SUMO modified Ubc9 is dependent on the presence of a SUMO interaction motif (SIM) on Sp100. The crystal structure of SUMO modified Ubc9 demonstrates how the newly created binary binding interface can provide the gain in affinity leading to enhanced modification of certain targets, thereby providing a unique way of regulating sumoylation.

A third example of regulation by SUMO differs from the previous ones since it does not involve a covalent attachment of SUMO but a non-covalent interaction between SUMO and Ubc9 and is described in **chapter 5**. Here we have shown that the non-covalent interaction between Ubc9 and SUMO promotes the formation of SUMO chains, a phenomenon that has been shown to occur for SUMO isoforms 2 and 3 *in vivo*. The crystal structure of Ubc9 bound to SUMO non-covalently shows that this interaction takes place at a site distinct from the catalytic site at the back of the Ubc9 molecule. Disruption of this interaction strongly reduces the formation of SUMO chains on several targets. Similar interactions between ubiquitin(-like) proteins and ubiquitin were shown for UbcH5c and Mms2, and we propose a model in which SUMO2 chain formation occurs through a mechanism similar to ubiquitin chain formation by the Mms2-Ubc13 heterodimer.

In this thesis we describe various aspects of both regulation by sumoylation as well as the regulation of the process of sumoylation itself. We found several ways in which SUMO can regulate the process of sumoylation and determined the function of sumoylation of two distinct targets.

Samenvatting

Eiwitten behoren tot de belangrijkste bestanddelen van levende organismen aangezien bijna alle cellulaire processen afhankelijk zijn van een of meerdere eiwitten. De bouwstenen van eiwitten zijn de aminozuren die ketens vormen in een volgorde gebaseerd op de basenvolgorde van ons DNA. Er zijn dus nagenoeg evenveel verschillende eiwitten als er genen zijn. De aminozuurvolgorde samen met de drie-dimensionele vouwing geven ieder van deze eiwitten een specifieke structuur en functie. Alle processen in de cel waarbij eiwitten betrokken zijn dienen op een gecontroleerde manier te verlopen. Daarom bestaan er verschillende mechanismen om eiwitten te reguleren. Dit gebeurt meestal op het niveau van eiwit-synthese, -afbraak of -modificatie. Post-translationele modificatie van eiwitten is een belangrijk mechanisme in bijvoorbeeld signaal transductie, aangezien het een snelle, doch omkeerbare, verandering in functie kan veroorzaken. In dit proces worden eiwitten covalent gemodificeerd. In veel gevallen, zoals bij fosforylering, methylering of acetylering, wordt er een kleine functionele groep aan het eiwit gekoppeld, er bestaan echter ook modificaties bestaand uit hele eiwitten.
Het meest bekende voorbeeld van deze laatste modificaties is de ubiquitinering, dit is een modificatie met het 76 aminozuren tellende eiwit ubiquitine. Modificatie van substraateiwitten met ubiquitine kan resulteren in verschillende effecten waarvan de afbraak van het substraat het bekendste voorbeeld is. Echter, ubiquitinering speelt ook een rol bij reparatie van DNA schade, endocytose en vele andere cellulaire processen. Naast ubiquitine bestaan er ook een aantal modificerende eiwitten die qua structuur gelijkenis vertonen met ubiquitine, maar een andere rol in de cel vervullen. SUMO is een van deze ubiquitine-achtige eiwitten en functioneert in cellulaire processen zoals intracellulair transport, regulatie van de celcyclus en DNA transcriptie. SUMO wordt covalent aan substraten gekoppeld door middel van een serie enzymatische reacties gelijkend op die in ubiquitinering, waarbij achtereenvolgens drie enzymen betrokken zijn. In de eerste stap activeert het E1 enzym SUMO door de vorming van een thioester binding tussen het actieve cysteïne residu van het enzyme en de C-terminus van SUMO. Vervolgens wordt SUMO overgedragen aan een E2 enzym waarbij het karakter van de binding behouden blijft, een thioester, maar nu tussen SUMO en het actieve cysteïne residu van het E2 enzyme. Tenslotte wordt SUMO, meestal met behulp van een E3 enzyme, overgezet naar het substraat waarbij een isopeptide binding wordt gevormd tussen de C-terminus van SUMO en een lysine van het substraat.
Aangezien vele biologische processen gereguleerd worden door middel van SUMO modificatie is het niet verwonderlijk dat sumoylering ook een rol speelt bij bepaalde ziektes. Er zijn sterke aanwijzingen dat SUMO een rol kan spelen in virale infecties, kanker en neurondegeneratieve aandoeningen zoals de ziekte van Alzheimer, Parkinson en Huntington. Om het mechanisme van sumoylering en de rol van SUMO in deze aandoeningen beter te begrijpen is het belangrijk om de eiwitten en eiwitcomplexen die hierbij betrokken zijn tot in detail te analyseren.
Het werk beschreven in dit proefschrift biedt nieuwe inzichten in de regulatie van sumoylering door het bestuderen van biochemische en structurele aspecten van SUMO modificatie. Het beschrijft het katalytisch mechanisme van een SUMO E3 enzym, de structuur en functie van twee gesumoyleerde substraten en het geeft nieuwe inzichten in het mechanisme van SUMO ketenvorming.

