Targeting SUMO conjugates for degradation: The human RING finger RNF4 as a specialized ubiquitin ligase

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ABBREVIATIONS

AML	Acute myeloid leukemia
Amp^R	Ampicillin resistence
AOS	Activation of Smt3p
APL	Acute promyelocytic leukemia
APS	Ammonium peroxydisulfate
ARNTL1	Aryl hydrocarbon receptor nuclear translocator-like protein 1
ATO, As_2O_3	Arsenic trioxide
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
B	B-Box
B1	B-Box 1
BB	Binding buffer
BSA	Bovine serum albumin
Cam^R	Chloramphenicol resistence
CC	Coiled coil
CENP-I	Centromere protein I
CK2	Casein kinase 2
Coomassie	Coomassie Brilliant Blue
DAPI	4',6-Diamidine-2'-phenylindole dihydrochloride
Daxx	Death associated protein 6
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethylsulfoxide
DTT	Dithiotreithol
DUB	Deubiquitylase
EB	Elution buffer
ECL	Enhanced Chemiluminescence
EMEM	Eagle's Minimal Essential Medium
ERK	Extracellular signal-regulated kinase
FAT10	F-locus associated transcript 10
FCS	Fetal calf serum
FPLC	Fast Protein Liquid Chromatography
FT	Flow through
GMP1	GAP modifying protein 1
GSH	Glutathione
GST	Glutathione-S-transferase
HDAC	Class IIa histone deacetylase
HECT	Homologous to E6AP C-terminus
HIF1 α	Hypoxia inducible transcription factor alpha
HIP1α	Huntingtin interacting protein 1 alpha
HMW-SC	High Molecular Weight SUMO Conjugate
HP1	Heterochromatin protein 1
HRP	Horse-radish peroxidase
HSV-1	Herpes simpex virus 1
ICP0	Infected Cell Polypeptide 0
IFN	Interferon
IKK	I kappa B kinase
IPI	International Protein Index

IPTG	Isopropyl-β-D-thiogalactopyranosid
ITC	Isothermal titration calorimetry
ITC	Isothermal Titration Calorimetry
IκBα	Inhibitor of NFκBα
K_A	Association constant
Kan^R	Kanamycine resistence
K_D	Dissociation constant
KSHV	Kaposi's sarcoma associated herpesvirus
LB	Luria-Bertani
LIC	Leukemia initiating cells
MCAF1	MBD1-containing chromatin associated factor
MCS	Multiple cloning site
MS	Mass spectrometry
M_W	Molecular weight
N	Stoichiometry
NAA	Non-essential amino acids
NEM	N-ethylmaleimide
NEMO	NFκB essential modulator
NFκB	Nuclear factor κB
NLS	Nuclear localization signal
Nmi	N-myc-interactor
NMR	Nuclear magnetic resonance
NPC	Nuclear pore complex
o/n	Over night
ORF	Open reading frame
ori	Origin of replication
P	Pellet
PBS	Phosphate buffered saline
Pc2	Polycomb protein 2
PCNA	proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD	Pulldown
PDSM	Phosphorylation-dependent sumoylation motif
PFA	Paraformaldehyde
PIAS	Protein inhibitors of activated STAT
PIC1	PML-interacting clone 1
PML	Promyelocytic leukemia protein
PML-NB	PML nuclear body
polyUb	Poly-ubiquitin
PROX1	Prospero-like protein
PVDF	Polyvinylidene fluoride
R	RING
RA	Retinoid acid
RanBP2	Ran binding protein 2
RanGAP1	Ran-GTPase activating protein
RARE	RA response elements
RARα	retinoid acid receptor alpha
RBCC motif	RING, B-Box(es), coiled-coil domain
Rfp	RING finger protein
RING	really interesting new gene
RL	Raw lysate

RNF4	RING finger 4
RT	Room temperature
RU	Response units
S	Supernatant
S200	Superdex 200
S75	Superdex 75
SAE	SUMO activating enzyme
SBM	SUMO binding motif
SDS	Sodium dodecyl sulfate
SDS-PA	SDS polyacrylamide
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SENP	Sentrin specific protease
SIM	SUMO interaction motif
SMT3, smt3	Suppressor of Mif Two 3, gene, protein
SPR	Surface Plasmon Resonance
SP-RING	Siz/PIAS-RING
STAT	Signal transducer and activator of transcription
StUbL	SUMO-targeted ubiquitin ligase
SUMO	Small ubiquitin related modifier
SUMOc./conj.	SUMO conjugated
SUMOylation	SUMO conjugation to substrates
SUSP	SUMO specific protease
TEMED	N,N,N‘,N‘-Tetra-methylethylendiamine
TEV	Tobacco etch virus protease
TGF	Transforming growth factor
TOPORS	Topoisomerase I and p53 binding protein
TRIM	Tripartite motif
TUBEs	Tandem-repeated ubiquitin-binding entities
UBA	Ubiquitin-associated
Uba/Ube1	Ubiquitin activating enzyme/Ubiquitin enzyme 1
Ubc/Ube2	Ubiquitin-conjugating enzyme/Ubiquitin enzyme 2
UBD	Ubiquitin binding domain
UbE2	Ubiquitin E2
UBL	Ubiquitin-like modifier
Ulp	Ubiquitin-like protease
ULS	Ubiquitin ligase for SUMO conjugates
UPS	Ubiquitin proteasome system
Usp25	Ubiquitin specific protease 25
VHL	Von Hippel-Lindau factor
wt	Wildtype
ZNF	Zinc finger

1. INTRODUCTION

1.1 Relevance of posttranslational modifications in living cells

Cells need to face and react to influences in their environment irrespective of being a protozoon or part of a higher organism to ensure their viability or to fulfill specialized tasks of a united cell-structure in a higher organism.
Therefore highly organized interconnected signaling networks evolved sensing extra- or intracellular stimuli and induce diverse changes ranging from altered transcription to the activation or repression of protein functions. The bases of these signaling networks are small molecules that can be attached to other molecules such as proteins, RNA or lipids to induce altered affinities or binding properties for other molecules as well as to regulate functional aspects such as enzymatic activity or cellular localization. These so-called posttranslational modifications permit much faster signaling and reaction than for example any altered transcription/translation modules could (Walsh, 2007).
Posttranslational modifications of proteins also greatly extend the functional diversity and dynamics of the proteome. The usually transient attachment of small molecules such as phosphate, methyl or acetyl groups to certain proteins is well established (Cheung, 2000; Cohen, 2001; Walsh, 2007; Yang and Seto, 2008; Ng, 2009). Additionally, there are small proteins in eukaryotic cells that can be covalently attached to other proteins. The most prominent member of these modifiers is ubiquitin, after which this group is named. Other Ubiquitin-like modifiers (UBLs) are SUMO, NEDD8, ISG15, Fat10, Atg8 or Atg12 (Kerscher, 2006; Hochstrasser, 2009).

1.2 Ubiquitin modification of proteins

Since ubiquitin was discovered in 1975, it became apparent that posttranslational modification of proteins by ubiquitin plays an important role in diverse cellular processes, including cell division, differentiation, signal transduction, protein trafficking, and quality control (Schlesinger, 1975; Hershko, 1983; Ciechanover, 2005; Mukhopadhyay and Riezman, 2007). It is a highly conserved protein ubiquitously expressed in all eukaryotes with a size of 8,5 kDa and can be covalently attached to proteins by free lysine, cysteine, or N-terminal residues by specific enzymatic cascades. These cascades typically involve E1, E2 and E3 enzymes (see section 1.2.1). The most abundant attachment form of ubiquitin is the conjugation to lysine (K) residues in its substrate molecules (Glickman and Ciechanover, 2002; Bloom, 2003; Cadwell and Coscoy, 2005). Mono-ubiquitylation, the addition of a single ubiquitin moiety per targeting site, was shown to modulate protein endocytosis, intracellular transport, and DNA repair (see section 1.2.2.1; Haglund, 2003; Ulrich, 2005; Acconcia, 2009; Thompson

and Hinz, 2009). Attached mono-ubiquitin can be further modified to generate poly-ubiquitin (polyUb) chains via linkages between several ubiquitin molecules, of which the most prominent is the K48-linked chain that targets the conjugated substrate for proteasomal degradation (see section 1.2.2.2; Hochstrasser, 2006).

1.2.1 Mechanistic aspects of Ubiquitin conjugation

Ubiquitin forms a stable β-grasp fold which is shared by all UBLs despite low sequence homologies. This suggests a common ancestry and indeed, the β-grasp fold may have arisen as an RNA-binding module in a primitive protein-translation system (Burroughs, 2007). Furthermore, the UBL conjugation pathway shows some similarity to bacterial sulphur transfer enzymes within the biosynthesis pathways of the molybdenum cofactor and thiamine (Iyer, 2006; Hochstrasser, 2009).

Four ubiquitin genes in humans encode a total of 14 copies of ubiquitin, so that it is synthesized as a linear poly-ubiquitin chain (Wiborg, 1985). To generate free cellular mono-ubiquitin molecules, theses chains require proteolytic processing by deubiquitylases (DUBs; Komander, 2009b). The single ubiquitin molecules then terminate with the typical Gly-Gly motif, which is common to all UBLs and which is indispensible for substrate modification (Hershko, 1981; Dye and Schulman, 2007).

Mechanistically, the covalent attachment of ubiquitin to substrate proteins involves several enzymatic steps, carried out by three types of enzymes – E1, E2 and E3 (see Figure 1.1; Hershko, 1983). The E1 enzyme (Ubiquitin activating enzyme, e.g. Ube1) activates ubiquitin for transfer by adenylating its C-terminus in an ATP-dependent step. Ubiquitin is then coupled to the E1 active site cysteine, forming a reactive thioester between E1 and the C-terminal glycine of ubiquitin (Ciechanover, 1981; Hershko, 1981; Ciechanover, 1982). The activated ubiquitin is subsequently transferred to one of the distinct ubiquitin-conjugating enzymes (Ubc's; E2s) by transthiolation to a conserved cysteine of the E2 (Pickart and Rose, 1985; Haas and Bright, 1988). The E2 proteins catalyze substrate ubiquitylation in conjunction with an ubiquitin-protein ligase (E3). The concerted action of different E2 and E3 enzymes provide substrate specificity to the ubiquitin conjugation reaction (Reiss, 1989; Sung, 1991; Pickart and Eddins, 2004; Hochstrasser, 2006; Christensen, 2007). During this last step of the enzymatic cascade, the ubiquitin molecule is usually transferred to an ε-amino group of a lysine side chain in the substrate, thereby forming a peptide-like amide bond (isopeptide bond; Goldknopf and Busch, 1977; Hershko, 1983; Pickart and Eddins, 2004). By adding activated ubiquitin moieties to internal lysine residues on the previously conjugated ubiquitin molecule, poly-ubiquitin chains are synthesized (see section 1.2.2.2; Chau, 1989; Hershko and Ciechanover, 1998).

Whether ubiquitin chain assembly is mediated via a simple sequential addition of ubiquitin moieties to the nascent chain on the substrate or via pre-assembly on E2/E3 active sites and subsequent transfer onto the substrate is currently under debate (Hochstrasser, 2006; Li, 2007; Ye and Rape, 2009).

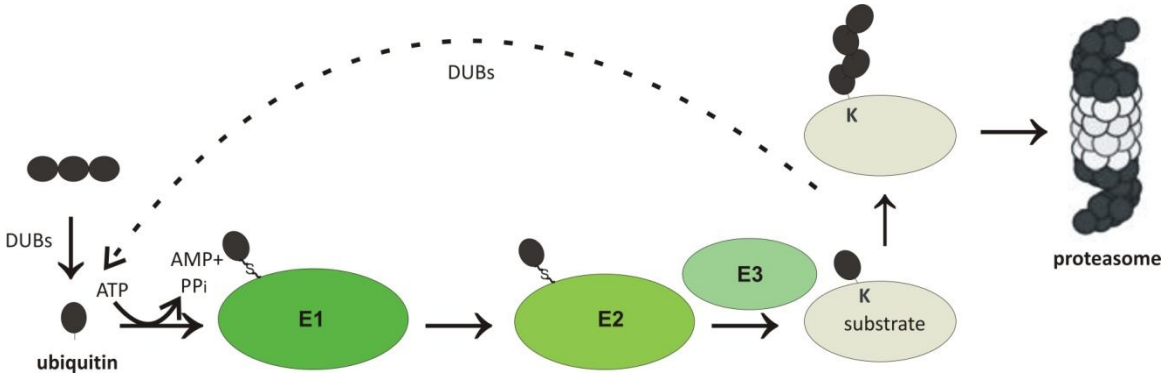


Figure 1.1: ubiquitin conjugation cascade
Ubiquitin is expressed as polyprotein; the C-terminal di-glycine motif of individual ubiquitin moieties is exposed after cleavage mediated by deubiquitylating enzymes (DUBs). Ubiquitin is activated by E1 enzymes under ATP consumption and forms a thioester to the E1 active cysteine. Activated ubiquitin is then transferred to an E2 enzyme. E2 and E3 enzymes ubiquitylate the substrate in a concerted action. Attachment to other ubiquitin molecules forms poly-ubiquitin chains. Some types of ubiquitin chains are recognized by the proteasome that degrades the substrate. Modified from (Miteva, 2010).

1.2.1.1 E1 enzymes

There are two different E1 enzymes for ubiquitin activation in vertebrates, Ube1/UBA1 and Ube1L2/UBA6. They share 40 % of sequence homology to each other (Ciechanover, 1981; Ciechanover, 1982; Chiu, 2007; Jin, 2007; Pelzer, 2007). Ube1L2 was identified as an ubiquitin E1 in 2007, so before it was assumed that Ube1 is the only ubiquitin activating enzyme in vertebrates as it is in lower eukaryotes (Schulman and Harper, 2009). Ube1 charges most known ubiquitin E2 enzymes whereas Ube1L2 has a selective ubiquitin E2 enzyme, Ube2Z/Use1 (Jin, 2007). Furthermore, Ube1L2 is capable to activate F-locus associated transcript 10 (FAT10), a UBL, *in vivo* and *in vitro* (Chiu, 2007).

1.2.1.2 E2 enzymes

Family members of the ubiquitin conjugating enzymes (E2s) all possess a highly conserved ubiquitin-conjugating (Ubc) catalytic fold (van Wijk and Timmers, 2009). These 14-16 kDa domains have an approx. 35 % sequence conservation among different family members and provide a binding platform for E1s, E3s, and the activated ubiquitin or UBL (Burroughs, 2008). The catalytic cysteine, which accepts the activated ubiquitin molecule, is embedded within this domain (van Wijk and Timmers, 2009).
Until now, 38 E2s have been identified in humans and some of them promote specifically the conjugation of ubiquitin-like modifiers other than ubiquitin (Ye and Rape, 2009). Together with the respective E1 enzymes, this specificity results in parallel conjugation pathways of ubiquitin and UBLs, although there are some cases of crosstalk (e.g. the ubiquitin E2 Ube2L6/UbcH8 is shared by ISG15 or the before mentioned use of Ube1L2 by ubiquitin as well as by FAT10; Kim, 2004; Zhao, 2004; Chiu, 2007). Several E2s play distinct roles in the ubiquitylation of a substrate. Although most E2s ubiquitylate substrates without any intrinsic selectivity for a specific acceptor lysine, some stimulate ubiquitin chain assembly through a defined lysine in ubiquitin. In these cases, the next

ubiquitin molecule being assembled is positioned in such a way that only the respective acceptor lysine side chain can attack the thioester bond to the E2 (Ye and Rape, 2009). Examples are the E2 MMS2/Ubc13 complex in the assembly of K63-linked chains (Hofmann and Pickart, 1999; Deng, 2000; VanDemark, 2001) or the UbcH10/Ube2S complex in the assembly of K11-linked chains (Williamson, 2009).
Other E2s, such as UbcH5, catalyze the formation of ubiquitin chains that lack specificity for any lysine residue of ubiquitin (Brzovic and Klevit, 2006).

1.2.1.3 Ubiquitin E3 ligases

Approximately 600-1000 E3s exist in the human genome, some of which belong to a special class like the RING (really interesting new gene), the HECT (homologous to E6AP C-terminus) or the U-Box domain family (Hatakeyama and Nakayama, 2003; Li and Ye, 2008; Deshaies and Joazeiro, 2009; Rotin and Kumar, 2009).
For the HECT-domain family of E3s, ubiquitin is first transferred to a conserved cysteine of the E3 before it is finally transferred to a substrate group (Huibregtse, 1995; Rotin and Kumar, 2009). For most other ubiquitylation reactions, the E3 rather functions as an adaptor that positions the substrate in close proximity to the reactive E2~ubiquitin thioester. The majority of such E3s belong to the RING-based domain family (~616 proteins; Freemont, 1991; Deshaies and Joazeiro, 2009). In addition to substrate recognition, E3s might have other roles in the catalytic cycle, such as allosteric activation of the E2 as well as mediating linkage-specific poly-ubiquitin chain assembly together with the respective E2 enzymes (Huang, 2004; Pickart and Eddins, 2004; Deshaies and Joazeiro, 2009).

1.2.1.3.1 RING/U-Box ligases

The RING domain was first described in 1991 and was thought to mediate DNA binding or protein dimerization (Freemont, 1991; Joazeiro and Weissman, 2000; Deshaies and Joazeiro, 2009). Only later it became clear, that many RING domain containing proteins serve as ubiquitin ligases and bind to E2 enzymes (Bailly, 1997; Zachariae, 1998; Joazeiro, 1999; Kamura, 1999; Lorick, 1999; Deshaies and Joazeiro, 2009). The consensus sequence of a RING domain is

C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-C-X2-C-X(4-48)-C-X2-C

(where X is any amino acid). The conserved cysteine and histidine residues complex two zinc atoms while forming an interleaved globular structure, the RING domain (see Figure 1.2; Barlow, 1994; Borden, 1995). RING domain E3 ligases are either part of a multi-subunit complex or act as homo- or heterodimers to mediate substrate ubiquitylation (Sharp, 1999; Hashizume, 2001; Xia, 2003; Thornton and Toczyski, 2006; Kawai, 2007). Other domains such as the B-Box of the tripartite motif (TRIM) subfamily of RING proteins (see section 1.6; Tao, 2008) and the U-Box are structurally related to the RING (Aravind and Koonin, 2000).

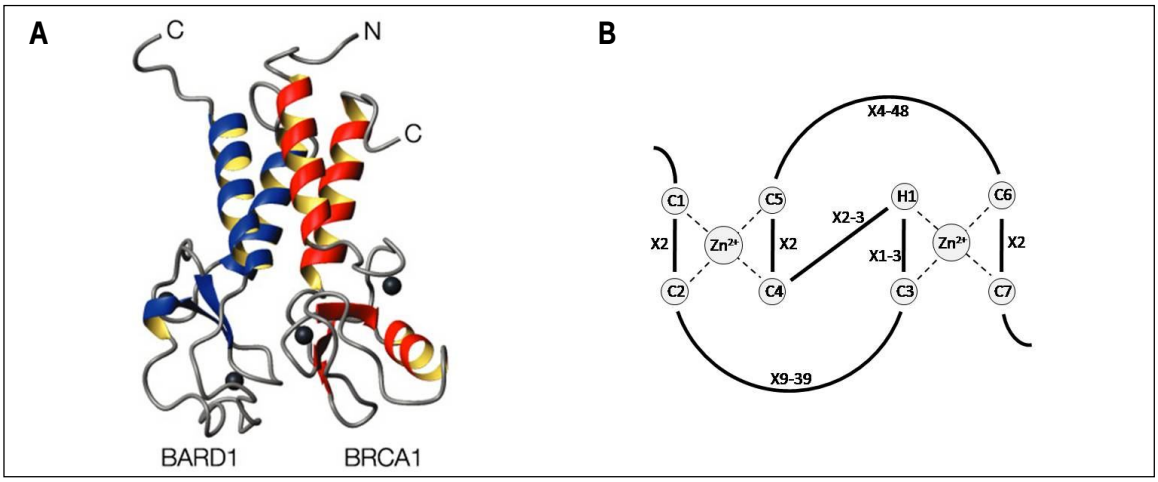


Figure 1.2: RING domain – Dimer and cross-brace structure
A) Structure of the RING heterodimer BARD1/BRCA1. The dimer interface is further stabilized through α-helical interactions (Brzovic, 2001).
B) Schematic representation of a RING domain. The primary sequence organization of the RING domain is folded in a cross-brace structure to coordinate the two zinc atoms. The first cysteine that coordinates zinc is labeled as C1, and so on. H1 denotes the coordinating histidine. Xn refers to the number of amino acid residues in the spacer regions between the zinc coordinating residues (modified from Deshaies and Joazeiro, 2009).

1.2.1.4 Deubiquitylating enzymes

Deubiquitylases (DUBs) exhibit three major functions: They generate the pool of free ubiquitin molecules by cleaving the newly translated linear ubiquitin chains into single molecules. Then, they can remove ubiquitin (chains) from modified substrates. Additionally, DUBs associated with the proteasome are responsible for ubiquitin recycling from proteins prone for degradation (Lam, 1997; Park, 1997; Borodovsky, 2001; Verma, 2002; Komander, 2009b). Furthermore, some DUBs can edit the form of ubiquitin modification by trimming ubiquitin chains (Wertz, 2004). In the human genome, approx. 79 genes encode for deubiquitylases of which nearly all family members are cysteine proteases while the members of one subgroup are zinc metalloproteases (Nijman, 2005).

1.2.1.5 Ubiquitin binding domains

Diverse proteins in the ubiquitin signaling pathway need to recognize and distinguish ubiquitin and its diverse chained forms from each other and from other UBLs. Therefore, a range of ubiquitin binding domains (UBDs) exist (UIMs, UBXs, UBAs, etc.), most of which recognize a specific hydrophobic patch around ubiquitin's Ile44. The diverse UBDs differ in their structure and do not necessarily share a common motif (Dikic, 2009). Furhtermore, some UBDs have been shown to act as a cis-E3 mono-ubiquitin ligase, promoting self-ubiquitylation of the protein they are part of (Hoeller, 2007).

1.2.2 Mono-ubiquitylation and polymeric ubiquitin chains

Ubiquitin can be attached to the substrates in multiple ways.

The following scheme (Figure 1.3) gives an impression of the variety of signals achieved by different ubiquitylation types.

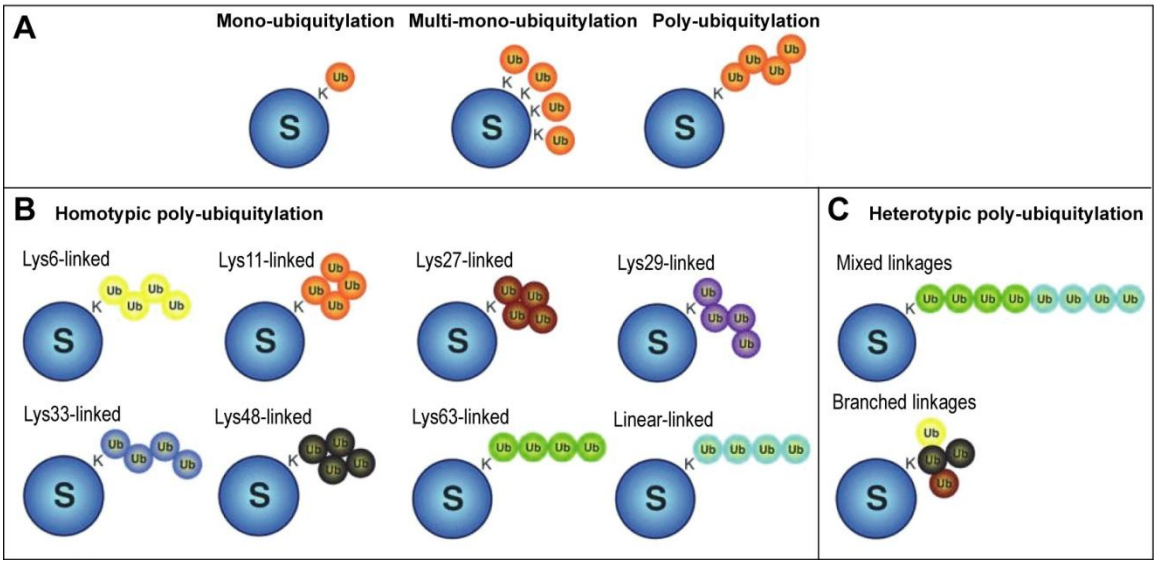


Figure 1.3: Different forms of ubiquitylation

A) The three general forms of ubiquitylation: mono-ubiquitylation, multiple mono-ubiquitylation and poly-ubiquitylation. B) Forms of homotypic poly-ubiquitylation. Each ubiquitin chain contains a single linkage type which may lead to distinct ubiquitin chain conformations. Multiple homotypic ubiquitin chains on the same substrate are possible. C) Forms of heterotypic poly-ubiquitylation. In mixed linkages, a ubiquitin chain has alternating linkage types. In branched or forked poly-ubiquitin chains, a single ubiquitin is extended at two or more lysine residues. The scheme was modified from (Komander, 2009a).

1.2.2.1 Mono-ubiquitylation

Mono-ubiquitylation in general serves as a signaling module to confer additional protein-protein interaction properties to its substrate proteins. In the case of receptor endocytosis, it regulates the internalization and sorting of the modified receptor that is either recycled or further transported to the endo-lysosomal compartment for degradation (Levkowitz, 1998; Levkowitz, 1999; Joazeiro, 1999; Acconcia, 2009).

In the case of the processivity factor “Proliferating cell nuclear antigen” (PCNA) monoubiquitylation results in the recruitment of a damage-tolerant polymerase and translesion synthesis while the modification of the same residue with a K63-linked chain results in an error-free damage avoidance pathway. Its modification with the small ubiquitin related modifier (SUMO) facilitates DNA damage tolerance upon replication fork stalling by recruitment of Srs2 (Hoege, 2002; Stelter and Ulrich, 2003; Kannouche, 2004; Pfander, 2005; Ulrich, 2005).

1.2.2.2 Poly-ubiquitin chains

Ubiquitin possesses seven lysine residues on its surface, namely K6, K11, K27, K29, K33, K48 and K63 that can all serve as acceptor sites for additional ubiquitin molecules *in vitro* and *in vivo*, thereby forming poly-ubiquitin chains (see Table 1.1; Arnason and Ellison, 1994; Johnson, 1995; Baboshina and Haas, 1996; Peng, 2003). Poly-ubiquitin chains consisting of four ubiquitin moieties attached through K48 linkages typically mark a protein for proteasomal degradation (Hershko, 1983; Chau, 1989; Hershko and Ciechanover, 1998; Pickart and Fushman, 2004). Chain formation through other lysine residues of various lengths and shapes results in various chain conformations and create a range of molecular signals in the cell (Kim, 2007; Ikeda and Dikic, 2008). In addition, linear chains are also assembled *in vivo* by head-to-tail arrangement of ubiquitin moieties through the α-amino group at the N-terminus (Kirisako, 2006). Recently, it has been postulated, that all non-K63-linked ubiquitin chains target proteins for degradation (Xu, 2009).

Table 1.1: Relative cellular abundance and form of homotypic poly-ubiquitin chains

Chain via	abundance	Cellular functions	Chain form
Lys48	29 %	Protein degradation via the UPS (Pickart and Eddins, 2004)	zig-zagged compact helix, lefthanded (Eddins, 2007)
Lys63	17 %	modulation of protein-protein interactions (Wang, 2008; Komander, 2009c)	pseudo-linear; open, left-handed helix (Komander, 2009c)
Lys11	28 %	Protein degradation in response of ER stress (Xu, 2009)	tube or helix (Bremm and Komander, 2009)
Lys6	N.D.	inhibits ubiquitin-dependent proteolysis (Shang, 2005)	N.D.
Lys33	3 %	N.D.	N.D.
Lys29	3 %	linked to lysosomal degradation (Chastagner, 2006)	N.D.
Lys27	9 %	N.D.	N.D.
Met1	N.D.	TNF/NFκB signaling (Haas, 2009; Iwai and Tokunaga, 2009)	linear (Komander, 2009c)

1.2.2.2.1 K48-linked chains and the Ubiquitin-Proteasome system

The majority of intracellular proteins are degraded via the ubiquitin proteasome system (UPS) (Lee and Goldberg, 1998). The 'housekeeping' 26S proteasomes are ATP-driven, multi-subunit proteolytic complexes that preferentially degrade proteins tagged with K48-linked poly-ubiquitin chains (Hershko, 1980; Chau, 1989; Hershko, 1991; Seufert and Jentsch, 1992; Hershko and Ciechanover, 1998; Voges, 1999; Elsasser and Finley, 2005). 26S proteasomes are comprised of a 20S core component and two flanking 19S regulatory complexes that regulate substrate specificity and access to the catalytic chamber of the 20S core. The 20S core is highly conserved from yeast to humans; simpler prototypes are also found in prokaryotes (Löwe, 1995). Four α- and β-rings surround a barrel-shaped cavity in the 20S core (Groll, 1997). The two inner β-rings form a central chamber that harbors the proteolytic centers containing chymotryptic-, tryptic- and caspase-like activities (Marques, 2009). Tetra-ubiquitin is the minimum signal for efficient proteasomal targeting. The mechanism of targeting involves an increase in substrate affinity that is brought about by autonomous binding of the K48-linked tetra-ubiquitin chain. Binding is mediated by the UBDs (see section 1.2.1.5) of the 19S subunits Rpn10/S5a and Rpn13 (Deveraux, 1994; Lam, 2002; Groll, 1997; Husnjak, 2008; Schreiner, 2008). Recognition of this signal is followed by substrate unfolding and translocation into the degradation chamber while ubiquitin itself is recycled. After degradation, the resultant peptides are released through the entry channel (Finley, 2002; Hutschenreiter, 2004; Komander, 2009b).

1.3 Ubiquitin-like modifiers

Ubiquitin is the most prominent and most abundant member of a diverse group of evolutionarily conserved small proteins that are covalently conjugated to target proteins, the ubiquitin-like modifiers (UBLs). Although the other members vary in their degree of sequence similarity to ubiquitin, they all share the typical β-grasp fold and seem to be conjugated through an analogous enzymatic cascade of specific E1, E2 and E3 enzymes (see table A.1; Kerscher, 2006). Each UBL contains one or two glycines at the C-terminus that is used to form the isopeptide bond with target proteins. Most UBLs (with the exception of FAT10, ATG12 and URM1) are synthesized as precursors whose conjugation requires C-terminal cleavage at this glycine through specific processing proteases (Groettrup, 2008). Barring ubiquitin, the so far best studied UBLs are the family members of small ubiquitin related modifiers (SUMO) which have a wide range of substrates and functions (Hochstrasser, 2009). As this thesis is mainly dealing with a new function for the SUMO modification of proteins, this group is described in more detail (see section 1.4). Functions of other UBL family members are summarized in Appendix table A.1; a phylogenetic tree for all human UBLs is displayed in Figure 1.4.

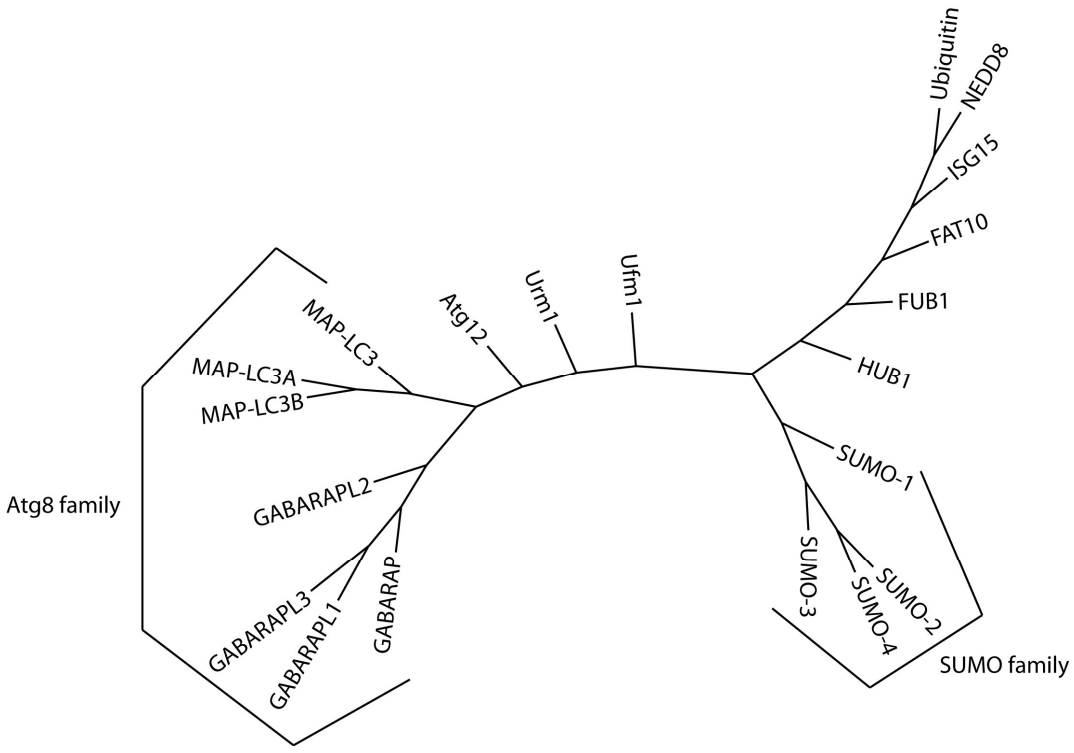


Figure 1.4: **Phylogenetic tree of human Ubiquitin-like modifiers**
The tree was derived through human UBL sequence comparison using MAFFT and virtualized with its associated software ArchaeopterixA (Katoh and Toh, 2008). Urm1 represents the closest relative to the ancestors of UBLs; the human Atg8 homologues as well as the human SUMOs are sub grouped.

1.4 SUMO modification of proteins

An increasing number of proteins have been shown to be SUMO modified and many of them are tumor suppressor proteins, transcription factors or nuclear body proteins. SUMO is an ubiquitin-like protein and its conjugation to substrates (SUMOylation) is thus a reversible process including the typical enzymatic cascade with a SUMO-specific set of E1, E2 and E3 enzymes and a special set of proteases (see section 1.4.2 and Figure 1.7 therein; Xu, 2009).

SUMO is highly conserved in all eukaryotes, but lower eukaryotes like the yeasts *S.cerevisiae* and *S. pombe* as well as the nematode *C. elegans* possess only a single gene, while in mammalian cells four SUMO paralogs exist (Xu, 2009). Plants even have up to eight SUMOs (Kurepa, 2003; Lois, 2003). All SUMO paralogs are translated as precursors that need to be processed to produce the mature di-glycine motif at the C-terminus (Xu, 2009). Compared to ubiquitin, all SUMOs possess a flexible N-terminal extension (see Figure 1.5; Bayer, 1998; Huang, 2004).

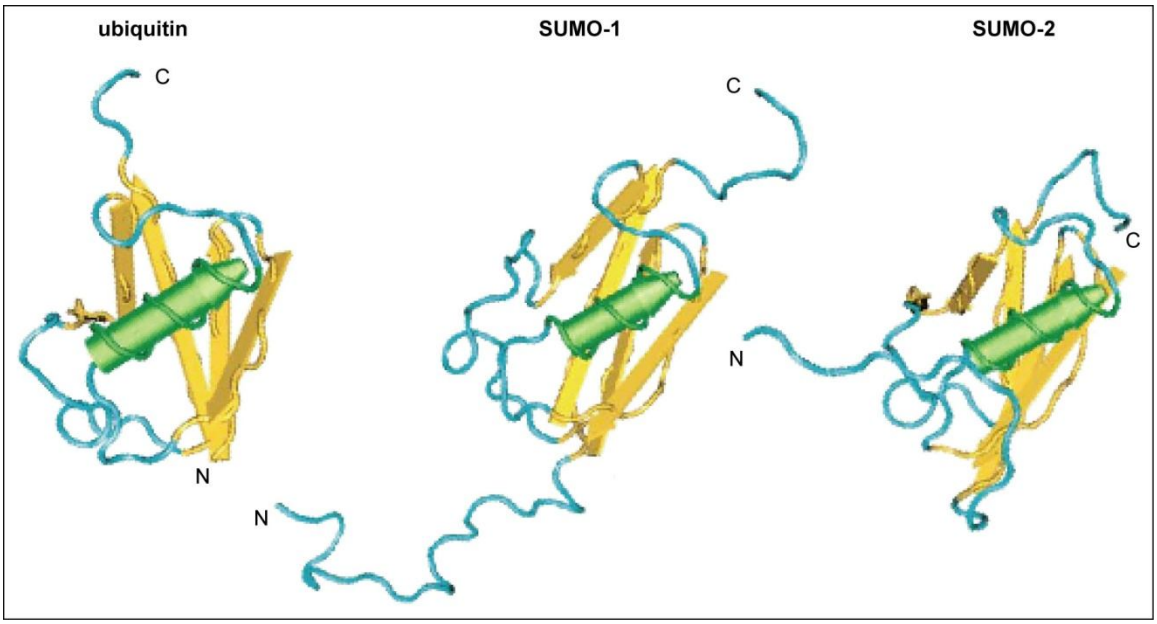


Figure 1.5: Comparison of the three-dimensional structures of ubiquitin, SUMO-1 and SUMO-2
NMR structures of human ubiquitin (PDB ID: 1D3Z), SUMO-1 (PDB ID: 1A5R) and SUMO-2 (PDB ID: 2AWT) as ribbon diagrams. Despite their low sequence homology, ubiquitin and the SUMO paralogues share the highly conserved three-dimensional structure of the β-grasp fold (α-helices are depicted in green, β-strands in yellow). Structures were displayed using Cn3D 4.1.

Out of the four mammalian SUMOs, SUMO-4 is probably not able to be conjugated to substrates (Owerbach, 2005), whereas SUMOs 1-3 are conjugated to lysines in their substrate proteins forming a covalent amide bond (SUMOylation; Johnson and Blobel, 1997; Johnson, 1997; Sampson, 2001). SUMO-1 shares 44 % sequence identity with SUMO-2 and SUMO-3 which are 97 % identical in their processed and conjugatable form (see Figure 1.6). Therefore, they are commonly seen as entity (Lapenta, 1997; Chen,

1998; Maticvan Hagen, 2008). Yeast SUMOs (Bencsath, 2002; Bylebyl, 2003; Skilton, 2009) as well as SUMO-2/3 harbor a consensus SUMOylation motif ΨKxE/D (where Ψ represents a hydrophobic and x any amino acid) and are able to form polymeric chains via a lysine residue in their N-terminus (K11) (Tatham, 2001). For SUMO-1 this has not been observed *in vivo*.

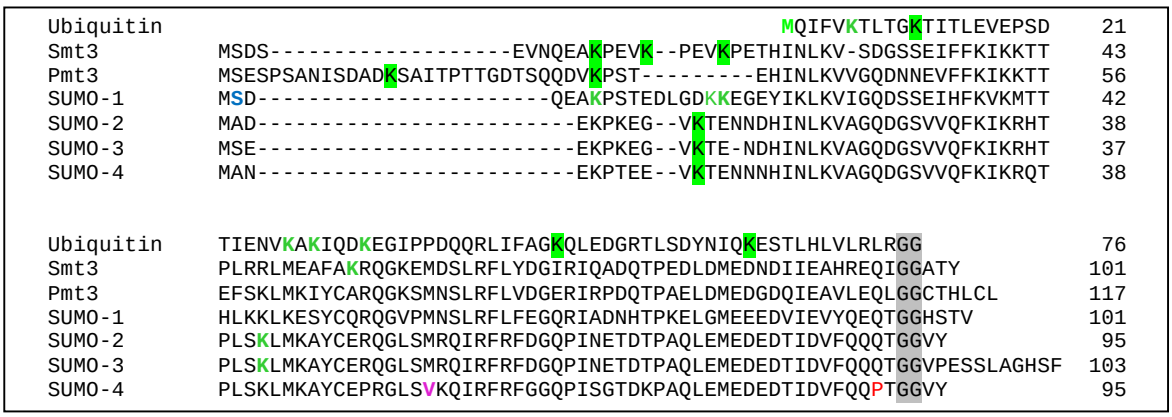


Figure 1.6: Multiple sequence alignment of human and yeast SUMO isoforms compared to ubiquitin
All SUMOs display an N-terminal extension missing in ubiquitin. Additional residues C-terminally of the di-glycine motif (which is highlighted in grey) have to be processed to form mature SUMOs. SUMO-4 cannot be processed to its mature form due to a proline residue (displayed in red) which prevents access to the catalytic center of the SUMO proteases (Owerbach, 2005). The SUMO-4 M→V substitution at position 55 associated with a higher susceptibility for type 1 diabetes is depicted in magenta. Conjugatable lysine and methionine residues are highlighted in green, boxes represent the main attachment sites for polymeric chain assembly. The K42, K41 and K54 attachment sites in SUMO-2, SUMO-3 and Smt3, correspondingly, as well as the respective SUMO1 lysines have been shown *in vitro* only (Pedrioli, 2006; Jeram, 2010). SUMO-1 was shown to be phosphorylated *in vivo* at S2 (depicted in blue). Sequences were aligned using MAFFT version 6 (Katoh and Toh, 2008).

Non-covalent interactions of proteins with SUMO or SUMOylated proteins, respectively, are conferred via SUMO interaction motifs (SIMs) (Minty, 2000; Song, 2004; Hannich, 2005; Song, 2005; Hecker, 2006; see section 1.4.3). The majority of SUMO targets is modified at very low steady state levels *in vivo*. However, SUMOylation-deficient mutants can have striking effects, probably due to dynamic SUMOylation/deSUMOylation cycles (Johnson, 2004).

1.4.1 SUMO proteins

1.4.1.1 Yeast SUMOs: Smt3 and Pmt3

In *S.cerevisiae*, SUMO is encoded by a single gene that was originally isolated as a suppressor of mutations in a centromeric protein and was therefore named *suppressor of Mif Two 3* (*SMT3*) (Meluh and Koshland, 1995; Mannen, 1996). The *SMT3* gene is essential for viability while its orthologue *pmt3* (for *S. pombe* homologue of *SMT3*) in *S. pombe* is not, although mutants lacking *pmt3* grow poorly and display severe defects in genome maintenance (Tanaka, 1999).

1.4.1.2 Mammalian SUMO isoforms

Human SUMO-1 was identified as a homologue to the *S.c.* Smt3 protein and shares only 18 % sequence identity to ubiquitin. It was the first SUMO paralog discovered - independently from several groups - and therefore was also aliased as hSmt3, Ubiquitin-like modifier 1 (UBL1), PML-interacting clone 1 (PIC1), Sentrin or GAP modifying protein 1 (GMP1) (Boddy, 1996; Mannen, 1996; Matunis, 1996; Okura, 1996; Shen, 1996).

It is expressed in many different cell types, predominantly localized to the nucleus, where it generally remains in the conjugated form (Kamitani, 1997; Saitoh and Hinchey, 2000). The first identified and best characterized substrate is the Ran-GTPase activating protein (RanGAP1). Conjugation with SUMO-1 localizes RanGAP1 to the nuclear pore complex (NPC) where it is involved in nuclear import/export (Matunis, 1996; Mahajan, 1997). Another prominent substrate in mammalians is the promyelocytic leukemia protein (PML), the scaffold protein for PML nuclear bodies (PML-NBs) which are large nuclear protein complexes (Sternsdorf, 1997; Müller, 1998; Ishov, 1999). SUMOylation of PML is a prerequisite for the formation of PML-NBs and many proteins that are localized to PML-NBs are also SUMOylated (Zhong, 2000; Matunis, 2006; Bernardi and Pandolfi, 2007). A more detailed description of PML-NBs is given in section 1.7.