Van de drie enzymen betrokken bij SUMO modificatie zijn de E1 en E2 homoloog aan hun equivalenten in ubiquitinering, dit is echter niet het geval voor alle E3 enzymen of ligases. Doordat ze in staat zijn de modificatie vele malen efficiënter te laten verlopen, spelen E3's een belangrijke rol in sumoylering. Een van deze E3 enzymen, RanBP2, vormt een onderdeel van een groot eiwit complex dat betrokken is bij transport van cytosolaire eiwitten naar de celkern, het nuclear pore complex (NPC). RanBP2 is een langgerekt eiwit dat het cytosol insteekt en waarvan het C-terminale gedeelte actief is als SUMO E3 ligase. Het vertoont echter geen enkele gelijkenis met andere bekende SUMO of ubiquitine ligases. In **hoofdstuk 2** beschrijven we de biochemische karakterisatie van dit SUMO E3 enzym. We tonen aan dat RanBP2 geen thioester met SUMO vormt als tussenproduct en daarom niet verwant is aan de HECT-type ubiquitine E3 ligases. Vervolgens

laten we zien dat RanBP2 niet fungeert als adapter tussen de E2 en het substraat en daarom ook niet lijkt op de RING-type E3-ligases. RanBP2 is echter in staat de sumoylering te versnellen zonder een directe interactie aan te gaan met het substraat. De data gepresenteerd in hoofdstuk 2 leiden tot een model waarbij de interactie van RanBP2 met de E2, Ubc9, in staat is de reactie te beïnvloeden via een allosteer mechanisme. Het principe van dit model is later bevestigd door een andere groep, met de opheldering van de kristalstructuur van het katalytische gedeelte van RanBP2 in complex met Ubc9 en een gesumoyleerd substraat. Deze structuur laat zien dat RanBP2 waarschijnlijk verantwoordelijk is voor het optimaal positioneren van de Ubc9~SUMO thioester voor snelle overdracht van SUMO naar het substraat.