Additionally, many transcription factors and chromatin-associated proteins are transient SUMO substrates (as, for example the before mentioned modification of PCNA in DNA repair pathways), so that SUMOylation is required for a variety of cellular processes (Zhao, 2007; see also section 1.4.4). In some cases, SUMO-1 modification is thought to counteract ubiquitylation and subsequent degradation of the substrate, as the modifications occur on the same lysine residue and exclude each other. An example for that is the Inhibitor of NFκB alpha (IκBα) whose SUMOylation stabilizes the protein, thereby inhibiting nuclear factor kappa B (NFκB) transcriptional activity (Desterro, 1998).

SUMO modification often occurs on specific lysine residues within the before mentioned consensus motif, ΨKxE/D, which can be directly recognized by the SUMO conjugating enzyme Ubc9/Ube2I (Sampson, 2001). SUMO-1 has also been shown to be phosphorylated *in vivo* at the very N-terminus (serine 2), but a function linked to that modification has not been identified yet (Matic, 2008).

SUMO-1 deficient mice are viable despite a first observation in which SUMO-1 deletion was lethal (Alkuraya, 2006). They lack any apparent phenotype, due to a compensatory utilization of SUMO-2/3 for SUMOylation of SUMO1 targets (Evdokimov, 2008; Zhang, 2008). However, on the cellular level, a decreased localization of SUMO-2/3 modified RanGAP1 to the nuclear pore was observed and PML nuclear body formation was reduced. Interestingly, the amount of PML protein as well as its modification by SUMO-2/3 was significantly reduced (Evdokimov, 2008).

Generally, SUMO-2/3 supply the main reservoir of free SUMO that is used for conjugation in response to certain stress stimuli like heat and ethanol as well as oxidative and osmotic stress, resulting in high molecular weight SUMO conjugates

(HMW-SCs). This response involves the modification of many proteins as well as SUMO chain formation and is reversed upon recovery; partly due to the action of SUMO-specific proteases (see section 1.4.2.4; Saitoh and Hinchey, 2000; Haindl, 2008; Golebiowski, 2009). SUMO chain formation through the consensus lysine 11 in both SUMO-2 and SUMO-3 results in mixed chains *in vivo* (Matic, 2008). The exact roles of SUMO conjugation in the cellular stress response is not fully understood; it might help to eliminate otherwise toxic proteins as indicated by a newly discovered pathway (see section 1.5).

The non-conjugatable SUMO-4 isoform was originally characterized as a susceptibility gene for type 1 diabetes (Guo, 2004; Owerbach, 2005). This susceptibility is somehow limited to Asian populations as it is not found in Caucasians (Ikegami, 2008). Correlated to that was a polymorphism in SUMO-4 at position 55, with an amino acid substitution from methionine to valine (as depicted in Figure 1.6).

1.4.2 Mechanistic aspects of SUMO conjugation

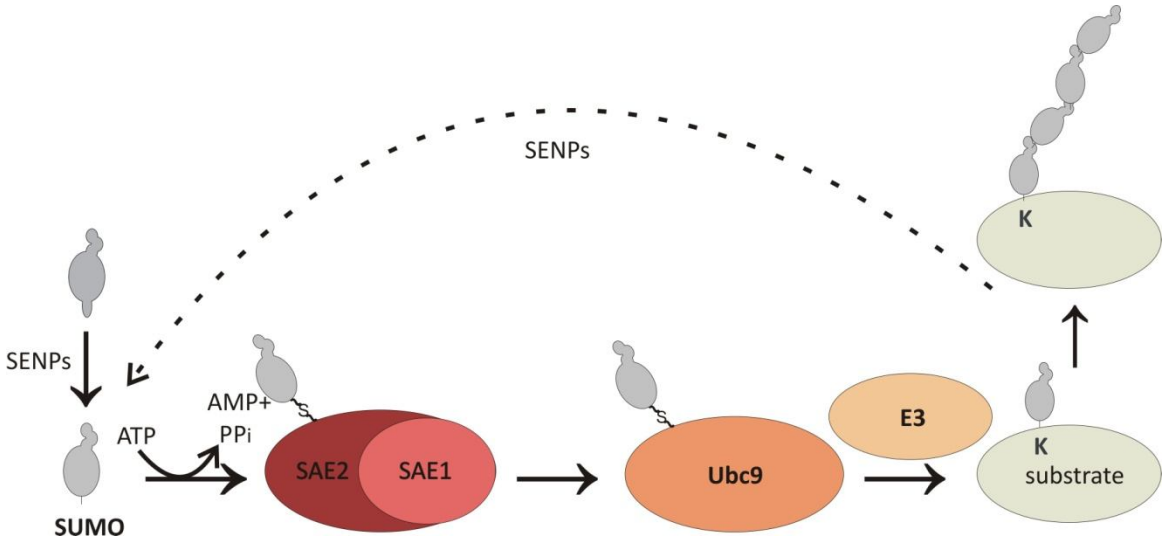


Figure 1.7: SUMOylation cascade
Before SUMO can be conjugated it needs to be processed to its mature form by SUMO proteases (SENPs/Ulps). The SUMO-E1 heterodimer SAE1/SAE2 activates SUMO and transfers it from the active site cysteine in SAE2 to the SUMO-E2 enzyme Ubc9. Ubc9 either directly recognize substrates with the SUMO consensus site ΨKxE (Ψ, hydrophobic; x any amino acid) or in conjunction with a SUMO-E3 ligase. In contrast to SUMO-1, SUMO-2, SUMO-3 and yeast SUMOs are able to form chains that can be reversed by SENP/Ulp activity. Modified from (Miteva, 2010).

1.4.2.1 SUMO E1 enzymes

SUMO conjugation follows in principle the same mechanism as ubiquitin conjugation. The SUMO E1 enzyme is a heterodimer composed of the proteins activation of Smt3p and the ubiquitin activating protein 2, short AOS1/UBA2, in yeasts or its mammalian homologues SUMO activating enzymes 1 and 2 (SAE1/SAE2). AOS1/SAE1 as well as UBA2/SAE2 display sequence similarities to the N- and C-terminal parts, respectively, of ubiquitin-activating enzymes (Dohmen, 1995; Johnson, 1997; Azuma, 2001). Both AOS1

and UBA2 are essential for viability in *S. c.*, consistent with an essential function for SUMO modification (Dohmen, 1995; Johnson, 1997).
A specific binding of the SUMO E2 enzyme Ubc9 is mediated by a ubiquitin-like domain in UBA2/SAE2 and that this binding involves a folding-upon-binding process of unstructured parts within the E1 enzyme (Lois and Lima, 2005; Wang, 2009). UBE1DC1, the E1 enzyme for Ufm1 modification, has been reported to also activate SUMO-2 *in vitro* and to colocalize with overexpressed SUMO-2 *in vivo* (Zheng, 2008).

1.4.2.2 E2 enzyme Ubc9

The E2 enzyme Ube2I/Ubc9 is the specific conjugation enzyme for SUMO (Desterro, 1997; Johnson and Blobel, 1997; Schwarz, 1998; Knipscheer, 2007).
Ubc9 is able to directly recognize substrates harboring the SUMOylation consensus site ΨKxE/D, to which it can bind without the help of an additional E3 enzyme (Sampson, 2001). An extended motif, ΨKxExxS/T(P) induces phosphorylation-dependent SUMOylation of substrates harboring this motif, also termed the phospho-SUMOyl-switch or for phosphorylation-dependent sumoylation motif (PDSM) (Hietakangas, 2003; Hietakangas, 2006; Yang and Gregoire, 2006).
To promote chain formation, Ubc9 has a non-covalent binding site for SUMO (around residue H20) that is used to recruit another Ubc9-SUMO thioester intermediate (Tatham, 2003; Knipscheer, 2007). Ubc9 is essential for viability in most species, probably because Ubc9 deficiency leads to a complete abrogation of SUMOylation (Seufert, 1995; Jones, 2002; Johnson, 2004). An exception is *S. pombe* where SUMOylation itself is also not essential (Ho and Watts, 2003).

1.4.2.3 SUMO E3 ligases

Only a few SUMO E3 ligases have been identified so far. Substrate specificity is partly linked to the distinct intracellular localization of the ligases and of the substrates, respectively. Ran binding protein 2 (RanBP2), for example is associated with the NPC (Wu, 1995; Yokoyama, 1995); the Polycomb protein 2 (Pc2) is mainly found in large complexes on chromatin (Wotton and Merrill, 2007). Some SUMO E3 ligases have dual functions, for instance the Class IIa histone deacetylases (HDACs). HDAC7 has been shown to promote SUMOylation of PML independently of its deacetylase activity (Gregoire and Yang, 2005; Zhao, 2005; Gao, 2008; Martin, 2009). TOPORS, a Topoisomerase I and p53 binding protein (Haluska, 1999; Zhou, 1999), is the first example of a protein that possesses both ubiquitin and SUMO E3 ligase activity. The ubiquitination activity maps to a conserved RING domain in the N-terminal region of the protein and is regulated by phosphorylation, both of which are not required for SUMOylation activity (Rajendra, 2004; Weger, 2005; Park, 2008).

1.4.2.3.1 PIAS proteins

The protein inhibitors of activated STAT (PIAS) were initially named for their ability to interact with and inhibit the signal transducer and activator of transcription (STAT) factors (Chung, 1997; Liu, 1998). PIAS proteins are evolutionarily conserved in eukaryotes with mammals encoding four PIAS genes, PIAS1, PIAS2 (PIASx), PIAS3 and PIAS4 (PIASy). Homologues of mammalian PIAS proteins are found in non-vertebrates, plants and yeast, including the *S. cerevisiae* proteins Siz1 and Siz2/Nfi1 (Johnson and Gupta, 2001; Palvimo, 2007).

The Siz/PIAS (SP)-RING displays structural similarity to RING and U-Box domains and is, together with the C-terminal SIM, required for activation of the Ubc9~SUMO thioester for conjugation to substrates (Yunus and Lima, 2009). Thus, similarly to the function of RING-type ubiquitin E3 ligases, PIAS proteins are likely to act as adaptors between the Ubc9~SUMO intermediate and the SUMO substrate.

1.4.2.3.2 RanBP2

In vertebrate cells, the nucleoporin 358 (Nup358)/Ran binding protein 2 (RanBP2) is a major component of the nuclear pore complex (Wu, 1995; Yokoyama, 1995). RanBP2 forms a complex with SUMOylated RanGAP1, the GTPase activating protein for Ran, thereby tethering it to the cytoplasmic filaments of the NPC (Matunis, 1996; Mahajan, 1997). Despite being equally well modified by SUMO-1 and SUMO-2 *in vitro*, RanGAP1 is primarily modified by SUMO-1 *in vivo*. This paralog-selective modification is due to a more stable, higher affinity complex of RanBP2 and SUMO-1-modified RanGAP1 that preferentially protects it from SUMO isopeptidases (Zhu, 2009). Moreover, RanBP2 functions as SUMO E3 ligase for RanGAP1 as well as for many other proteins *in vitro* and *in vivo* and is itself SUMOylated (Saitoh, 1998; Pichler, 2002; Kirsh, 2002; Dawlaty, 2008). A C-terminal domain, which is characterized by the presence of two internal repeats, is sufficient to efficiently promote SUMOylation in the presence of E1, E2, SUMO-1 and ATP *in vitro* (Pichler, 2002; Kirsh, 2002).

1.4.2.4 SUMO proteases

The first SUMO-specific proteases, the distantly related Ubiquitin-like proteases Ulp1 and Ulp2, were discovered in *S. cerevisiae* (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000; Schwienhorst, 2000). Ulp1 is essential for the G2/M transition in the cell cycle and its inactivation as well as overexpression of its catalytic domain is lethal, pointing towards the essential function of a balanced SUMOylation/deSUMOylation (Li and Hochstrasser, 1999; Takahashi, 2000; Mossessova and Lima, 2000). Ulp1 functions both in SUMO maturation as well as in substrate deconjugation and is located at the NPC (Li and Hochstrasser, 1999; Takahashi, 2000). Ulp2 instead is localized predominantly in the nucleoplasm and functions in the disassembly of SUMO chains (Li and Hochstrasser, 2000; Schwienhorst, 2000; Bylebyl, 2003) with implications for the restart of the cell cycle after DNA repair and the checkpoint-induced metaphase arrest

(Schwartz, 2007). Ulp2 deletion strains are viable despite showing an abnormal phenotype including chromosome mis-segragation and hypersensitivity to DNA damaging agents (Li and Hochstrasser, 2000; Schwienhorst, 2000).
To date, six human SUMO specific proteases (SUSPs) or Sentrin specific proteases (SENPs) - SENP1, 2, 3, 5, 6 and 7 - have been discovered with diverse activities and localizations within the cell (listed in Figure 1.8; Xu, 2009). An additional family member is SENP8, which has been shown to be a deNEDDylating enzyme (Gan-Erdene, 2003). All SUMO proteases share a C-terminal ~ 250 amino acid long catalytic domain and differ in their N-termini which probably confer substrate specificity and intracellular localization.

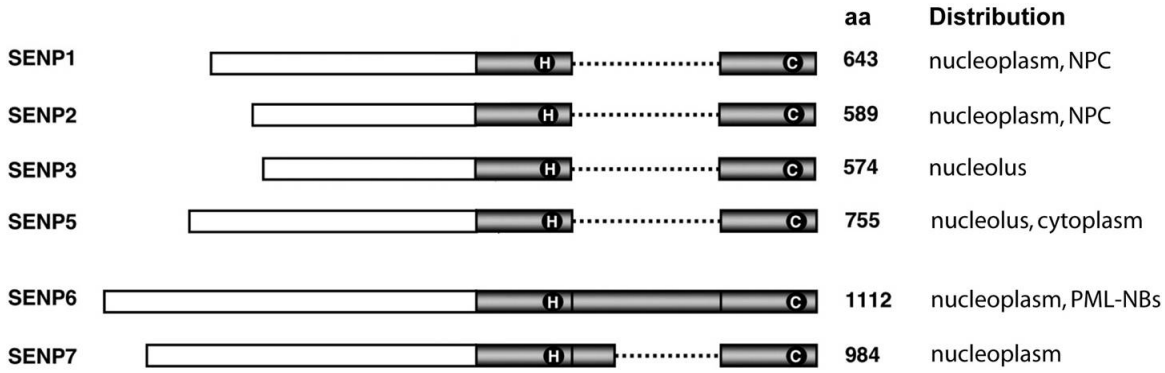


Figure 1.8: Schematic representation of human SUMO proteases and their cellular distribution
The conserved SENP catalytic domain is represented in grey with catalytic residues in circles. The dotted line illustrates insertions within the catalytic domains of SENP6 and SENP7 (aa, amino acids; NPC, nuclear pore complex). Modified from (Mikolajczyk, 2007 and Xu, 2009).

SENP1 has been shown to mediate particularly the deconjugation of SUMO-1 modified substrates within the nucleus, while SUMO-2/3 substrates remain largely unaffected (Yamaguchi, 2005). Moreover, it exhibits the highest endopeptidase activity among the SENPs with its processing activity during SUMO maturation follows the order SUMO-1 > SUMO-2 > SUMO-3 (Xu and Au, 2005). SENP2 instead has the highest maturation efficiency for SUMO-2 (Reverter and Lima, 2004). Both SENP1 and SENP2 have been located to the nucleoplasm and to the NPC (Gong, 2000; Hang and Dasso, 2002; Bailey and O'Hare, 2004). SENP3 and SENP5 are localized in the nucleolus (Nishida, 2000; Di Bacco, 2006; Gong and Yeh, 2006), though SENP5 might also function within the cytosol, where it has been shown to deSUMOylate a dynamin related protein, DRP1 that is involved in mitochondrial fission (Zunino, 2007). SENP3 and SENP5 show a preference for SUMO-2/3 over SUMO-1 maturation and deconjugation (Di Bacco, 2006; Gong and Yeh, 2006).
SENP6 and SENP7 both contain an insertion within the conserved catalytic domain and have been found to disassemble specifically SUMO-2/3 chains analogous to Ulp2 cleaving SUMO chains in *S. cerevisiae*. Partly deletions of the insertion in SENP7 reduce the affinity of SENP7 to bind SUMO-2/3 chains (Lima and Reverter, 2008). Endopeptidase activity towards the SUMO precursors has not been detected for any of the both enzymes (Mikolajczyk, 2007; Lima and Reverter, 2008). SENP6 and SENP7 have

been both located to the nucleoplasm where they might exert overlapping functions. RNAi mediated knockdown of SENP6 resulted in a drastic increase of PML-NB size and number, suggesting a function for SENP6 in the disassembly of SUMO-2/3 chains in these structures (Mukhopadhyay, 2006).

1.4.3 SUMO interaction motifs

Similar to the ubiquitin system, SUMO binding proteins display a common motif recognizing SUMO non-covalently. Unlike the interaction of ubiquitin and UBDs, binding to SUMO is mediated through a short motif, the SUMO interaction motif (SIM) or SUMO binding motif (SBM) (see Figure 1.9.A; Minty, 2000; Song, 2004; Hannich, 2005; Song, 2005; Hecker, 2006).

Three types of SIMs have been identified so far (see Figure 1.9.B). SIMa is characterized by a core consisting of four hydrophobic residues, immediately followed by a mixed cluster of S/D/E residues, the so-called acidic stretch. The third hydrophobic position in SIMa motifs is less conserved than the other hydrophobic positions and instead, also non-hydrophobic, even acidic residues may be present (Song, 2004; Hannich, 2005; Miteva, 2010).

In a reversed orientation of that motif, SIMr, the acidic stretch precedes the hydrophobic core (Song, 2005; Hecker, 2006).

The type b SIM usually sticks to the consensus sequence V-I-D-L-T, with some variations in the first two hydrophobic amino acids (Uzunova, 2007; Miteva, 2010).

Mixed SIM types are also present, the PIAS SIMs, for example are of type b, followed by an acidic stretch. Whether these types of SIMs have different binding capacities towards different SUMO isoforms remains to be elucidated, although there is a hint that SIM type b binds SUMO-2/3 with a higher affinity than SUMO-1 (Sekiyama, 2008).

Binding occurs upon insertion of the hydrophobic SIM core into a hydrophobic cleft between the β2-strand and helix α1 of the SUMO molecule, forming a parallel β-sheet together with the β2-strand (Reverter and Lima, 2005; Song, 2005; Hecker, 2006; Sekiyama, 2008). An example is depicted in Figure 1.9.A; the SIMb from PIAS2 bound to SUMO-1.

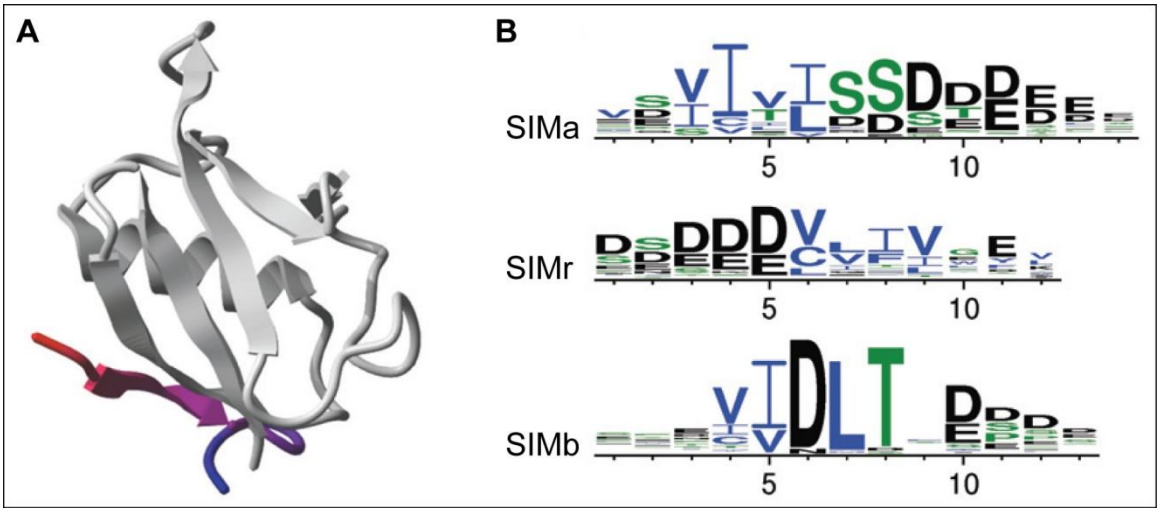


Figure 1.9: SUMO interaction motifs (SIMs)
A) Ribbon diagram of the SIMb from PIAS2 bound to human SUMO-1 (based on structure PDB ID: 2ASQ). B) Residue conservation of the three SIM types is shown in a sequence logo representation. Overall height of a position indicates its information content; height of individual residues indicates their frequency at that position (charged amino acids are displayed in black, polar in green and hydrophobic in blue). Pictures were taken from (Miteva, 2010).

Furthermore, SIMs often contain serine and threonine residues that are able to be acidified by phosphorylation (Hecker, 2006). A recent publication showed that binding to SUMO by the SIM of PIAS1 is enhanced after Casein kinase II (CK2) phosphorylation of serine residues adjacent to the SIM core domain (Stehmeier and Müller, 2009). Serine residues in similar SIMs, for example within the PML protein, were also shown to be phosphorylated by CK2 after osmotic stress induction (Scaglioni, 2008; Stehmeier and Müller, 2009). This indicates an integrative crosstalk function for SIM motifs between the phospho- and SUMO-regulated signaling pathways within mammalian cells.
In analogy to the activity of ubiquitin/substrate non-covalent interactions leading to mono-ubiquitylation (Hoeller, 2007), SIMs have also been shown to non-covalently recruit Ubc9~SUMO thioesters and serve as a cis-regulatory SUMO-E3 ligase modules (Lin, 2006; Knipscheer, 2008; Meulmeester, 2008; Cho, 2009).

1.4.4 Cellular roles of SUMO modification

Roles of SUMO modification in the cellular context are as multifaceted as the proteins subjected to SUMOylation. Some SUMO substrates are modified in the cytosol such as the inhibitor of NFκB, IκBα, thereby inhibiting cytokine or innate immune receptor signaling, or the mitochondrial fission GTPase DRP1 whose activity is linked to its SUMOylation (Figueroa-Romero, 2009).
However, the vast majority of substrates are nuclear proteins, highlighting the primary nuclear functions of SUMOylation. As already mentioned above, SUMOylation is involved in DNA repair, transcriptional regulation, chromosome maintenance and mitosis, but also in inflammation, nuclear organization, protein localization and stress pathways, as reviewed in Gill, 2005; Ulrich, 2005; Matunis, 2006; Heun, 2007; Palvimo,

2007; Dasso, 2008; Tempe, 2008; Bergink and Jentsch, 2009; Garcia-Dominguez and Reyes, 2009. Deregulated SUMOylation may lead to cancer and is implicated in neurodegenerative diseases (Kim and Baek, 2006; Martin, 2007; Sarge and Park-Sarge, 2009).

Generally, SUMOylation is important for nuclear organization as many substructures as the nuclear envelope, Nucleoli and PML nuclear bodies are disrupted when the SUMO pathway is defective (Nacerddine, 2005; Heun, 2007).

Proper protein localization upon SUMOylation is not only known for RanGAP1 and PML, but for many nuclear proteins and is mainly achieved by mono-SUMOylation or multiple mono-SUMOylation of a given protein and its interaction with SIM-containing partners (Matunis, 2006). Many transcription factors, for instance, relocalize to PML-NBs when SUMOylated (Johnson, 2004; Palvimo, 2007). Transcriptional regulation is also mediated through SUMOylation of transcriptional co-repressors or transcriptional activators (Palvimo, 2007; Garcia-Dominguez and Reyes, 2009).

Dynamic SUMOylation/deSUMOylation events are also indispensable for sister chromatid separation in mitosis. The process is not fully understood but many centromeric proteins are SUMOylated, including yeast mif-2, whose mutant phenotype led to the discovery of SUMO (Dasso, 2008). Furthermore, many factors involved in DNA replication, telomere elongation and DNA repair are SUMOylation targets, thereby revealing also emerging connections between the SUMO and the ubiquitin pathways (Bergink and Jentsch, 2009; Galanty, 2009; Morris, 2009).

A connection of the pathways was also discovered in the cellular response to stress.

As already mentioned for SUMO-2/3 in humans (see section 1.4.1.2; Saitoh and Hinchey, 2000), diverse stress stimuli upregulate SUMO-modification, generating HMW-SCs (Zhou, 2004). SUMO-targeted ubiquitin ligases (StUbLs) - or else - ubiquitin ligases for SUMO conjugates (ULS) recognize these HMW-SCs and target them for degradation via the proteasome. The next section will describe the discovery of these ligases in yeasts and their functional requirements.

1.5 ULS proteins

During the initial phase of this work, ULS proteins have been just identified in *S. cerevisiae* and in *S. pombe*.

ULS proteins target SUMO-modified proteins for ubiquitylation and subsequent degradation. They contain a RING domain and one or several SIMs (as shown schematically in Figure 1.10).

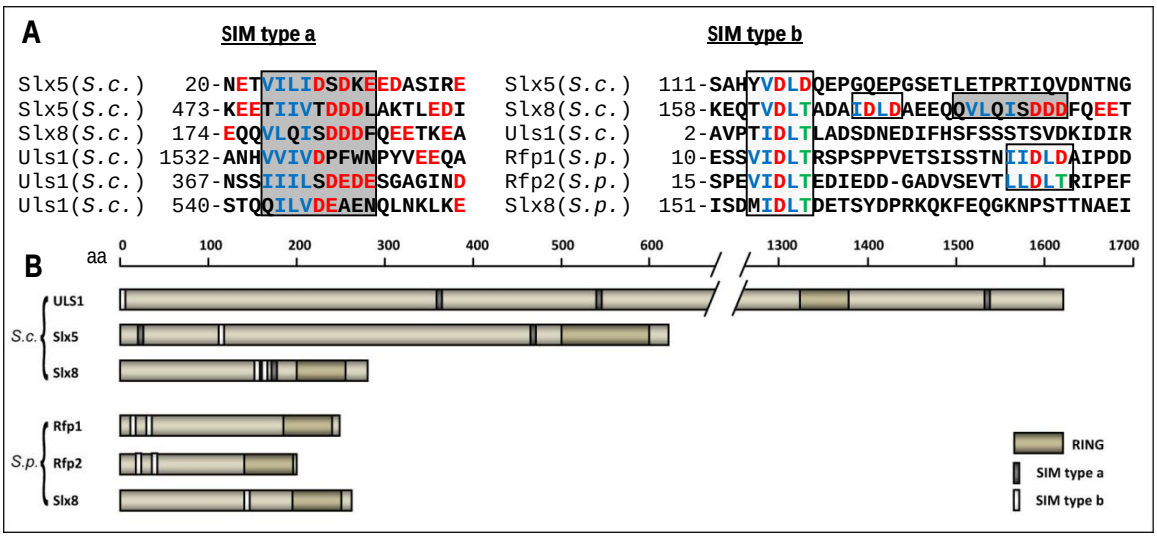


Figure 1.10: Yeast ULS proteins
A) Alignment of proved and putative SUMO interaction motifs type a (boxed in dark grey) and type b (boxed in light grey) in ULS proteins from *S. cerevisiae* and *S. pombe* (hydrophobic residues within the SIM are displayed in blue, acidic in red and threonines in green).
B) Schematic representation of yeast ULS proteins. Domains/Motifs are depicted relative to the total protein size (ULS, ubiquitin ligase for SUMO conjugates; SIM, SUMO interaction motif; aa, amino acids).

1.5.1 *S. cerevisiae* Slx5-Slx8 and Uls1

The *S. cerevisiae* ULS proteins, the RING containing proteins Slx5 (alias Hex3) and Uls1 (alias Ris1 or Dis1) have been identified as non-covalent SUMO (Smt3) interacting proteins with each harboring several SIMs (for SIM type refer to Figure 1.10; Uzunova, 2007). Slx5 was also isolated as a high-copy suppressor of a temperature-sensitive Ulp1 mutant (Xie, 2007). Slx5 forms a RING dimer with Slx8 and the complex was shown to be an active ubiquitin ligase involved in genome stability (Mullen, 2001; Ii, 2007). Slx5-Slx8 and ULS1 bind especially to HMW-SCs (Uzunova, 2007). Both Slx5-Slx8 and ULS1 target HMW-SCs for proteasomal degradation by a concerted action together with the redundant ubiquitin E2s Ubc4/Ubc5 (Uzunova, 2007). Further, an *in vitro* model substrate, a Rad52-SUMO fusion, was shown to be preferentially modified with ubiquitin by Slx5-Slx8 compared to Rad52 alone (Xie, 2007).

1.5.2 *S. pombe* Rfp1/Rfp2-Slx8

ULS proteins were also identified in *S. pombe*, where Slx8 forms a dimer with the redundant RING finger proteins Rfp1 and Rfp2. These are not related to Slx5 but also harbor several SIMs (see
Figure 1.10) and were found to interact with *S. pombe* SUMO (Pmt3; Kosoy, 2007; Prudden, 2007; Sun, 2007). As Slx5, Rfp1 and Rfp2 lack intrinsic ubiquitin ligase activity but in a complex with Slx8 they form an active ubiquitin ligase targeting HMW-SCs and thus serve as functional homologues for Slx5. Cells lacking these complexes are sensitive to genotoxic stress and show genomic instability (Kosoy, 2007; Prudden, 2007; Sun, 2007). Rfp1-Slx8 and Rfp2-Slx8 stimulate both the *in vitro* ubiquitylation of a GST-SUMO

fusion as well as of a di-SUMO mimicry, Rad60, which is involved in DNA repair (Prudden, 2007; Sun, 2007; Prudden, 2009).

1.5.3 RNF4, a putative human ULS

Proteasomal inhibition of HeLa cells showed a specific stabilization of SUMO-2/3 conjugates while the SUMO-1 conjugate pattern appeared normal (Uzunova, 2007). Therefore, it was likely that also the ULS pathway is conserved to humans. Indeed, some of the before-mentioned ULS studies identified a small nuclear RING finger protein, RNF4, which could alone complement for the deletion phenotypes of the yeast ULS proteins (Kosoy, 2007; Prudden, 2007; Sun, 2007; Uzunova, 2007).

RNF4 comprises 190 amino acids (aa) and contains a RING domain at its C-terminus and three to four putative SIMs in its short N-Terminus. It homodimerizes through its RING domain and undergoes self-ubiquitylation *in vitro* (Häkli, 2004).

In human cells, RNF4 has been localized to the nucleus and there predominantly to PML nuclear bodies. Furthermore, it was shown to interact with PML when free SUMO-1 was added to the reaction, a behavior that is thought to be the basis of PML nuclear body formation (Häkli, 2005; for a detailed description of PML and PML-NBs refer to sections 1.6 and 1.7). Altogether, these data point to a role for RNF4 as ULS but experimental data in a mammalian system as well as putative substrates are still missing.

1.6 The promyelocytic leukemia protein PML

PML was first identified as part of an oncogenic fusion protein causing acute promyelocytic leukemia (APL, see section 1.8; Borrow, 1990; de The, 1990). It belongs to the TRIM family of proteins and is expressed in several isoforms (Jensen, 2001; Condemine, 2006). Post-translational modifications regulate PML stability, PML localization and PML nuclear body formation (see sections 1.6.1 and 1.7.1). The *PML* gene is located on chromosome 15q22 and consists of nine exons (Fagioli, 1992). Alternative splicing of exons 5 to 9 yields in at least eleven isoforms, of which most are nuclear and only two are cytoplasmic (see Table 1.2 and Figure 1.11; Fogal, 2000; Jensen, 2001).

Table 1.2: Human PML isoforms

Name	Alternative name(s)*	Protein ID (NCBI database)	Length [aa]	Size [kD]	Cellular localization
PML1	PML I[1], TRIM19 α[2]	NP_15023841	882	97.5	Nuclear
PML2	PML V[1], TRIM19 β[2], PML12	NP_150243/48	611	67.4	Nuclear
PML3	PML II[1], TRIM19 γ[2]	NP_150245	824	90.2	Nuclear
PML4	-	-	-	-	-
PML5	PMLVI[1]; TRIM19 ε[2]	NP_150247	560	62.0	Nuclear
PML6	PML IV[1]; TRIM19 ζ[2]; PML3[3]	NP_002666	633	70.0	Nuclear
PML7	PMLVIb[1]; TRIM19 η[2]	NP_150249	423	47.6	Cytoplasmic
PML8	PML VIIb[1]; TRIM19 θ[2]	NP_150250	435	48.6	Cytoplasmic
PML9	PML II[1]; TRIM19 κ[2]	NP_150242	829	90.7	Nuclear
PML10	PMLIVa[1]; TRIM19 λ[2]	NP_150252	585	65	Nuclear
PML11	-	NP_150253	781	85.7	Nuclear
PML-L[3]	PML III[1]	AAB19601	641	70.4	Nuclear

*Alternative PML names from different publications: 1) Jensen, 2001; 2) Nisole, 2005; 3) Fogal, 2000

At the N-terminus, coded by exons 1-3, all PML isoforms contain a tripartite motif that defines the TRIM family of proteins (see Figure 1.11; Condemine, 2006). It is characterized by a sequential arrangement of a RING domain, one or two B-Boxes and a coiled-coil domain, the RBCC motif (Reymond, 2001). PML (also aliased TRIM19) comprises two zinc-binding B-Boxes, B1 and B2, that are implicated in protein-protein interaction (Borden, 1998). The α-helical coiled coil domain has been shown to mediate PML multimerization (Kastner, 1992; Reddy, 1992). Exon 6 contains a nuclear localization signal that is not present in the alternatively spliced and therefore

cytoplasmic PML isoforms 7 and 8 (Jensen, 2001; Lin, 2004). A SIM 'type a' is coded by exon 7 and is not present in isoforms 5, 7 and 8 (see Figure 1.11; Shen, 2006).

The differentially spliced C termini are relatively young in terms of evolution as mice only express two PML isoforms homologous to human PML1 and PML2 and might reflect the ability of different PML isoforms to interact with distinct binding partners (Condemine, 2006; Bernardi and Pandolfi, 2007).

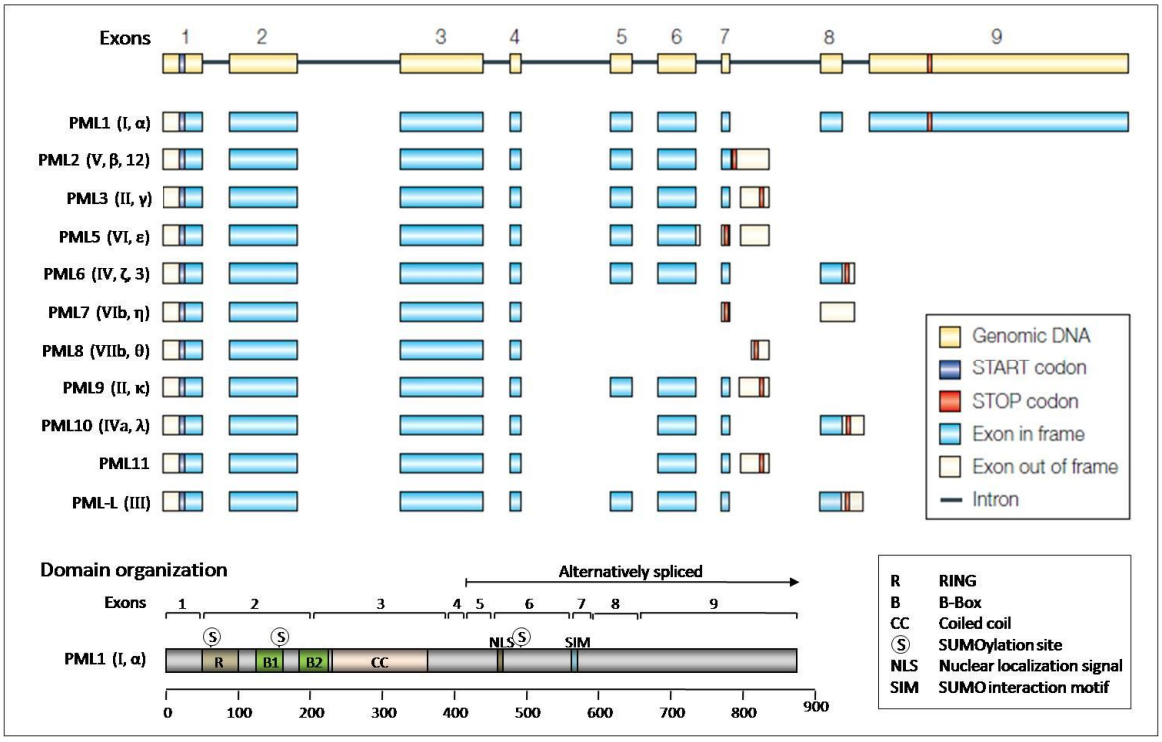


Figure 1.11: Human PML splice variants and domain organization
Schematic representation of human PML gene (yellow), transcripts (blue) and protein (grey). The domain organization of the PML protein isoforms is exemplified for isoform 1; other isoforms vary in their C-termini according to their alternatively spliced transcripts. Exons in frame (blue) represent translation in the same reading frame as isoform 1; out of frame (pale pink) the use of an alternative frame. Modified from (Nisole, 2005) and (Shen, 2006).

The cellular expression levels vary between the different isoforms with PML1 and PML3 being the most abundant ones (Condemine, 2006; Bernardi and Pandolfi, 2007).

Overall PML expression is strongly induced by interferons (IFNs) type I and type II and the tumor suppressor p53 (Lavau, 1995; Stadler, 1995; de Stanchina, 2004). Furthermore, PML is frequently lost or downregulated in various solid tumors (Gurrieri, 2004; Bernardi and Pandolfi, 2007).

1.6.1 Posttranslational modifications of PML

Apart from transcriptional control and alternative splicing, PML is highly regulated at the posttranslational level. The most prominent posttranslational modification of PML is the one with SUMO. PML colocalizes with and is covalently modified by all three conjugatable SUMOs: SUMO-1, SUMO-2 and SUMO-3 (Boddy, 1996; Sternsdorf, 1997; Kamitani, 1998a). PML contains three SUMOylation sites, namely K65 within the RING

domain, K160 at the end of B-Box 1 and K490 (K442 in PML 10 and 11) which is localized between the NLS and the SIM (see Figure 1.11; Kamitani, 1998b). The SUMO-1 modification of PML is essential for PML nuclear body formation (see section 1.7.1; Müller, 1998; Duprez, 1999; Zhong, 2000). Other posttranslational modifications such as acetylation and phosphorylation have been shown to induce SUMOylation of PML upon diverse stimuli or to regulate other effects of PML (Hayakawa, 2008; Scaglioni, 2008; Stehmeier and Müller, 2009).

PML can be phosphorylated by several kinases at multiple sites (Yang, 2002; Bernardi, 2004; Scaglioni, 2008; Stehmeier and Müller, 2009). For instance, the extracellular signal-regulated kinase (ERK) has been shown to phosphorylate PML at several serine and threonine residues in the N-terminus and between the K442/490 SUMOylation site and the SIM after treatment with arsenic trioxide (As_2O_3, ATO), a therapeutic drug used in the treatment of APL (see section 1.8). This phosphorylation induces SUMO modification of PML and apoptosis (Hayakawa and Privalsky, 2004).

1.6.2 PML functions

PML is mainly seen as 'the' scaffold protein of PML nuclear bodies, sequestering the resident as well as the transient proteins to these structures through SUMO/SIM interactions as described in section 1.7 (Shen, 2006; Matunis, 2006). Therefore, most functions assigned to PML as the already mentioned one in apoptosis are mediated through the dynamic PML nuclear body network and are discussed in section 1.7.2. Additionally, PML might act as a SUMO E3 ligase, as PML self-SUMOylation was observed when expressed in *S. cerevisiae* (Quimby, 2006).

Cytoplasmic PML has been found to be involved in the transforming growth factor (TGF) β signaling pathway as it interacts with the downstream regulators Smad2/3 and Smad anchor for receptor activation (SARA). PML deficient mice cells are resistant to TGFβ-dependent growth arrest, underlining the growth suppressive function of PML (Lin, 2004; Le Roy and Wrana, 2004).

1.7 PML Nuclear Bodies

PML nuclear bodies are dynamic protein complexes within the nuclei of higher eukaryotic organisms with an average size of 0.2 µm to 1.0 µm diameter (see Figure 1.12). They are defined by the presence of the PML protein that is thought to be the scaffold protein of these subnuclear domains (also aliased as nuclear domain 10, Kremer bodies or PML oncogenic domains; Bernardi and Pandolfi, 2007).

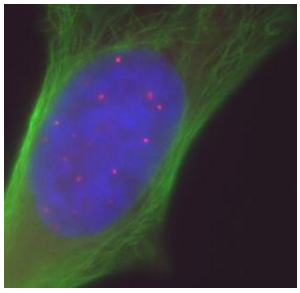


Figure 1.12: PML nuclear bodies
Fluorescent microscopy image of PML-NBs (red) in a HeLa cell nucleus. Chromatin is stained with DAPI (blue), the cytoplasmic microtubular network with anti-tubulin (green).

To date, 166 proteins have been found to associate to PML nuclear bodies, constitutively or transiently in a dynamic interplay of the individual molecules (Weidtkamp-Peters, 2008; Van Damme, 2010). Therefore, it is not surprising that they are implicated in the regulation of diverse cellular functions (see section 1.7.2).

1.7.1 PML nuclear body formation

The formation of PML-NBs is SUMO- and cell cycle dependent. Both, PML SUMOylation and the PML SIM are required for PML nuclear body formation (Müller, 1998; Zhong, 2000; Shen, 2006). SUMO modification of many nuclear proteins results in the translocation to PML-NBs, for instance Sp100, Daxx, zinc finger proteins like ZNF198 or transcription factors like HSF2 (Sternsdorf, 1997; Goodson, 2001; Jang, 2002; Kunapuli, 2006). However, mutant PML that lacks its SUMOylation sites fails to form the distinct PML-NB pattern. Moreover, other proteins usually located to PML-NBs do not accumulate in nuclear dots but display aberrant localization patterns (Zhong, 2000). Additionally, also the SIM of PML has been shown to be involved in PML-NB formation because a SIM-defective mutant localized in aberrant patterns that differed from the usual PML-NB pattern (Shen, 2006). The current model of PML nuclear body formation involves multiple SUMO-SIM interactions: First, between different PML dimers or multimers; second, between PML and other proteins and third between different PML-NB proteins (Matunis, 2006; Shen, 2006). For instance, Daxx is recruited to PML-NBs through its C-terminal SIM (Lin, 2006). The interaction of Sp100 to the heterochromatin protein 1 (HP1) depends on the SUMOylation of Sp100 and both proteins are sequestered in PML-NBs (Seeler, 1998; Sternsdorf, 1999; Seeler, 2001). The association and dissociation of different proteins to and from the PML-NB network is a dynamic process with component exchange rates ranging from a few seconds, e.g. Daxx, to a minute. Most PML isoforms exchange every 10 minutes while isoform 2 exchanges at a

slower rate of about one hour. These observations point towards a special role for PML 2 being the main scaffold form for PML-NBs (Weidtkamp-Peters, 2008).
During mitosis, PML-NBs are disassembled due to PML deSUMOylation. The resulting PML accumulations contribute in G1 to the reestablishment of PML-NBs (Dellaire, 2006).
Another factor that influences PML-NB integrity is chromatin. Several studies suggest that PML-NBs may have a direct connection with chromatin (Eskiw, 2003; Luciani, 2006; Lang, 2010). Upon radiation stress and nuclease treatments, PML-NBs increase their mobility immediately (Maul, 1995; Eskiw, 2004; Ching, 2005) and a recent study revealed that PML-NBs are formed de novo at telomeric DNA during interphase (Brouwer, 2009).