Al zijn er honderden SUMO substraten geïdentificeerd, de biochemische consequentie van hun modificatie is in veel gevallen niet tot in detail bekend. Echter, over het algemeen heeft sumoylering een verandering in eiwit interacties, activiteit, locatie of stabiliteit tot gevolg. In **hoofdstuk 3** beschrijven we de identificatie van een nieuw SUMO substraat, het ubiquitine conjugerende enzym E2-25K. We tonen aan dat de sumoylering van dit enzym resulteert in een afname van enzymatische activiteit. De kristalstructuur van het SUMO geconjugeerde E2-25K, tevens de eerste substraat*SUMO kristalstructuur die bepaald is, laat zien dat SUMO gekoppeld wordt aan een lysine in de N-teminale helix van E2-25K. Naast deze covalente binding is er een klein maar goed gedefinieerd bindingsoppervlak tussen beide eiwitten. Het feit dat de sumoylering plaatsvindt op een lysine gesitueerd in een α-helix is bijzonder aangezien de meeste SUMO sites zich bevinden in ongestructureerde delen van een eiwit. In het geval van E2-25K is deze secundaire structuur echter cruciaal voor de herkenning van deze lysine voor sumoylering. De observatie dat secundaire structuur van belang is voor de herkenning van een SUMO site kan leiden tot een betere voorspelling van nieuwe SUMO modificatie sites.
In **hoofdstuk 4** laten we zien dat E2-25K niet het enige E2 enzym is dat gereguleerd wordt door sumoylering door aan te tonen dan het SUMO conjugerende enzym Ubc9 zelf ook covalent gemodificeerd kan worden met SUMO. De kristalstructuur van Ubc9 gemodificeerd met SUMO demonstreert dat de positie van SUMO ten opzichte van Ubc9 verschilt van SUMO ten opzichte van E2-25K. Dit was een onverwacht resultaat aangezien Ubc9 en E2-25K op elkaar lijken qua structuur en in beide gevallen SUMO gekoppeld is aan een equivalente lysine in de N-terminale helix van de E2's. Het verschil in de positie van SUMO kan verklaard worden door een extra interactie van SUMO met een Ubc9-specifieke β-hairpin die afwezig is in ubiquitine E2's als E2-25K. Wellicht mede door het verschil in oriëntatie van SUMO, heeft de sumoylering van Ubc9 een compleet ander effect dan sumoylering van E2-25K. SUMO modificatie van Ubc9 reduceert niet de activiteit van het enzym maar heeft een verandering in substraat specificiteit tot gevolg. De modificatie van sommige substraten wordt geremd en van anderen juist versneld. Een specifiek substraat waarvan de sumoylering versneld wordt als Ubc9 zelf gemodificeerd is met SUMO, is de transciptionele regulator Sp100. In dit geval is de verhoogde sumoylering afhankelijk van de aanwezigheid van een SUMO interactie motief (SIM) op Sp100. De kristalstructuur van gesumoyleerd Ubc9 laat zien hoe de interactie van SUMO gekoppeld aan Ubc9 met de SIM op Sp100 kan leiden tot een tweedelig bindingsoppervlak. Dit resulteert in een efficiëntere binding tussen E2 en substraat en daardoor een versnelde reactie. Hiermee presenteren we een unieke manier van regulatie van sumoylering.
Het derde voorbeeld van regulatie door SUMO is beschreven in **hoofdstuk 5** en verschilt van de voorgaande aangezien er geen sprake is van een covalente verbinding maar een niet-covalente. We laten hier zien dat de niet-covalente interactie tussen Ubc9 en SUMO de vorming van SUMO ketens bevordert. De details van deze interactie demonstreren we in de kristalstructuur van het niet-covalente Ubc9-SUMO complex. Deze structuur laat zien dat de binding plaatsvindt ver van het katalytische cysteïne residu en tevens niet overlapt met de covalente bindingsplaats. Kennis van de structuur heeft het mogelijk gemaakt mutaties te vinden die de niet-covalente binding verbreken maar geen invloed hebben op de eerste twee stappen in het sumoyleringsproces. Met behulp van deze mutaties tonen we aan dat het verstoren van de interactie tussen SUMO en Ubc9 de vorming van SUMO ketens op verschillende substraten remt. Vergelijkbare interacties zijn ook gevonden tussen ubiquitine en zowel de ubiquitine E2 UbcH5c als het E2-achtige eiwit Mms2. Vergelijking met deze structuren leidt tot

een model waarin het mechanisme van SUMO ketenvorming gelijkenis vertoont met de vorming van ubiquitineketens door de Mms2-Ubc13 heterodimeer.

In dit proefschrift worden verscheidene aspecten van zowel regulatie door sumoylering als regulatie van het proces van sumoylering zelf beschreven. We hebben verschillende manieren gevonden waarop SUMO het proces van sumoylering kan reguleren en de functie beschreven van sumoylering van twee specifieke SUMO substraten.

Curriculum Vitae

Puck Knipscheer werd geboren op 4 december 1974 te Nijmegen. Zij behaalde haar VWO diploma in 1993 aan de Nijmeegse Scholengemeenschap Groenewoud in Nijmegen. In 1993 begon zij met de studie moleculaire wetenschappen aan de Wageningen Universiteit. Haar eerste afstudeerstage vond plaats bij de vakgroep biochemie in Wageningen in de groep van Prof. Ivonne Rietjens. Een tweede afstudeerstage deed zij in Palmerston North, Nieuw Zeeland aan de Massey University, Centre of Structural Biology onder supervisie van Prof. Geoffrey Jameson. Haar derde stage vond plaats in de groep van Prof. Anton Berns op de afdeling moleculaire genetica aan het Nederlands Kanker Instituut. Sinds december 1999 is zij werkzaam geweest als onderzoeker in opleiding in de groep van Prof. Titia Sixma op de afdeling moleculaire carcinogenese van het Nederlands Kanker Instituut. Per 1 april 2007 zal zij haar onderzoek voortzetten in de groep van Prof. Johannes Walter aan Harvard Medical School in Boston.

List of Publications

Puck Knipscheer, Willem J van Dijk, Jesper V Olsen, Matthias Mann and Titia K Sixma (2006) Non-covalent interaction between Ubc9 and SUMO promotes SUMO chain formation. *Submitted for publication.*

Puck Knipscheer, Willem J van Dijk, Jesper V Olsen, Matthias Mann, Frauke Melchior, Titia K Sixma and Andrea Pichler (2006) Ubc9 sumoylation regulates SUMO target discrimination. *Submitted for publication.*

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* these authors contributed equally to this work

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I hope I have had enough time to teach you some of the 'basics' for survival in this lab.

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Het was super!

Puck