1.7.2 Functions of PML nuclear bodies

The PML nuclear body network has been implicated in a variety of functions due to the numerous associated proteins. They are involved in such diverse cellular processes as transcription, DNA repair, apoptosis, senescence, cell cycle and anti-viral defense (Dellaire and Bazett-Jones, 2004; Everett and Chelbi-Alix, 2007; Bernardi and Pandolfi, 2007; Bernardi, 2008; Bourdeau, 2009). Deregulation of PML-NBs might induce carcinogenesis (Gurrieri, 2004). An overview of the functional distribution of PML-NB components is given in Figure 1.12.
Despite the actual function of PML-NBs remains to be determined, three non-mutually exclusive hypotheses for the function of PML-NB network have been proposed:
1) As ‘nuclear storage depot’ in which association to PML-NBs regulates the nucleoplasmic protein levels, for instance of transcription factors (Negorev and Maul, 2001).
2) As ‘sites of nuclear activity’ such as transcription and DNA repair (Ching, 2005).
3) As a ‘nuclear platform for posttranslational modifications’ such as phosphorylation, acetylation, SUMOylation and ubiquitylation.
Indeed, many kinases, phosphatases, acetylases, deacetylases and enzymes of the ubiquitin and SUMO pathway have been found to associate to PML-NBs (Boddy, 1996; Duprez, 1999; Engelhardt, 2003; Möller, 2003; Bailey and O'Hare, 2005; Saitoh, 2006; Yang, 2006; Bernardi and Pandolfi, 2007; Gao, 2008; Song, 2008; XuChan, 2009; Van Damme, 2010).

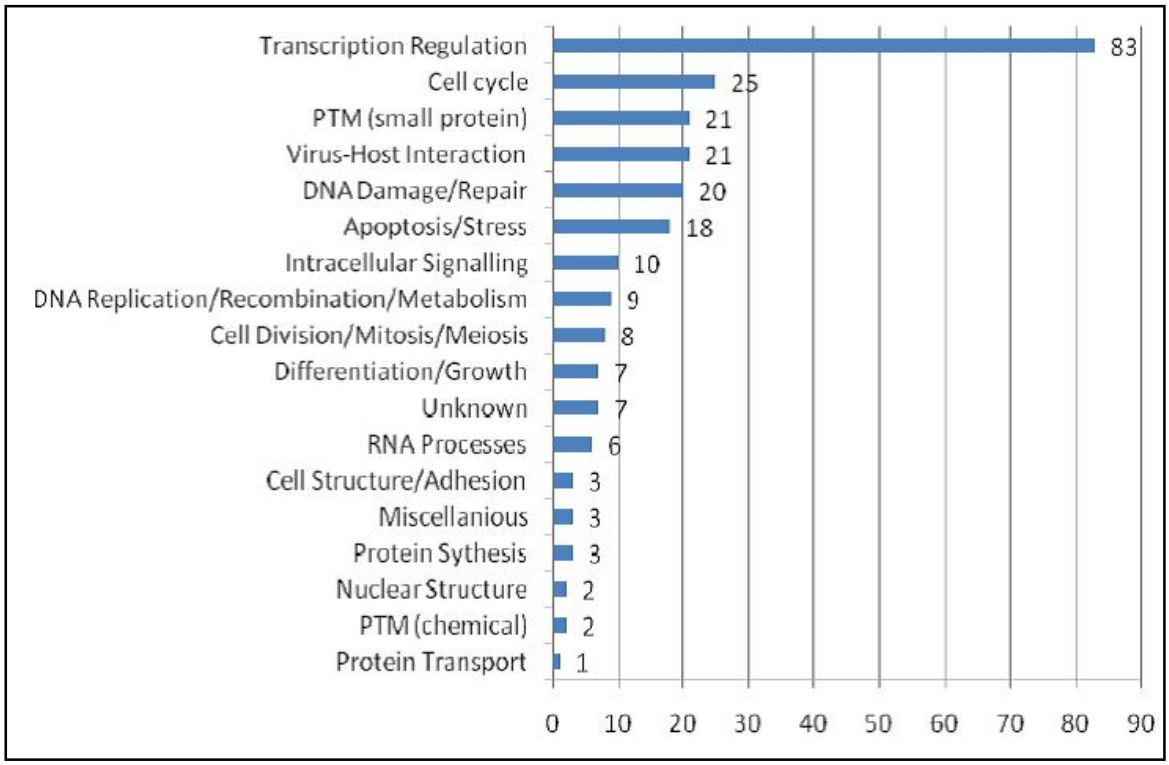


Figure 1.12: Functional distribution of PML-NB components
For each category the number of proteins found to associate with PML-NBs during the given processes is plotted. The plot was generated by (Van Damme, 2010) and is based on the 166 proteins they identified to participate in the PML-NB network (PTM, posttranslational modification). The categories were merged to facilitate interpretation.

PML-NBs are also involved in innate anti-viral mechanisms. This is already implicated by the IFN-induced upregulation of PML, Sp100 and Daxx which results in larger PML-NBs (Guldner, 1992; Lavau, 1995; Stadler, 1995; Shimoda, 2002). Further, many viruses evolved strategies to escape the PML-NB mediated defense and replicate slower when deficient for these factors (Everett and Chelbi-Alix, 2007). An example is the viral ubiquitin ligase ICP0 from Herpes simpex virus 1 (HSV-1). ICP0 targets PML and Sp100 for degradation (Chelbi-Alix and de The, 1999). With an ICP0-deficient HSV-1 mutant it was shown that PML-NB proteins Daxx, ATRX, PML and Sp100 relocate to the entry sites of viral DNA and are reorganized in putative PML-NBs at these DNA molecules (Everett and Murray, 2005; Everett, 2008; Lukashchuk and Everett, 2010). In analogy to PML-NB formation at sites of DNA repair and at telomeres (see section 1.7.1), it is tempting to speculate that some PML-NB proteins might somehow recognize naked DNA or DNA endings and relocate to these places, thereby initiating de novo PML-NB formation. Other viruses exploit PML-NBs through relocalization of PML-NB associated transcription factors to their genomes (Evans and Hearing, 2005; Everett and Chelbi-Alix, 2007).
Together, PML-NBs might be an integrative network, sensing different cellular conditions and exerting many different functions by modulating the respective proteins in their activities through posttranslational modifications. That deregulation of such a

network might contribute to aberrant effects and cancerogenesis, is dramatically demonstrated in acute promyelocytic leukemia, in which PML-NBs are dispersed.

1.8 Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML) and is morphologically characterized by a differentiation block of the granulocytic lineage at the promyelocytic stage which results in faster cell divisions and cancer (de The, 1991; Goddard, 1991; Kakizuka, 1991; Pandolfi, 1991). In most cases, the genetic cause is a specific t(15;17) chromosomal translocation that results in a fusion of PML and the retinoid acid receptor alpha (RARα) genes and proteins, the PML-RARα fusion (Rowley, 1977; de The, 1990; Alcalay, 1991; Goddard, 1991; Kakizuka, 1991; Pandolfi, 1991; Chang, 1992). A proper assembly of PML-NBs is prevented in these cells and therefore, a large number of proteins mislocalize and contribute to the aberrant cell function in the leukemic cells (Melnick and Licht, 1999).
Treatment of APL with chemotherapies used to have a poor prognosis, but over the past 20 years, two novel agents, all-trans retinoic acid (ATRA) and ATO, have radically changed the prognosis of this disease. By now, APL is one of the rare malignancies that may be cured by targeted agents (Estey, 2006; Wang and Chen, 2008; Ravandi, 2009).

1.8.1 Aberrant functions of the PML-RARα fusion protein

PML-RARα possesses diverse oncogenic properties, which is due to the aberrant functions of both fusion partners. The oncogenic fusion protein PML-RARα is able to bind not only to the consensus RA response elements (RARE), but also to sites, in which the two AGGTCA half sites of the RARE can be in any orientation or spacing. This additional property is conferred by the homodimerization of its PML part (Perez, 1993; Grignani, 1998; Lin, 1998). PML-RARα also enhances co repressor recruitment (Melnick and Licht, 1999; Hoemme, 2008) and the transcriptional silencing involves also the SUMOylation of the PML part, RXR binding and the recruitment of the Polycomb complex (Zhu, 2005; Sternsdorf, 2006; Zhu, 2007). Moreover, off-target gene expression is altered due to binding of PML-RARα to other transcription factors (Reineke, 2007; van Wageningen, 2008).
Apart from transcriptional regulation, the PML-RARα fusion disrupts the formation of PML nuclear bodies and therefore many cellular processes that are associated with these structures (Daniel, 1993; Dyck, 1994; Koken, 1994; Weis, 1994).

1.8.2 Treatment of APL

The target-directed treatment with ATRA leads to a cell differentiation of the myeloids along the granulocytic lineage as it releases the co-repressors from the complex and recruits transcriptional co-activators to the target genes (Ohnishi, 2007). However, with ATRA as a single agent, the majority of the patients experienced a relapse within a few months (Wang and Chen, 2008). This is probably due to the survival of Leukemia

initiating cells (LICs) that are then responsible for the relapses. LICs are a minority of tumor cells that are able to self-renew, but usually do not cycle (Wang and Dick, 2005).
Another effective therapy of APL is the application of arsenic trioxide, which in low dosages renders the leukemic cells to differentiation or in higher dosages triggers their apoptosis (Chen, 1996; Chen, 1997; Shen, 1997). As one of the oldest drugs known, it was reintroduced as a drug against APL in the 1990's (Zhu, 2002). It has improved the clinical outcome of APL with a complete remission rate of up to 90 % of patients with both newly diagnosed and relapsed APL or of patients resistant to ATRA (Wang and Chen, 2008).
In contrast to ATRA, ATO targets the PML part of the PML-RARα fusion for degradation, while wild-type PML relocates from aberrant nuclear structures and reforms PML nuclear bodies (Chen, 1996; Zhu, 1997; Müller, 1998; Lallemand-Breitenbach, 2001). Treatment of cells with ATO resulted in an upregulated SUMO-1 modification of PML or PML-RARα at K160 and these SUMOylated forms were stabilized during proteasome inhibition (Lallemand-Breitenbach, 2001), pointing to a regulation of PML via the then unknown ULS pathway. The observed differences in degradation of the fusion protein and the wild-type (wt) copy are probably due to a slower turnover of wild-type PML (Müller, 1998).
More recent clinical studies suggest that a combination therapy of ATRA and ATO has synergistic effects on APL, as it results in higher rates of complete remission as well as lower rates of relapse within a five year range as it occurred with traditional chemotherapy or either agent alone (Shen, 2004; Wang and Chen, 2008; Hu, 2009). This is accompanied by an effective down regulation of the PML-RARα transcript and protein (Jing, 2001; Zhu, 2001; Nasr, 2008) and can be explained by the fact that ATRA and ATO use different target sites within the fusion protein and act via distinct pathways (Benoit, 2001; Zhu, 2002; Zhao, 2004; Tarkanyi, 2005; Tarrade, 2005; Zheng, 2005; Joe, 2006; Mathieu and Besancon, 2006; Leung, 2007). Furthermore, in an APL mouse model, it could be clearly shown that high ATRA dosages or an ATRA/ATO combinational therapy led to the eradication of LICs through the degradation of the PML-RARα fusion protein (Nasr, 2008).

1.9 Aims of this thesis

There was evidence that the ULS pathway is conserved in humans as SUMO-2/3 conjugation is generally upregulated after diverse cell stresses and conjugates are proteasomal targets in mammalian cells (Saitoh and Hinchey, 2000; Uzunova, 2007).
However, a specialized ubiquitin ligase targeting these SUMO conjugates had not been identified yet. Therefore, the main aim of this thesis was to identify a mammalian ubiquitin ligase for SUMO conjugates (ULS).
First, it should be investigated, whether SUMO-2/3 conjugates accumulate under proteasome inhibition at all and in which part of the cell. This attempt intended to minimize the candidates out of the numerous ubiquitin E3 ligases to those being localized to a certain cellular compartment or substructure. Also, it had already been shown that SUMO-1 conjugates accumulate around PML nuclear bodies under proteasome inhibition (Bailey and O'Hare, 2005) and it was tempting to speculate that this might also happen to SUMO-2/3. Possible candidate ULS proteins had to be investigated, however, for their ability to specifically recognize and ubiquitylate SUMO modified proteins in order to prove that they indeed act as ULS proteins. For that purpose, an *in vitro* ubiquitylation assay for SUMO conjugates should be developed.
In order to obtain appropriate *in vitro* substrates – SUMO modified proteins – several known SUMO substrates should be SUMOylated in *E. coli* by making use of a trimeric vector system expressing the SUMOylation machinery in *E. coli* (Uchimura, 2004a; Uchimura, 2004b). This method should be optimized such that especially the SUMO modified species of a protein substrate would be enriched.
Finally, the SUMO/SIM dependent recruitment of the identified ULS to its substrates should be investigated in more detail in order to specify the prerequisites for the recognition of SUMOylated proteins. This is especially interesting as most proteins are not targeted to degradation when conjugated by SUMO under normal cellular conditions. For some, even stabilization has been observed (Desterro, 1998). Experiments in yeast had already shown that specifically high molecular weight conjugates are targets of yeast ULS proteins, emphasizing a mechanism in which multi- or polySUMOylation serves as signal for ULS recruitment (Uzunova, 2007; Xie, 2007; Prudden, 2007).
Knowing the prerequisites of mammalian ULS recruitment, it should also be possible to identify further cellular substrates regulated via the ULS pathway.
As mentioned, earlier studies indicated that the promyelocytic leukemia protein PML might be a target of that SUMO-dependent degradation pathway in mammalian cells (Müller, 1998; Lallemand-Breitenbach, 2001). Indeed, PML is mainly conjugated by SUMO-2/3 upon ATO treatment. Isolated PML-SUMO-2/3-ubiquitin hybrid conjugates from ATO-treated cells further supported the idea that PML is regulated via the ULS pathway in mammalian cells upon ATO treatment (Weißhaar, 2008). PML was therefore an ideal substrate candidate for *in vitro* studies.

2. MATERIALS & METHODS

2.1 Materials

2.1.1 Chemicals & Solutions

Chemicals used were graded p.A. and purchased from Merck (Darmstadt), Sigma (Sigma-Aldrich, Seelze) or Roth (Karlsruhe) unless otherwise specified. All solutions and culture media were prepared with ultrapure water derived from a combined reverse osmosis/ultrapure water system equipped with UV and ultrafiltration (Milli-Q system; Millipore, Billerica, USA).

2.1.2 Kits

DNA purification and gel extraction were carried out using the NucleoBond PC 100 or the NucleoSpin Extract II Kits (Macherey-Nagel, Düren), respectively.
Sequencing reactions were carried out using the BigDye Terminator v 3.1 Sequencing Kit from Applied Biosystems (Darmstadt).

2.1.3 Plastic ware

Plastic ware was purchased from Sarstedt (Nümbrecht) with exceptions for Cryotubes (Greiner Bio-one, Frickenhausen), 15 cm cell culture dishes (TPP; Trasadingen, Switzerland) and pipet tips (Starlab, Ahrensburg). Sterile filters (0,2 µm) were purchased from VWR (Darmstadt) or Whatman (Dassel), protein concentrators (Amicon Ultra) from Millipore (Billerica, MA, USA).

2.1.4 Standards

MassRuler Express DNA Ladder Mix #SM1283	Fermentas (St. Leon-Rot)
PageRuler Prestained Protein Ladder #SM0671	Fermentas (St. Leon-Rot)
Unstained Protein Molecular Weight Marker #SM0431	Fermentas (St. Leon-Rot)

2.1.5 Enzymes

Restriction enzymes, DNA polymerases and DNA ligases have been purchased from New England Biolabs (Ipswich, USA), Jena Biosciences (Jena) or AB Biosciences (Foster City, USA).

Other Enzymes:

DNaseI	Applichem (Darmstadt)
Creatine phosphokinase	Sigma (Sigma-Aldrich, Seelze)
Trypsin	Sigma (Sigma-Aldrich, Seelze)
Thrombin	Serva (Heidelberg)
Ube1	BIOMOL (Enzo Life sciences, Lörrach)
His_6-UbcH5b	BIOMOL (Enzo Life sciences, Lörrach)
Ubc4	Kind gift of Maria Miteva and Jürgen Dohmen, Cologne
Slx5-Slx8	Kind gift of Maria Miteva and Jürgen Dohmen, Cologne

Further enzymes applied in *in vitro* assays have been purified from *E. coli* expression cultures (see section 2.2.4.3).

2.1.6 Antibodies

Primary antibodies:

anti-GST (Z-5; sc-459)	1:2000	Santa Cruz Biotechnology (Santa Cruz, USA), rabbit polyclonal
anti-His_5 (Penta-His)	1:2000	Qiagen (Hilden), mouse monoclonal
anti-myc (ab9106)	1:1000	Abcam (Cambridge, UK), rabbit polyclonal
anti-PML (5E10)	1:100	kind gift of Roel van Driel, mouse monoclonal
anti-PML (A301-167A, -168A)	1:2000 each	Bethyl Laboratories (Montgomery, TX, USA), rabbit polyclonal
anti-PML RING-B1 (3004)	1:1000	Biogenes (Berlin), rabbit polyclonal
anti-RNF4 (3005)	1:100	Biogenes (Berlin), rabbit polyclonal
anti-Sp100 (N-20; sc-16328)	1:500	Santa Cruz Biotechnology (Santa Cruz, USA), goat polyclonal
anti-SUMO2 (ab22654)	1:2000	Abcam (Cambridge, UK), rabbit polyclonal
anti-Tubulin (DM1A; T6199)	1:500	Sigma (Sigma-Aldrich, Seelze), mouse monoclonal
anti-Ubiquitin (P4D1; sc-8017)	1:2000	Santa Cruz Biotechnology (Santa Cruz, USA), mouse monoclonal

Secondary antibodies:		
donkey-anti-mouse Alexa 488	1:1000	Molecular probes (Invitrogen, Karlsruhe)
donkey-anti-goat Alexa 546	1:1000	Molecular probes (Invitrogen, Karlsruhe)
donkey-anti-rabbit Alexa 546	1:1000	Molecular probes (Invitrogen, Karlsruhe)
goat-anti-mouse HRP	1:10000	BioRad (Munich)
goat-anti-rabbit HRP	1:10000	BioRad (Munich)
rabbit-anti-goat HRP	1:10000	Santa Cruz Biotechnology (Santa Cruz, USA)

2.1.7 Plasmid vectors

All listed plasmids have been generated during this work unless otherwise stated.

Table 2.1: Constructs for transient expression in mammalian cells

Construct	*Restriction sites used*	*Resistance*
pCMV3b-myc-SUMO1†	BamHI/XhoI	Kan^R
pCMV3b-myc-SUMO2†	BamHI/EcoRI	Kan^R
pCMV3b-myc-SUMO3†	BamHI/ EcoRI	Kan^R

† kindly generated by Christiane Horst

Table 2.2: Expression constructs used in the *E. coli* SUMOylation system

Construct	*Restriction sites used*	*Resistance*
pACYCDuet-1-6H-hSAE1/hSAE2†	BamHI/NotI and AflII/XhoI of pCDF-Duet-1-hSAE1/hSAE2	Cam^R
pRSFDuet-1-6H-hSUMO1/mUbc9	BamHI/NotI and NdeI/XhoI	Kan^R
pRSFDuet-1-6H-hSUMO2/mUbc9	BamHI/EcoRI and NdeI/XhoI	Kan^R
pRSFDuet-1-6H-hSUMO3/mUbc9	BamHI/EcoRI and NdeI/XhoI	Kan^R
pETDuet-1-GST-hPML 11 (50-179)†	BamHI/EcoRI	Amp^R
pETDuet-1-GST-hPML 11 (50-179)/ hPIAS3 (10-628)	BamHI/EcoRI and NdeI/BglII	Amp^R

Construct	*Restriction sites used*	*Resistance*
pETDuet-1-GST-hPML 11 (50-179)/ hPIAS4†	BamHI/EcoRI and NdeI/BglII	Amp^R
pETDuet-1-GST-hPML 11 (50-179)K65R/ hPIAS3 (10-628)	BamHI/EcoRI and NdeI/BglII	Amp^R
pETDuet-1-GST-hPML 11 (50-179) K65R/ hPIAS4	BamHI/EcoRI and NdeI/BglII	Amp^R
pETDuet-1-GST-hPML 11 (50-179) K160R/ hPIAS3 (10-628)	BamHI/EcoRI and NdeI/BglII	Amp^R
pETDuet-1-GST-hPML 11 (50-179) K160R/ hPIAS4	BamHI/EcoRI and NdeI/BglII	Amp^R
pETDuet-1-GST-hPML 11 (1-503)/ hPIAS4†	BamHI/EcoRI and NdeI/BglII	Amp^R
pETDuet-1-GST-hPML 11 (1-522)/ hPIAS4†	BamHI/EcoRI and NdeI/BglII	Amp^R
pETDuet-1-GST-hSp100 (1-332)†	BamHI/EcoRI	Amp^R
pETDuet-1-GST-hSp100 (1-332)/ hPIAS3 (10-628)	BamHI/EcoRI NdeI/BglII	Amp^R
pETDuet-1-GST-hSp100 (1-332)/hPIAS4†	BamHI/EcoRI and NdeI/BglII	Amp^R
pETDuet-1-GST-hSp100 (1-332)/ hRanBP2 (2553-2711)†	BamHI/EcoRI and NdeI/BglII	Amp^R
pETDuet-1-GST-hSp100 (1-332)/ hRanBP2 (2633-2711)†	BamHI/EcoRI and NdeI/BglII	Amp^R
pETDuet-1-GST-hSp100 (1-480)/hPIAS4†	BamHI/EcoRI and NdeI/BglII	Amp^R
pETDuet-1-GST-hSp100 (31-273)/hPIAS4†	BamHI/EcoRI and NdeI/BglII	Amp^R

* mUbc9 and hUbc9 share 100 % protein sequence identity
† kindly generated by Christiane Horst

Tabelle 2.1: *E. coli* expression constructs

Construct	*Restriction sites used*	*Resistance*
pET9d-TEV protease[1]	NcoI/PmlI	Kan^R
pGEX-TN-hSENP1 (415-643)	EcoRI/NotI	Amp^R
pGEX-TN-hSENP6 (625-1112)	BamHI/XhoI	Amp^R

Construct	*Restriction sites used*	*Resistance*
pGEX-4T2-hSUMO1[2]	BamHI/HindIII	Amp^R
pGEX-4T2-hSUMO2[2]	BamHI/EcoRI	Amp^R
pGEX-4T2-hSUMO3[2]	BamHI/EcoRI	Amp^R
pGEX-4T2-hSUMO1ΔN15	BamHI/XhoI	Amp^R
pGEX-4T2-hSUMO2 ΔN11	BamHI/XhoI	Amp^R
pGEX-4T2-hSUMO3 ΔN11	BamHI/XhoI	Amp^R
pGEX-4T2-2 x hSUMO1ΔN15	BamHI/XhoI	Amp^R
pGEX-4T2-2 x hSUMO2 ΔN11	BamHI/XhoI	Amp^R
pGEX-4T2-2 x hSUMO3 ΔN11	BamHI/XhoI	Amp^R
pGEX-4T2-3 x hSUMO1ΔN15	BamHI/XhoI	Amp^R
pGEX-4T2-3 x hSUMO3 ΔN11	BamHI/XhoI	Amp^R
pGEX-4T2-4 x hSUMO1ΔN15	BamHI/XhoI	Amp^R
pGEX-4T2-4 x hSUMO2 ΔN11	BamHI/XhoI	Amp^R
pGEX-TN-hUbiquitin (1x) (1st part of the human polyprotein)	EcoRI/XhoI	Amp^R
pET3a-hUbcH5B[3]	NdeI/EcoRI	Amp^R
pGEX-TN-hRNF4 wt	BamHI/EcoRI	Amp^R
pGEX-TN-hRNF4 (1-105)[†]	BamHI/EcoRI	Amp^R
pGEX-TN-hRNF4 (1-125)[†]	BamHI/EcoRI	Amp^R
pGEX-TN-hPML SIM (419-522) wt	BamHI/EcoRI	Amp^R
pGEX-TN-hPML SIM (419-522) S→A	BamHI/EcoRI	Amp^R
pGEX-TN-hPML SIM (419-522) S→D	BamHI/EcoRI	Amp^R
pGEX-TN-hSp100 (274-332)	BamHI/EcoRI	Amp^R
pGEX-TN-hDaxx (625-740)	BamHI/EcoRI	Amp^R
pGEX-TN-hPIAS3 (394-470)	BamHI/EcoRI	Amp^R
pGEX-TN-hPIAS4 (445-570)	BamHI/EcoRI	Amp^R
pGEX-TN-hZNF198 wt[††]	BamHI/NotI	Amp^R
pGEX-TN-hTIF1α wt[††]	NcoI/NotI	Amp^R

1) kind gift of Ingrid Schwienhorst, 2) kind gift of Martin Scheffner, Konstanz;
3) kind gift of Jörg Höhfeld, Bonn
[†] kindly generated by Christiane Horst; [††] kindly generated by Anke Krause

The cDNAs for SAE1, SAE2 and RanBP2 were kind gifts of Frauke Melchior. The mUbc9 cDNA was kindly provided by Martin Scheffner, Konstanz. All other cDNAs have been obtained from the German Resource Center for Genome Research (RZPD), now ImaGenes (Berlin).

2.1.8 Oligonucleotides

Oligonucleotides for PCR and site-directed mutagenesis have been obtained from biomers (Ulm). A full list of oligonucleotides is given in the Appendix.

2.1.9 *E. coli* strains and human cell lines

Escherichia coli (*E.coli)* DH5α	*Φ80dlacZMΔM15, recA1,endA1, gyrA96, thi-1, hsdR17(r_K^-, m_K^+), supE44, relA1, deoR, Δ(lacZYA-argF)U169* (Woodcock, 1989)
E.coli BL21 (DE3)	B, F^-, *dcm*, *ompT*, *hsdS* (r_B^- m_B^-), galλ (DE3) (Weiner, 1994)
E.coli Rosetta 2 (DE3) pLysS	*F^-, ompT, hsdSB (r_B^- m_B^-), gal, dcm* (DE3) pLysSpRARE (CamR) (Novagen, Schwalbach)
HeLa B cells (ECACC No: 85060701)	Human Negroid cervix carcinoma (European Collection of Cell Cultures; Health Protection Agency, Salisbury, UK)
Hs27 cells (ECACC No: 94041901)	Human foreskin, fibroblast (European Collection of Cell Cultures; Health Protection Agency, Salisbury, UK)

2.1.10 Sorbents and FPLC columns

Protino Glutathione Agarose 4B	Macherey-Nagel (Düren)
Ni-NTA Superflow	Qiagen (Hilden)
Q Sepharose Fast Flow	GE Healthcare (Munich)
SP Sepharose	GE Healthcare (Munich)
Superdex 75, 10/300 GL	GE Healthcare (Munich)
HiLoad 16/60 Superdex 75 prep grade	GE Healthcare (Munich)
HiLoad 26/60 Superdex 200 prep grade	GE Healthcare (Munich)
FPLC columns XK16 and XK26	GE Healthcare (Munich)
200µL StageTips, C18 material	Proxeon (Odense C, Denmark)

2.1.11 Instruments

Agarose gel electrophoresis chamber:	
Blue Marine 100	Serva (Darmstadt)
Balances	Sartorius (Goettingen)
Biacore T100	Biacore (GE Healthcare; Munich)
Blotting Chamber TE 77 RWR	Amersham Biosciences (GE Healthcare; Munich)
Cary 100 Bio Spectrometer	Varian (Mulgrave, Victoria, Australia)
Centrifuges:	
Eppendorf 5415R	Eppendorf (Hamburg)
Hermle Z383K	Hermle (Gosheim)
Avanti J-20 XP	Beckman Coulter USA (Brae, CA, USA)
Clean bench: LaminAir MA 2M 48 GS	Heraeus (Hanau)
CO_2 incubator C200	Labotect (Goettingen)
Developing machine: AGFA Curix 60	AGFA (Mortsel, Belgium)
Fluorescence microscope: Axioplan 2	(Zeiss, Jena)
FPLC: Äkta purifier	GE Healthcare (Munich)
LTQ-Orbitrap Discovery	Thermo Fisher Scientific (Ulm)
Milli-Q System	Millipore (Billerica, USA)
PCR-Thermocycler gradient	Eppendorf (Hamburg)
Power Supply: Consort E835	Consort (Tournhout, Belgium)
Protein gel systems:	
Mini-PROTEAN	Bio-Rad (Munich)
SE600-15-1.5	Hoefer (San Francisco, CA, USA)
Shaking incubators: innova 4230 & 4330	New Brunswick Scientific (Edison, NJ, USA)
Stirrer:	
VS-C7	VWR (Darmstadt)
IKAMAK RCT	IKA-WERKE (Staufen)
Thermomixer	Eppendorf (Hamburg)
Ultrasonic Homogeniser: Sonifier 250	Branson (Danbury, CT, USA)
UV agarose gel analyzer: BioDoc Analyze	Biometra (Goettingen)
Vortex Genie 2	Scientific Industries (Bohemia, NY, USA)
VP-ITC Microcalorimeter	MicroCal (GE Healthcare; Munich)
LTQ-Orbitrap Discovery	Thermo Fisher Scientific (Ulm)

2.2 Methods

2.2.1 Buffers, Solutions and Media

2.2.1.1 Buffers & Solutions

SDS-PAGE running buffer:	25 mM Tris 190 mM Glycine 0.1 % Sodium dodecyl sulfate (SDS) (w/v)
5 x SDS-PAGE sample buffer:	50 mM Tris pH 6.8 10 % Glycerol (v/v) 1 % SDS (w/v) 0.1 % bromphenol blue 7 % β-Mercaptoethanol (β-ME) (v/v)
Blotting buffer:	25 mM Tris 190 mM Glycine 10 % MeOH (v/v)
ECL1:	100 mM Tris pH 8.5 2.5 mM Luminol 400 µM p-Cumaric acid
ECL2:	100 mM Tris pH 8.5 1.8 % H_2O_2 (v/v)
PBS (phosphate buffered saline):	136.9 mM NaCl 2.7 mM KCl 8.1 mM Na_2HPO4 1.76 mM KH_2PO4
PBST:	PBS 0.1 % Tween 20 (v/v)
IF fixation buffer:	PBS 3 % Paraformaldehyde (PFA) (w/v)
IF wash buffer:	PBS 0.2 % Saponin (w/v)

IF block buffer:	IF wash buffer 3 % bovine serum albumin (w/v) (BSA; Fraction V, protease free; Roth)
RIPA buffer:	50 mM Tris pH 7.5 150 mM NaCl 5 % Glycerol 1 % Triton X-100 (v/v) 1 % Sodium desoxycholate (w/v) 0.1 % SDS (w/v) 1 mM N-ethylmaleimide (NEM; freshly added) 2 mM Dithiotreithol (DTT) Complete protease inhibitor cocktail (Roche)
GST1:	50 mM Tris pH 7.5 150 mM NaCl 2 mM DTT
GST2:	50 mM Tris pH 7.5 300 mM NaCl 2 mM DTT
GST Elution buffer:	50 mM Tris pH 8.0 150 mM NaCl 20 mM Imidazole 5 mM β-ME
Ni^{2+}-NTA Binding buffer (BB)	50 mM Tris pH 8.0 300 mM NaCl 20 mM Imidazole 5 mM β-ME 0.1 % Triton X-100 (v/v)
Ni^{2+}-NTA Elution buffer (EB)	50 mM Tris pH 8.0 300 mM NaCl 1 M Imidazole 5 mM β-ME 0.1 % Triton X-100 (v/v)

'Substrate buffer':	30 mM Tris, pH 7.5
	100 mM KCl
	5 mM $MgCl_2$
	2 mM DTT
DnaK depletion buffer:	50 mM Tris, pH 7.5
(modified from Rudolph, 1998)	150 mM NaCl
	60 mM KCl
	10 mM $MgCl_2$
	2 mM Adenosine triphosphate (ATP)
	2 mM DTT
	+/- 5 % Glycerol
In vitro ubiquitylation buffer:	40 mM Tris, pH 7.5
(Established final composition)	67 mM KCl
	2 mM $MgCl_2$
	1 mM DTT
	5 mM ATP
	200 ng/µl BSA (NEB, Frankfurt)
	200 µM Pefabloc
	1 ng/µl Leupeptin
	1 ng/µl Aprotinin
	1 ng/µl Pepstatin

All Tris based buffers with a defined pH were adjusted with HCl to their appropriate pH. To all buffers applied for protein purification from *E. coli* either 20 µM Pefabloc or 10 µM PMSF (for Ni^{2+}-NTA buffers and purifications of zinc complexing proteins) were added with a combination of 0.1 µg/ml Leupeptin, 0.1 µg/ml aprotinin, 0.1 µg/ml pepstatin.

2.2.1.2 Culture media for *E. coli*

LB (Luria-Bertani)-Medium:

1 % Baktotryptone (w/v)	(Gibco/Invitrogen, Paisley, UK)
0.5 % Yeast extract (w/v)	(Gibco/Invitrogen, Paisley, UK)
1 % NaCl (w/v)	

All media were sterilized for 20 min at 121 °C and stored at room temperature (RT). For LB Agar plates 2 % agar was added to LB medium before sterilization.
The selection of antibiotic resistant bacteria was carried out by addition of the appropriate antibiotic to the medium after sterilization (100 µg/ml Ampicillin, 30 µg/ml Chloramphenicol or 30 µg/ml Kanamycin).

2.2.1.3 Culture media for human cells

HeLa B medium:

EMEM (*Eagle's Minimal essentiell Medium*)	(Gibco/Invitrogen, Paisley, UK)
+ 10 % inactivated FCS (*foetal calf serum*)	(Sigma-Aldrich, Seelze)
+ 1 % non-essential amino acids (NAA)	(Gibco/Invitrogen, Paisley, UK)
+ 1 % Penicillin / Streptomycin	(Gibco/Invitrogen, Paisley, UK)

Hs27 medium:

DMEM (*Dulbecco´s Modified Eagle Medium*)	(Gibco/ Invitrogen, Paisley, UK)
+ 10 % inactivated FCS (*foetal calf serum*)	(Sigma-Aldrich, Seelze)
+ 1 % non-essential amino acids (NAA)	(Gibco/Invitrogen, Paisley, UK)
+ 1 % Penicillin / Streptomycin	(Gibco/Invitrogen, Paisley, UK)
+ 200 µM L-Glutamin	(Gibco/ Invitrogen, Paisley, UK)

2.2.2 Biomolecular methods

2.2.2.1 Standard techniques

Molecular cloning, polymerase chain reactions (PCR) and DNA sequencing were carried out according to standard procedures (Ausubel, 2010) using enzymes purchased from New England Biolabs (Ipswich, USA), Jena Biosciences (Jena) and AB Biosciences (Foster City, USA). DNA Sequencing was carried out by the Cologne Center for Genomics sequencing facility.
Site-directed mutagenesis was performed according to the "QuickChange Site-directed mutagenesis" (Stratagene, La Jolla, USA) protocol.

2.2.2.2 Generation of artificial poly-SUMO chains

Artificial SUMO chains have been generated by ligating PCR products encoding SUMO-2ΔN11 or SUMO-1ΔN15 while simultaneously digesting with restriction enzymes specific for *Bam*HI (N-terminal site) and *Bgl*II (C-terminal site) in T4 DNA ligase buffer, 150 mM NaCl, 0.1 mg/ml BSA at 22 °C o/n (Tatham, 2008). The next morning, additional units of all three enzymes were added for another 4 hours. This resulted in a range of cDNAs encoding SUMO-2ΔN11 or SUMO-1ΔN15 monomers and polymers that were separated by agarose gel electrophoresis and individually extracted into solution.
These were then subjected to a second PCR for generating restriction sites for BamHI (N-terminally) and XhoI (C-terminally) for cloning into pGEX-4T2 as well as a GG motif in front of a stop codon preceding the XhoI site. Expression from these plasmids resulted in GST-tagged artificial SUMO-2ΔN11 or SUMO-1ΔN15 monomers or linear chains with up to 4 monomers. Each monomer in these chains is separated from the other by Arg-Ser.

2.2.3 Cell biological methods

2.2.3.1 Cell cultivation

HeLa B (Cervix carcinoma) and Hs27 (primary human fibroblast) cells were cultivated in the respective media (see section 2.2.1) at 37 °C, 5 % CO_2 and 99 % humidity in a C200 incubator (Labotect, Göttingen). Long-term storage was carried out in inactivated FCS/10 % DMSO at around -110 and -150 °C in the vaporous phase of liquid N_2.
Cells were expanded at ~ 80 % confluency to other plates by incubating them with trypsin for 1-2 min after washing them twice with PBS. Usually, ~ 1 x 10^6 HeLa B or Hs27 cells were plated per 10 cm dish.

2.2.3.2 Cell lysis

Hs27 cells were washed twice with ice-cold PBS before collected from plates with cell scrapers and transferred to 15 or 50 ml reaction tubes depending on the volume. Then, they were centrifuged at 300 g, washed once more with PBS and after another centrifugation lysed on ice with RIPA buffer (100 µl/1 x 10^7 cells) for 1 h. Cell debris was removed from lysate by centrifuging the lysed cells at 3000 g. Only the supernatant was used for isolation experiments (see section 2.2.4.13).

2.2.3.3 Cell transfection

HeLa B cells were transfected for fluorescent microscopy with pCMV-3b-SUMO constructs using Gene Juice transfection reagent (Novagen, Schwalbach) according to the manufacturer's instructions.

2.2.3.4 Preparation of cells for fluorescent microscopy

HeLa B cells were grown on sterilized cover slips which were added to the usual culture plates and transfected as described in section 2.2.3.3. After 40 h, 20 µM MG 132 was added or not for additional 7 h. Cells on cover slips were fixed in PBS with 3 % paraformaldehyde. After permeabilization in PBS with 0.1 % saponin (Sigma-Aldrich, Seelze), cells were blocked in PBS, 3 % BSA, 0.1 % saponin. Primary and Alexa-labeled secondary antibodies (Invitrogen, Karlsruhe) as well as 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI; Roche, Penzberg) were applied in blocking buffer. Cover slips were embedded in ProLong Gold antifade (Invitrogen, Karlsruhe) and examined using a Zeiss Axioplan2 fluorescence microscope (Zeiss, Göttingen).

2.2.4 Biochemical methods

2.2.4.1 SDS-PAGE and Western Blotting

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was applied to separate proteins according to their molecular size under reducing conditions unless otherwise stated (Laemmli, 1970). Table 2.4 summerizes the different SDS polyacrylamide (SDS-PA) gel compositions used. Two different protein gel systems from Bio-Rad and Hoefer have been utilized (see section 2.1.11).

Table 2.4: SDS gel compositions

	4 % stacking gel	*10 % seperating gel*	*15 % seperating gel*
Acrylamide (37,5:1)	4 %	10 %	15 %
Tris pH 8,8	-	390 mM	390 mM
Tris pH 6,8	150 mM	-	-
SDS	0.1 %	0.1 %	0.1 %
Ammoniumperoxo-disulfat (APS)	0.1 %	0.1 %	0.1 %
N,N,N‘,N‘-Tetra-methylethylendiamine (TEMED)	0.01 %	0.01 %	0.01 %
H_2O	add to final volume	add to final volume	add to final volume

After SDS-PAGE, separated proteins have been either stained with Coomassie Brilliant Blue (Coomassie; Serva, Heidelberg) in 20 % Ethanol, 10 % Acetic acid directly in SDS-PA gels or transferred to 0,2 µm Polyvinylidene fluoride (PVDF) membranes (Millipore, Schwalbach) by western blotting using a semi-dry system from Amersham Biosciences (GE Healthcare, Munich) (modified from Renart, 1979; Towbin, 1979).

2.2.4.2 Immunological detection of proteins on PVDF membranes

Detection of proteins has been carried out by incubating the PVDF membranes with specific primary and horse-radish peroxidase (HRP)-coupled secondary antibodies (see section 2.1.6) diluted in PBST/5 % milk powder. HRP-initiated Enhanced Chemiluminescence (ECL) exposed X-ray films (Super RX; Fujifilm, Düsseldorf) at sites of bound antibodies. ECL solution was either self-mixed 1:1 from ECL1 and ECL2 or purchased from Roche (Penzberg).

2.2.4.3 Protein purifications

Protein expression was usually carried out in *E. coli* Rosetta (DE3) pLys after induction with 0.1 mM Isopropyl-β-D-thiogalactopyranosid (IPTG) at individual temperatures. In some cases, *E. coli* BL21 (DE3) strains have been used to express proteins which is stated in the respective sections. Bacterial cultures (usually 6-10 l) were centrifuged at 6000 g for 10 min, resuspended in the buffers applied for the following first purification step and shock frozen in liquid nitrogen (liq. N_2).
Expressed proteins were isolated using different approaches (see the following sections). Lysis of bacteria was usually performed on ice with the following additives: 100 µg/ml Lysozym (for BL21 lysis), DNase I, protease inhibitors (either 200 µM Pefebloc or a combination of 100 µM PMSF, 1 µg/ml Leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin) and 1.5 % Sarkosyl (v/v) unless otherwise stated. To obtain fully lysed bacteria and sheared DNA, lysates were sonified 3 x for 30 s on ice at maximal constant pulse (resulting in the raw lysate, RL). Then the lysate was centrifuged at 50000 g for 1 h at 4 °C. The resulting supernatant (S) contained the expressed protein which was further purified. Supernatants containing Sarkosyl were substituted with 2 % Triton-X-100 (v/v) before subjected to following purification steps. Samples for RL, S, and Pellet (P) were taken for SDS-PAGE analysis. Usually, proteins were purified applying an affinity purification step followed by subsequent size exclusion chromatography. Fast Protein Liquid Chromatography (FPLC) was performed using an Äkta purifier (GE Healthcare). After gel filtration protein fractions of high purity were combined, concentrated using Amicon concentrators (Millipore) and aliquots were shock frozen in liq. N_2.

2.2.4.3.1 Affinity purification of GST-tagged proteins

GST-tagged proteins were glutathione (GSH) affinity purified performing batch purifications in 50 ml tubes. Cleared lysates (Supernatant S) were applied to the beads and incubated for 1 h at 4 °C on a rotating wheel. Unbound proteins were washed away by several washing steps: usually 5 x with GST1 buffer, 3 x with GST2 buffer and 3 x with GST1 buffer or later including 3 wash steps with DnaK depletion buffer (refer to section 2.2.4.6) before washing 3 x with GST1 buffer. Proteins that were purified from *E. coli* without the addition of Sarkosyl (like GST-SUMO-1, GST-SUMO-2, GST-SUMO-3, GST-ubiquitin and GST-SENP1 (415-643)) were additionally washed 3 x with GST1 buffer/0.1 % Triton X-100 (v/v). Elution was carried out using GST Elution buffer containing 10 - 20 mM GSH. When GST was cleaved off, instead of protein elution either TEV protease (for proteins expressed from pGEX-TN or pET-Duet vectors) or Thrombin (SERVA; for proteins expressed from pGEX-4T2 vectors) was added to the beads in appropriate buffers for following purification steps.

Individual GST buffers were applied for the purification of GST-ubiquitin (GST1: 30 mM Tris pH 7.5, 100 mM KCl, 5 mM EDTA, 2 mM DTT; GST2: 30 mM Tris pH 7.5, 300 mM KCl, 5 mM EDTA, 2 mM DTT), GST-SENP1 (415-643) (GST1: 20 mM Tris pH 8.0, 500 mM NaCl,

0.5 mM EDTA, 2 mM DTT; GST2: 20 mM Tris pH 8.0, 300 mM NaCl, 0.5 mM EDTA, 2 mM DTT) and GST-SIM constructs including all GST-RNF4 constructs purified (GST1 and GST2 buffer supplemented with 5 % Glycerol (v/v)).
The catalytic domain of SENP6 (aa 625-1112) was purified using 20 mM Tris pH 8.0, 300 mM NaCl, 0.1 % Triton X-100 (v/v), 2 mM DTT as GST buffer. As this protein had the tendency to precipitate upon concentration, ion exchange chromatography using Q Sepharose (GE Healthcare) was applied instead of Superdex 75 (S75) gel filtration as a second purification step after TEV cleavage (Q1 buffer: 20 mM Tris pH8.0, 300 mM NaCl, 0.1 % Triton X-100 (v/v), 2 mM DTT; Q2 buffer: 20 mM Tris pH 8.0, 1 M NaCl, 0.1 % Triton X-100 (v/v), 2 mM DTT).

2.2.4.3.2 Affinity purification of His_6-tagged proteins

His_6-tagged proteins were Ni^{2+}-NTA affinity purified by FPLC using a FPLC XK16 column (GE Healthcare) filled with a Ni^{2+}-NTA superflow affinity matrix (Qiagen). Proteins were applied to in Ni^{2+}-NTA BB, washed until baseline absorption and eluted with an Imidazole gradient from 20 mM in Ni^{2+}-NTA BB to 1 M Imidazole in Ni^{2+}-NTA EB.

An individual Ni^{2+}-NTA affinity purification protocol was applied for purifying His_6-tagged TEV protease: The BB was composed of 20 mM Tris pH 8.0, 20 mM Imidazole, 150 mM NaCl, 0.02 % Sodium desoxycholate, 2 mM β-Mercaptoethanol. An additional wash step was included using the BB/1 M NaCl before an elution gradient in 20 mM Tris pH 8.0, 20 mM Imidazole, 100 mM NaCl, 2 mM β-Mercaptoethanol, 20 % Glycerol was driven from 20 mM Imidazole to 300 mM Imidazole. Protein fractions of high purity were combined and added up to a final concentration of 50 % Glycerol.

2.2.4.3.3 Size exclusion chromatography

Size exclusion chromatography was carried out using a HiLoad 16/60 Superdex 75 prep grade gel filtration column (GE Healthcare). Before proteins were subjected to gel filtration, they were concentrated to a volume of 1-1.5 ml using Amicon protein concentrators (Millipore). Usually, proteins were filtrated into their respective GST1 buffers with exceptions for proteins that were utilized as *in vitro* ubiquitylation substrates and therefore were filtrated into 'substrate buffer' and SENP1 (415-643) which was filtrated into its special GST2 buffer (see section 2.2.4.3.1).

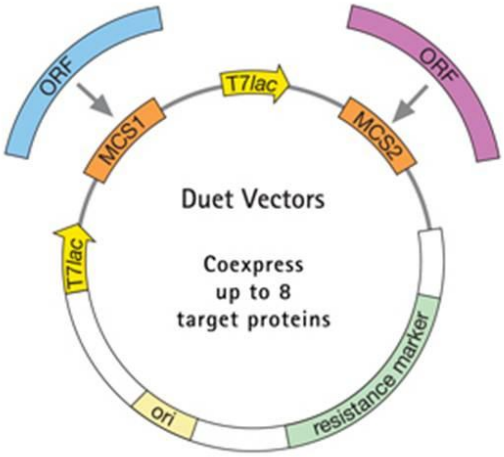
2.2.4.3.4 UbcH5b purification

Untagged UbcH5b was expressed in *E. coli* BL21 (DE3) for 5 h from pET3a-UbcH5b at 30 °C. Bacterial pellets were resuspended in 50 mM MES pH 6.0, 150 mM NaCl, 5 mM DTT, 100 µM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin and shock frozen in liq. N_2. Upon thawing, bacteria were stirred for 1 h at 4 °C and then centrifuged at 50000 g for 1 h at 4 °C. A classical lysis step was not performed, as UbcH5b leaks out from the bacterial periplasm into the buffer (the idea was taken from a purification protocol for Ubc9 by Frauke Melchior). Most *E. coli* proteins stay within the intact

bacterial membranes such that this step already yields in a fairly pure protein. It is therefore important not to use pLysS containing *E. coli* strains as, for instance, *E.coli* Rosetta 2 (DE3) pLysS. Further purification was achieved by applying the supernatant to ion exchange chromatography on SP sepharose (GE Healthcare) using a gradient from 150 mM to 1 M NaCl. UbcH5B containing fractions were subjected to size exclusion chromatography in 30 mM Tris pH 7.5, 300 mM KCl, 15 % Glycerol, 20 µM Pefabloc using a Superdex 75 gel filtration column.

2.2.4.4 Expression and purification of SUMOylated proteins from *E. coli*

To generate appropriate substrates for *in vitro* ULS studies, it is of particular importance to produce and enrich the SUMO-modified forms of the respective protein. For that reason, an efficient method to purify the SUMO-modified forms of a protein from an '*E. coli* SUMOylation system' was established during this work using the trimeric Duet vector system from Novagen (Merck, Darmstadt). Each of the three different vectors possesses two open reading frames (ORFs) as well as distinct origins of replication and resistance genes (see Figure 2.1).
Genes coding for the enzymes of the SUMOylation machinery, the SUMOs itself and possible GST-tagged substrates could therefore be introduced and expressed in parallel and in different combinations in *E. coli* such that the substrate is SUMOylated in *E. coli*.



vector	MCS1	MCS2
pACYCDuet1 (Cam^R)	6His-SAE1	SAE2
pRSFDuet1 (Kan^R)	6-His-SUMO (1/2/3)	Ubc9
pETDuet (Amp^R)	substrate	SUMO-E3

Figure 2.1: Trimeric vector system used to generate His_6-SUMOylated proteins in *E. coli*
Features of the trimeric vector system are shown in a schematic overview (Novagen /Merck, Darmstadt). All Duet vectors have two different Open Reading frames (ORFs) with inducible T7/lac promoters as well as distinct origins of replication (ori) and resitance markers (MCS, multiple cloning site). The table beneath summarizes the generated expression constructs for the production of SUMOylated proteins in *E. coli*.

The major advantage established here was to utilize His_6-tagged SUMOs instead of untagged versions. It was therefore possible to purify the SUMO-conjugated forms of a substrate by consecutive affinity purifications.
For each of the three conjugatable SUMOs, competent BL21 (DE3) strains were generated harboring two of the Duet vectors coding for the SUMO E1 enzyme and the E2 enzyme Ubc9 combined with one of the His_6-SUMOs: pACYCDuet1-SAE1 (1. MCS)|SAE2 (2. MCS) and pRSFDuet1-His_6-SUMO1/2/3 (1. MCS)|Ubc9 (2. MCS). These strains could then be transformed with a third vector coding for the GST-tagged substrate and occasionally for one of the tested SUMO E3 enzymes (pETDuet-GST-substrate (1. MCS)|E3 enzyme (2. MCS).
Expression in BL21 (DE3) cells was induced by 0.1 mM IPTG at 30 °C. His_6-SUMO-modified substrates were isolated by consecutive GSH and Ni^{2+}-NTA affinity purifications, subsequent TEV protease cleavage of the GST-tag and size exclusion chromatography using a Superdex 75 gel filtration column. Residual GST was removed after TEV cleavage by binding to fresh GSH affinity beads. Buffers used for GSH affinity purifications are GST1 and GST2, for Ni^{2+}-NTA affinity purifications Ni^{2+}-NTA BB and EB as well as 'substrate buffer' for gel filtrations. TEV cleavage was performed in Ni^{2+}-NTA BB at 4 °C o/n. Large scale purifications have been usually performed from 10 l *E. coli* expression cultures. Small scale (50 ml *E. coli* expression cultures) test purifications have been carried out similarly using each 50 µl affinity beads per lysate without subsequent cleavage and gel filtration.

During this work several batches of SUMOylated PML (50-179) have been purified that differ (slightly) in purity or SUMOylation efficiency.

2.2.4.5 PML (50-179) and RNF4 antibodies

In order to obtain efficient antibodies for western blotting against PML (50-179) and RNF4, both proteins were highly purified applying consecutive gel filtrations on HiLoad 16/60 Superdex 75 and 26/60 Superdex 200 prep grade columns after the described affinity purification (see section 2.2.4.3.1). PML (50-179) was sent in solution (native state) to a company producing antibodies (Biogenes). RNF4 had to be further purified from residual contaminants by SDS-PAGE and cut gel slices were sent to Biogenes. Therefore, the resulting polyclonal rabbit antiserum was directed against denatured RNF4. For both proteins, two antisera from different rabbits have been produced and were tested for application in Western Blots. One of each (serum 3004 for PML (50-179) and serum 3005 for RNF4) was efficiently and specifically recognizing its antigen (data not shown).

2.2.4.6 DnaK contamination and DnaK depletion

Purified RNF4, PML (50-179) K65R and K160R (unmodified and SUMO-3 modified) were usually contaminated by an unspecific band at ~ 70 kDa. This band could be identified as bacterial DnaK by anti-DnaK staining (data not shown; anti-DnaK was a kind gift of Thomas Langer, Cologne).
DnaK was also present in the sample sent for the PML (50-179) antibody production. Therefore, staining with anti-PML (50-179) often results in an additional band at ~ 70 kDa which represents DnaK and is thus unspecific for PML.
In later protein purifications, a DnaK depletion step was added to the purification procedure (see section 2.2.4.3.1) which resulted in a reduction of DnaK contamination up to 100 %.

2.2.4.7 *In vitro* ubiquitylation assays

For *in vitro* ubiquitylation assays, only purified proteins and enzymes have been utilized that were mixed together under certain conditions.
While establishing the *in vitro* ubiquitylation assay for SUMO conjugates, these conditions were varied in order to increase the specificity of the reaction. Therefore, at this place, the final reaction mixture is described that was used for all assays shown from Figure 3.17 B. All other *in vitro* ubiquitylation experiments shown were performed under varying conditions and are individually described beneath the according figures in section 3.4. When indicated, protease inhibitors were added in following concentrations: 200 µM Pefabloc, 1 ng/µl leupeptin, 1 ng/µl aprotinin and 1 ng/µl pepstatin.
The established standard *in vitro* ubiquitylation reaction was performed the following:
100 ng/µl unmodified or His_6-SUMO modified forms of a substrate protein (PML or Sp100) were subjected to *in vitro* ubiquitylation by RNF4 in 40 mM Tris pH 7.5, 67 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 200 ng/µl BSA (New England Biolabs, Frankfurt), protease inhibitors and 5 mM ATP containing 3 ng/µl Ube1, 3 ng/µl UbcH5b, 300 ng/µl ubiquitin +/- 30 ng/µl RNF4 for 7 h at 30 °C. Reactions were terminated by adding 2 x SDS sample buffer. Proteins were separated by SDS-PAGE, transferred to PVDF membranes and detected with the antibodies indicated.

The ubiquitin E2 activity test for UbcH5B was carried out using a modified protocol from (Lorick, 2005): 0.2 µg/µl Ube1, 1 µg/µl UbcH5b and 3 µg/µl ubiquitin were incubated at RT for 5 min in 50 mM Tris pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$ +/- 5 mM ATP. Reactions were divided and stopped by adding 2 x SDS sample buffer either containing the reducing agent β-Mercaptoethanol or not. E2~ubiquitin thioester formation was detected by anti-ubiquitin after SDS-PAGE in non-reduced samples only.

The ubiquitin E3 ligase activity of candidate ULS proteins was assessed under low salt conditions according to a modified protocol from (Joazeiro, 1999):
7.5 nM Ube1 (Biomol), 150 nM UbcH5b or *S. cerevisiae* Ubc4, 5 µM ubiquitin and 0.25 µM, 0.5 µM or 1 µM of the ULS candidates were mixed together in 50 mM Tris pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 10 U Creatinphosphokinase, 1 mM Creatinphosphate +/- 1 mM ATP and incubated for 7 h at 30 °C. The reactions were terminated by adding 2 x SDS sample buffer. Proteins have been separated by SDS-PAGE and detected with anti-ubiquitin.

2.2.4.8 Isolation of SUMO-ubiquitin-PML hybrid conjugates after in vitro ubiquitylation reactions

SUMO-ubiquitin-PML hybrid conjugates were isolated from the reaction mixture after RNF4-mediated *in vitro* ubiquitylation of His_6-SUMO modified PML (50-179) by applying denaturing Ni^{2+}-NTA affinity purification. The reaction mixture was diluted in 500 µl denaturing Ni^{2+}-NTA buffer (8 M Urea, 100 mM Na_2HPO_4/NaH_2PO_4, 2mM DTT) pH 8.0 and added to 50 µl Ni^{2+}-NTA affinity beads for 30 min at RT. Bound proteins were washed 5 x with denaturing Ni^{2+}-NTA buffer pH 6.3 and eluted with consecutive steps of 50 µl denaturing Ni^{2+}-NTA buffer pH 4.5 and 20 µl denaturing Ni^{2+}-NTA buffer supplemented with 1 M Imidazole. Eluted fractions were combined; proteins have been separated by SDS-PAGE and detected with anti-ubiquitin.

2.2.4.9 Assessing the ubiquitin attachment sites in His_6-SUMO-3 modified PML (50-179)

In order to investigate whether RNF4 attaches the ubiquitin molecules to SUMO, to PML or to both moieties of its substrate, the purified catalytic domains of SENP1 and SENP6 were utilized to reverse the SUMOylated state of PML (50-179).
After RNF4-mediated *in vitro* ubiquitylation of His_6-SUMO-3 modified PML (50-179) (see section 2.2.4.7), reactions were divided. To one half, purified catalytic domains of SENP1, SENP6 or both (each or together ~ 30 ng/µl, respectively) were added to the reactions, to the other half 1 x reaction buffer only. All reactions were then further incubated at 30 °C o/n before being terminated by adding 2 x SDS sample buffer. Proteins were separated by SDS-PAGE and detected with according antibodies.

Furthermore, a larger amount of ubiquitylated His_6-SUMO-3 modified PML (50-179) was separated by SDS-PAGE in order to analyze the ubiquitylated bands by mass spectrometry. Therefore, this gel was stained with Coomassie and bands corresponding to the first three ubiquitylated bands in the western blots were excised and in-gel digested with trypsin (Promega). Peptides were extracted and desalted using StageTips (Proxeon), separated on a reverse-phase C18 capillary using an Eksigent nLC-System connected on-line to the mass spectrometer (LTQ-Orbitrap Discovery) equipped with a nanoelectrospray source. Survey full scan MS spectra (400-2000) were acquired in the

Orbitrap, top five ions were sequenced by CID in the LTQ. Orbitrap measurements were performed enabling the lock mass option for high mass accurancy, which allowed direct determination of charge states. Mass spectromic analysis was carried out by the proteomics facility of the Cellular stress responses in Aging-Associated Diseases (CECAD) University of Cologne Cluster of Excellence. The individual peak lists have been searched in a ChopNSpice (Hsiao, 2009) data base generated from PML (50-179) and His_6-SUMO-3, respectively, using the SEQUEST program (Eng, 1994). ChopNSpice created databases for the ubiquitin-specific GlyGly modification on lysine residues each of PML (50-179) and His_6-SUMO-3. Data were double checked by searching the individual peak lists in the international protein index (IPI) database (IPIhuman 3.6.1; http://www.ebi.ac.uk/IPI/IPIhuman.html) allowing a dynamic GlyGly modification in the search parameter settings of the SEQUEST program.

2.2.4.10 Analytical gel filtrations of RNF4 (1-105) and artificial SUMO chains

50 µM RNF4 (1-105) has been incubated with either 200 µM monoSUMO-1 or monoSUMO-2, 100 µM diSUMO-1ΔN15 or diSUMO-2ΔN11 or 50 µM tetraSUMO-1ΔN15 or tetraSUMO-2ΔN11 o/n in GST1 buffer + 5 % Glycerol. Proteins have been subjected to analytical gel filtrations performed at 0.5 ml/min in GST1 buffer + 5 % Glycerol using an analytical Superdex 75 (10/300). Eluted protein fractions were separated by SDS-PAGE and stained with Coomassie. A special case represented the incubation of RNF4 (1-105) with tetraSUMO-1ΔN15, as protein fell out directly while mixing them. Overnight incubation led to a clear solution, but high-speed centrifugation (13000 g at 4 °C) resulted in a small protein containing pellet (examined with addition of Bradford solution). The supernatant was applied to analytical gel filtration.

2.2.4.11 ITC

Isothermal titration calorimetry (ITC) was performed using a VP-ITC (MicroCal). All titrations were carried out using purified artificial polySUMOs and RNF4 (1-125) in de-aerated GST1 buffer + 5 % Glycerol at 25 °C. All purified proteins were passed into the same buffer aliquot that was used for these experiments to avoid heat of dilution. Artificial polySUMOs were injected as ligands into the experimental cell containing the diluted RNF4 (1-125) solution. The ligand was injected into the cell in 7 µl steps (43 in total). After each individual injection, the released heat of binding was recorded for 3.5 min. All results were analyzed using the ORIGIN software package (MicroCal). The curve fit to the data describes a single site binding model dq/dLt = H (1/2 + (1 – XR –r)/(2 ((XR + r +1)2 – 4XR)1/2)) V, with XR = [LT]/[MT] and r = 1/KA[MT] (Wiseman, 1989; Indyk and Fisher, 1998). This fit optimizes parameters of stoichiometry (N), association constant (K_A), and the enthalpy of binding (ΔH). The dissociation constant (K_D) is the inverse of K_A.

2.2.4.12 Interaction of GST-SIM containing proteins with individual SUMO paralogs

500 µg purified SUMO-1, SUMO-2 and SUMO-3 have been each subjected to an interactions study (pulldown (PD) assay) with freshly GSH affinity purified and equalized GST-SIM constructs of PML (419-522), PML (419-522) S512-514, 517→A, PML (419-522) S512-514, 517→D, RNF4 (1-105), Sp100 (274-332), DAXX (625-740), PIAS3 (396-479) or PIAS4 (445-570) in GST1 + 5 % Glycerol, respectively. GSH affinity purification is described in section 2.2.4.3.1. Purified ubiquitin served as negative control. Proteins were eluted in 2 x SDS sample buffer, separated by SDS-PAGE and detected by SUMO-1, SUMO-2/3 or ubiquitin antibodies, respectively. Input of GST-SIM constructs were equalized by comparison of Coomassie stained protein bands after SDS-PAGE and is displayed as GST-SIM input.

2.2.4.13 Isolation of multi-/polySUMOylated proteins from cells treated with different stress stimuli by GST-RNF4 (1-105)

2 x 10^7 Hs27 human fibroblasts were either treated with DMSO (-) or 20 µM MG132 (Sigma-Aldrich, Seelze; solved in DMSO) (+) for 6 h, with 3 µM ATO -/+ 20 µM MG132 for 6 h, with 1 M Sorbitol -/+ 20 µM MG132 for 4 h or with 7.5 mM Canavanine -/+ 20 µM MG132 for 6 h.

While the Hs27 cells were treated with the different stress inducers, GST-RNF4 1-105 was freshly GSH affinity purified from *E.coli* Rosetta cells as described in section 2.2.4.3.1. Likewise, purified GST was bound in GST1 buffer + 5 % Glycerol as a control.

Samples of all purification and binding steps were analyzed by SDS-PAGE. Equal amounts of GSH-bound GST and GST-RNF4 were prepared for isolation of cellular proteins in 15 ml tubes (300 µl 1:1 suspension each).

After stress treatment, Hs27 cells were lysed in RIPA buffer (1 x 10^7 cells/ml) as described in section 2.2.3.2.

To shear DNA, cell lysates were additionally passed through a 20 Gauge needle before centrifuged to pellet cellular debris. Cleared lysates were divided equally and applied each to GST and GST-RNF4 1-105 beads in a 1/10 dilution in GST1 buffer + 5 % Glycerol.

The isolation of possible RNF4 substrates was carried out over night at 4 °C.

The next morning, Beads were washed once with 10 ml GST1 buffer/5 % Glycerol, followed by 2 wash steps with 5 ml GST1 buffer + 5 % Glycerol. Then, the beads were transferred to 1.5 ml tubes and further washed 5 x 1 ml GST1 buffer + 5 % Glycerol.

Proteins were eluted with 150 µl 2 x SDS sample buffer. 1/5 (30 µl) and 4/5 (120 µl) were applied on SDS-PA gels (Hoefer system) for western blot analysis and Coomassie staining, respectively. Individual lanes were excised into 10 different slices from the Coomassie-stained gel (> 50 kDa, including stack) and in-gel digested with trypsin (Promega). Peptide extraction and Orbitrap measurements were carried out by the CECAD proteomics facility as described in section 2.2.4.9.

The individual peak lists have been searched in the IPI database (IPIhuman 3.6.1; http://www.ebi.ac.uk/IPI/IPIhuman.html) using the MASCOT program (Matrix Science; Boston, MA, USA). Resulting protein hits were merged individually for each condition in a multiconsensus file and compared to the hits obtained for the DMSO negative samples. Protein hits that were also present in DMSO samples were eliminated from the result files. Filter settings allowed hits > 2 peptides and Ion Scores > 20.
The resulting proteins have been assessed for the presence of bona-fide SUMOylation sites using the SUMOplot SUMO site prediction program (Abgent; San Diego, CA, USA; accessible via the ExPASy proteomic tools server, http://www.expasy.ch/tools/) in order to increase the probability of the resulting proteins being regulated by the ULS pathway. Proteins that did not confer any predictable SUMOylation site were likewise eliminated from the result files. Proteins with low probability motifs were listed in grey.

2.2.4.14 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is a technique to study binding affinities between a ligand which is immobilized on a sensor chip in a flow cell and an analyte which is injected in aqueous solution through the flow cell under continuous flow. SPR-based instruments use an optical method to measure the refractive index near (within ~ 300 nm) the sensor surface (given in response units, RU).
SPR was performed at 25 °C on a Biacore T100 (Biacore). Roughly 2500 RU of ubiquitin, SUMO-1 and SUMO-2 were amino coupled to a CM5 sensor chip (CM5 matrix: carboxymethylated dextran covalently attached to a gold surface; Biacore). Several concentrations ranging from 25 µM-100 µM of PIAS3 (394-470) or RNF4 (1-105) were tested at 20 µl/min in 50 mM Tris pH 7.5/150 mM NaCl/5 % Glycerol. Association was recorded for 1 min followed by a dissociation period of 2 min. After this period, the surface was regenerated with a 5 s pulse injection of 50 mM NaOH.

3. RESULTS

3.1 Proteasome inhibition induces accumulation of SUMO-2/3 conjugates at PML-NBs

There are several hints that the ULS pathway is conserved to humans and that the PML protein might be a substrate for this pathway (Kosoy, 2007; Prudden, 2007; Sun, 2007; Uzunova, 2007; Weißhaar, 2008). In order to identify the according ULS enzyme, the first experiment attempted to test the hypothesis that SUMO-2/3 conjugates accumulate at PML-NBs under proteasome inhibition. That would limit the possible ULS candidates to proteins localizing to PML-NBs. Therefore, HeLa cells were transiently transfected with constructs expressing mature myc-tagged SUMO-1, SUMO-2 or SUMO-3 and treated with the proteasome inhibitor MG132. Figure 3.1 shows exemplarily the effects of proteasomal inhibition for cells expressing myc-SUMO-3.

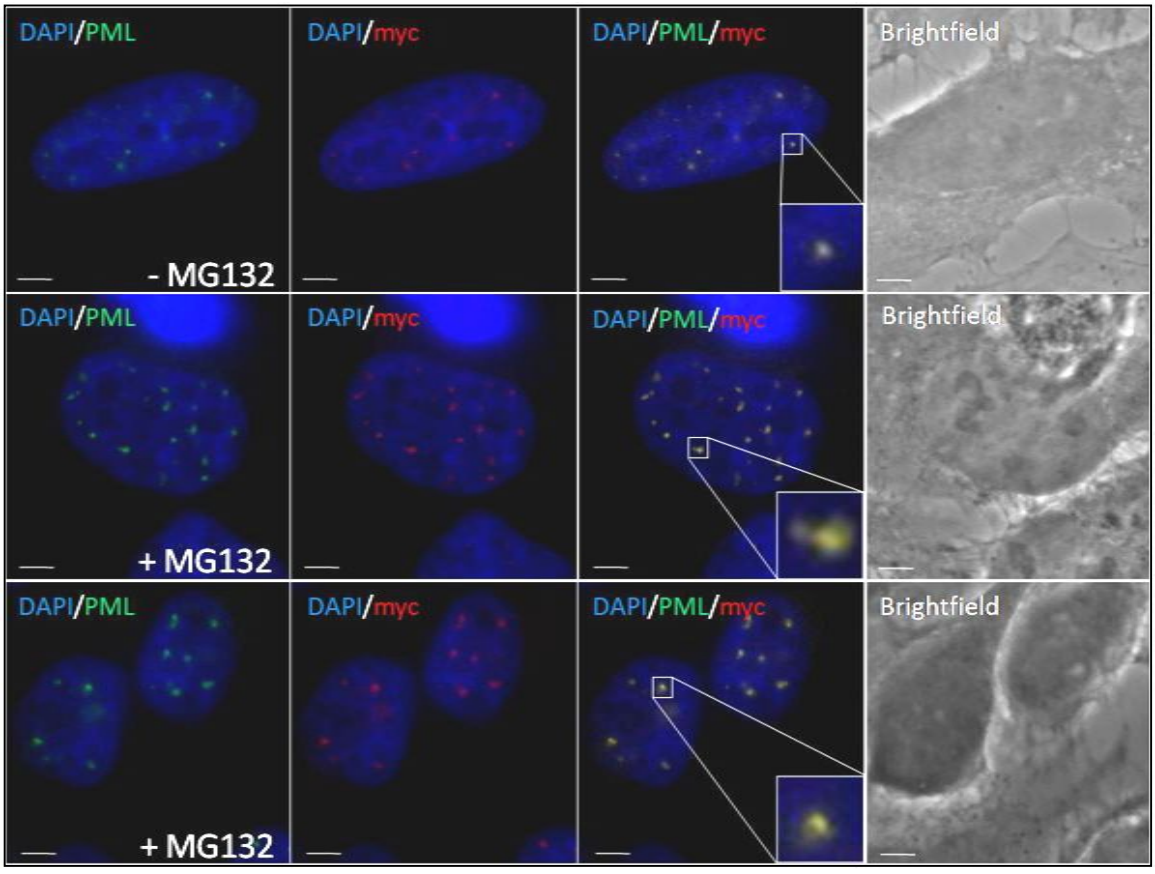


Figure 3.1: SUMO-3 conjugates accumulate at PML-NBs upon proteasome inhibition
Fluorescent microscopy image of HeLa cells transfected with an expression plasmid for mature myc-SUMO-3 40 h before treated with 20 µM MG132 for 7 h. Chromatin is stained with DAPI (blue), PML with the PML antibody 5E10 and the secondary donkey anti-mouse Alexa 488 (green). Myc-SUMO-3 is stained with anti-myc and the secondary donkey anti-rabbit Alexa 546 (red). Bar represents relative units.

The experiment demonstrated that upon proteasome inhibition, all three conjugatable SUMO paralogs accumulated at PML-NBs (data not shown for myc-SUMO-1 and myc-SUMO-2). Note that the general PML-NB size seems to increase upon proteasome inhibition, regardless of the size of the respective nuclei (bigger nucleus in the middle panel, smaller ones in the lower panel compared to the control nucleus without MG132 treatment). Cells transiently transfected with constructs expressing the chain-forming deficient myc-SUMO-2 K11R or myc-SUMO-3 K11R displayed a similar accumulation of SUMO conjugates at PML-NBs and as well an increase in PML-NB size and number (see exemplarily Figure 3.2 for myc-SUMO-3 K11R).

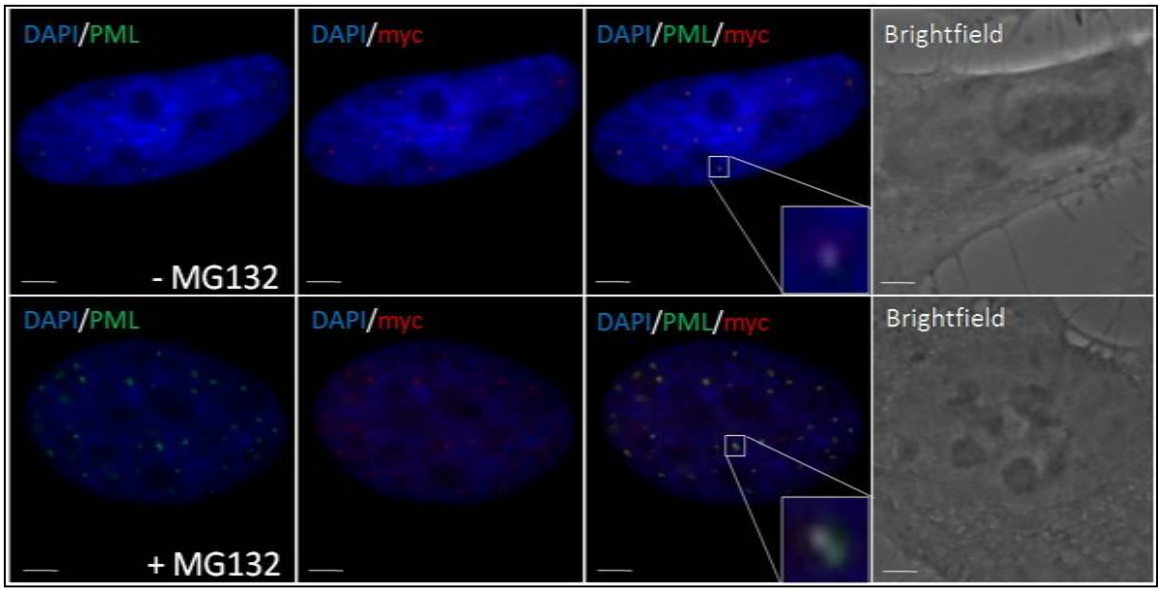


Figure 3.2: SUMO-3 K11R also accumulates at PML-NBs upon proteasome inhibition
Fluorescent microscopy image of HeLa cells transfected with an expression plasmid for mature myc-SUMO-3 K11R 40 h before treated with 20 µM MG132 for 7 h. Immunofluorescent staining was carried out as described in Figure 3.1. Bar represents relative units.

However, cells transiently transfected with the non-conjugatable myc-SUMOΔGG mutants had normal to small PML-NBs but the transfected SUMOΔGG mutants did not localize to them (data not shown). Therefore, the observed accumulation of mature SUMOs at PML-NBs was probably due to the accumulation of SUMOylated substrates. Together with the data from Bailey and O'Hare (2005), who observed the recruitment of SUMO-1 conjugates and moreover of proteasome subunits, it is reasonable to speculate that possible ULS substrates might accumulate at PML-NBs and that possible ULS proteins might be recruited to these structures in human cells.

In order to verify possible substrates and identify mammalian ULS proteins, it was necessary to reconstitute the ubiquitylation of SUMOylated conjugates *in vitro*.
Therefore, the efficient production and purification of SUMOylated proteins as well as the development of an *in vitro* ubiquitylation assay for SUMO conjugates was established and represents the main part of this work (see sections 3.2, 3.3 and 3.4).

3.2 Establishment of an efficient method to produce and purify SUMO-modified proteins from *E. coli*

Generating SUMO-modified proteins requires the enzymatic cascade of E1, E2 and E3 enzymes (as described in section 1.4). This process can be reproduced in *E. coli* (see section 2.2.4.4). First attempts to generate SUMO-modified proteins in *E. coli* have shown that the process is quite inefficient and the majority of the purified substrate is not SUMOylated (Uchimura, 2004a; Uchimura, 2004b; Weißhaar, 2008).

In order to obtain appropriate possible ULS substrates for *in vitro* studies, this part of the work aimed to improve the production and isolation for the SUMO-modified forms of possible substrates (all GST-tagged).

The major advantage established here was to utilize His_6-tagged SUMOs instead of untagged versions. It was therefore possible to purify the SUMO-conjugated forms of a substrate by consecutive affinity purifications (as described in section 2.2.4.4).

To test this method, several known SUMO substrates, e.g. the PML nuclear body proteins PML and Sp100, were subjected to small scale purifications.

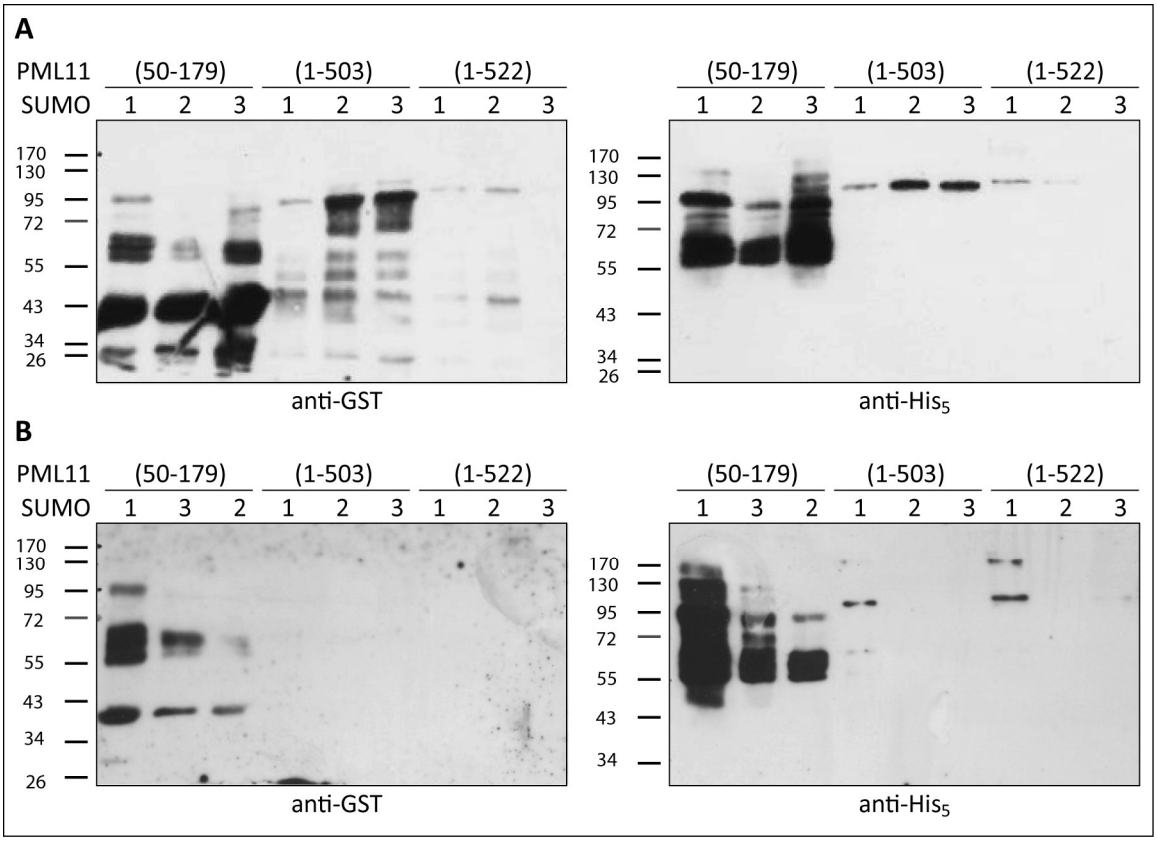


Figure 3.3: Small scale purification of His_6-SUMOylated GST-PML11 truncation variants
Different GST-PML truncation constructs were subjected to *E. coli* SUMOylation and then to subsequent GSH (A) and Ni^{2+}-NTA (B) affinity purification. PIAS4 was coexpressed as SUMO E3 enzyme.
Eluted proteins were separated by SDS-PAGE and detected with anti-GST or anti-His_5, respectively.

Three PML11 constructs were tested: PML11 (50-179), which consists of the RING and B1 domain with the first two SUMOylation sites, PML11 (1-503), which comprises all features common to all nuclear isoforms except of the SIM and PML11 (1-522) which additionally contains the SIM (see Figure 3.3).
E. coli preferentially expressed the smallest construct tested, GST-PML (50-179). Also, the SUMOylation of this protein was more efficient compared to the longer PML variants. Note that after GSH affinity purification most of the expressed GST-PML (50-179) was not modified with SUMO as the band migrating at ~ 43 kDa is only present in the anti-GST Blot (see Figure 3.3 A). SUMOylated forms were enriched upon Ni^{2+}-NTA affinity purification (compare respective ratios of Figure 3.3 A and B).

For Sp100 A, the full length construct (1-480), a truncated version that ends behind the SIM (1-332) and one that lacks the K297 SUMOylation site and the SIM (1-274) were tested (see Figure 3.4).

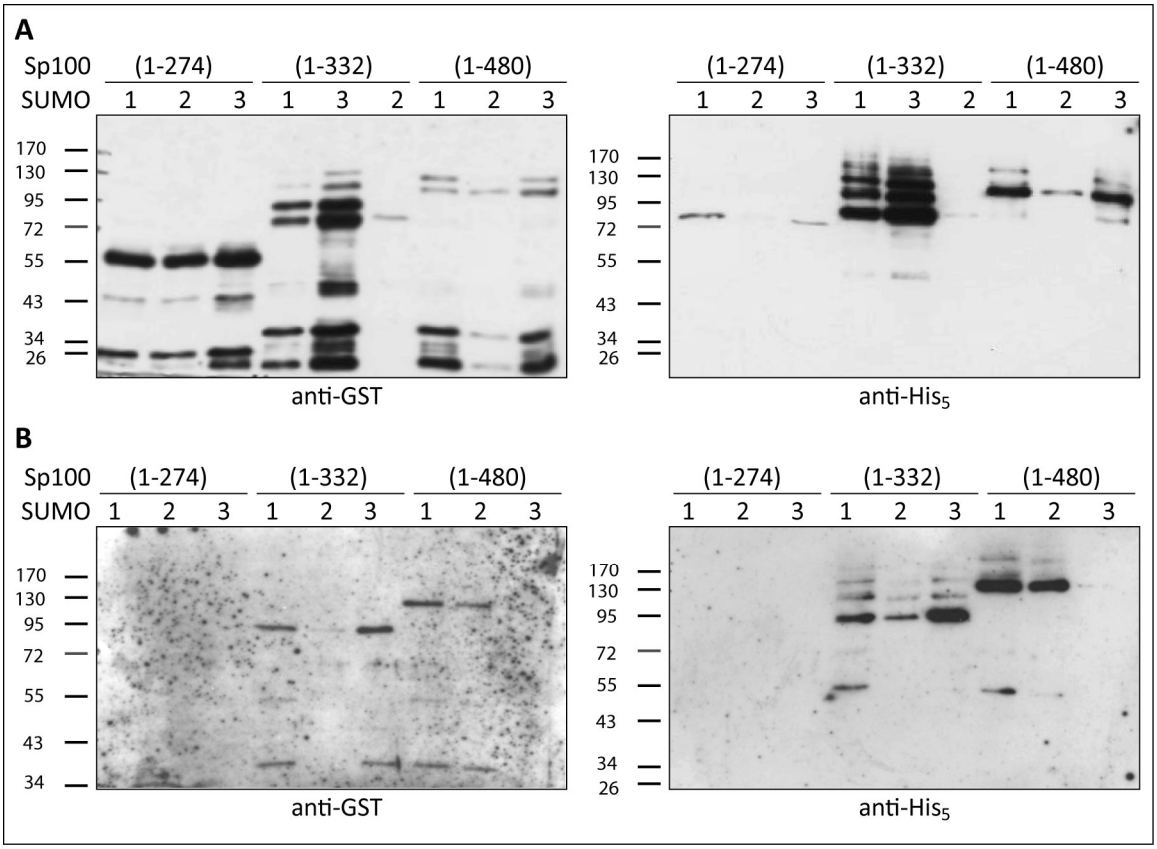


Figure 3.4: Small scale purification of His_6-SUMOylated GST-Sp100 truncation variants
Different GST-Sp100 truncation constructs were subjected to *E. coli* SUMOylation and then to subsequent GSH (A) and Ni^{2+}-NTA (B) affinity purification. PIAS4 was coexpressed as SUMO E3 enzyme.
Eluted proteins were separated by SDS-PAGE and detected with anti-GST or anti-His_5, respectively.

Upon the conditions applied, Sp100 (1-480) was not expressed in large amounts in *E. coli* and its SUMOylation was moderate. The shortest Sp100 construct (1-274) showed only

residual SUMOylation and demonstrated that the lost attachment site K297 is indeed the major one. Sp100 (1-332) is expressed and efficiently well SUMOylated.
Furthermore, although detection was limited with the anti-GST after Ni^{2+}-NTA affinity purification, SUMOylated forms were enriched; there is basically no band visible for unmodified Sp100 (1-332) which runs at ~ 72 kDa (compare protein running behavior between Figure 3.4 A and B).
In comparison to the test SUMOylation of PML constructs, it becomes apparent, that *E. coli* expressing the SUMO-2 system had difficulties not only to SUMOylate the substrates efficiently but in overall substrate expression which was always lower compared to the SUMO-1 and SUMO-3 expressing systems. Therefore, large scale purifications have been only performed for SUMO-1 or SUMO-3 conjugated substrates (see sections 3.2.1 and 3.2.2).

Additionally to PML and Sp100, non-PML-NB proteins, the NFκB essential modulator (NEMO) and the cytoplasmic-nuclear shuttling protein Huntingtin interacting protein 1 alpha (HIP1α) were subjected to small scale *E. coli* SUMOylation for comparison (see Figure 3.5 and Figure 3.6). NEMO has been shown to be specifically SUMO-1 modified by PIAS4 (Huang, 2003; Mabb, 2006). HIP1α displays several possible SUMOylation sites and was tested whether it could be a SUMO substrate in cooperation with Ian Mills (Cambridge).

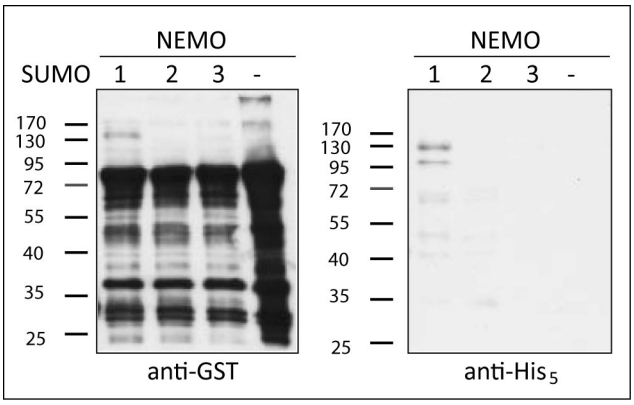


Figure 3.5: Small scale purification of His_6-SUMOylated GST-NEMO
GST-NEMO was subjected to *E. coli* SUMOylation and to subsequent GSH affinity purification. As SUMO E3 enzyme, PIAS4 was coexpressed.
Eluted proteins were separated by SDS-PAGE and detected with anti-GST or anti-His_5, respectively.

NEMO was not efficiently SUMO modified in *E. coli*. The only detectable SUMOylation occurred in the SUMO-1 system and is in line with the before mentioned literature.

For HIP1α, several possible SUMOylation sites exist (see Figure 3.6 A). To assess whether HIP1α is a SUMO substrate, two domains that comprise these sites were expressed separately in the SUMOylation systems (see Figure 3.6 B).
The HIP1α domain (722-974) was efficiently SUMOylated by SUMO-1 and to a lesser extent by SUMO-2 and SUMO-3 in the *E. coli* SUMOylation systems. Most likely, several sites in this domain served as SUMO acceptors. Notably, this substrate was also SUMOylated without the help of a SUMO E3 ligase. The other HIP1α domain (303-727) was probably not SUMOylated although there were slower migrating bands detected

with anti-GST compared to the non-modified form running at ~ 43 kDa. However, as these bands were not detected with the His_5-antibody, it is unlikely that they contain SUMO.

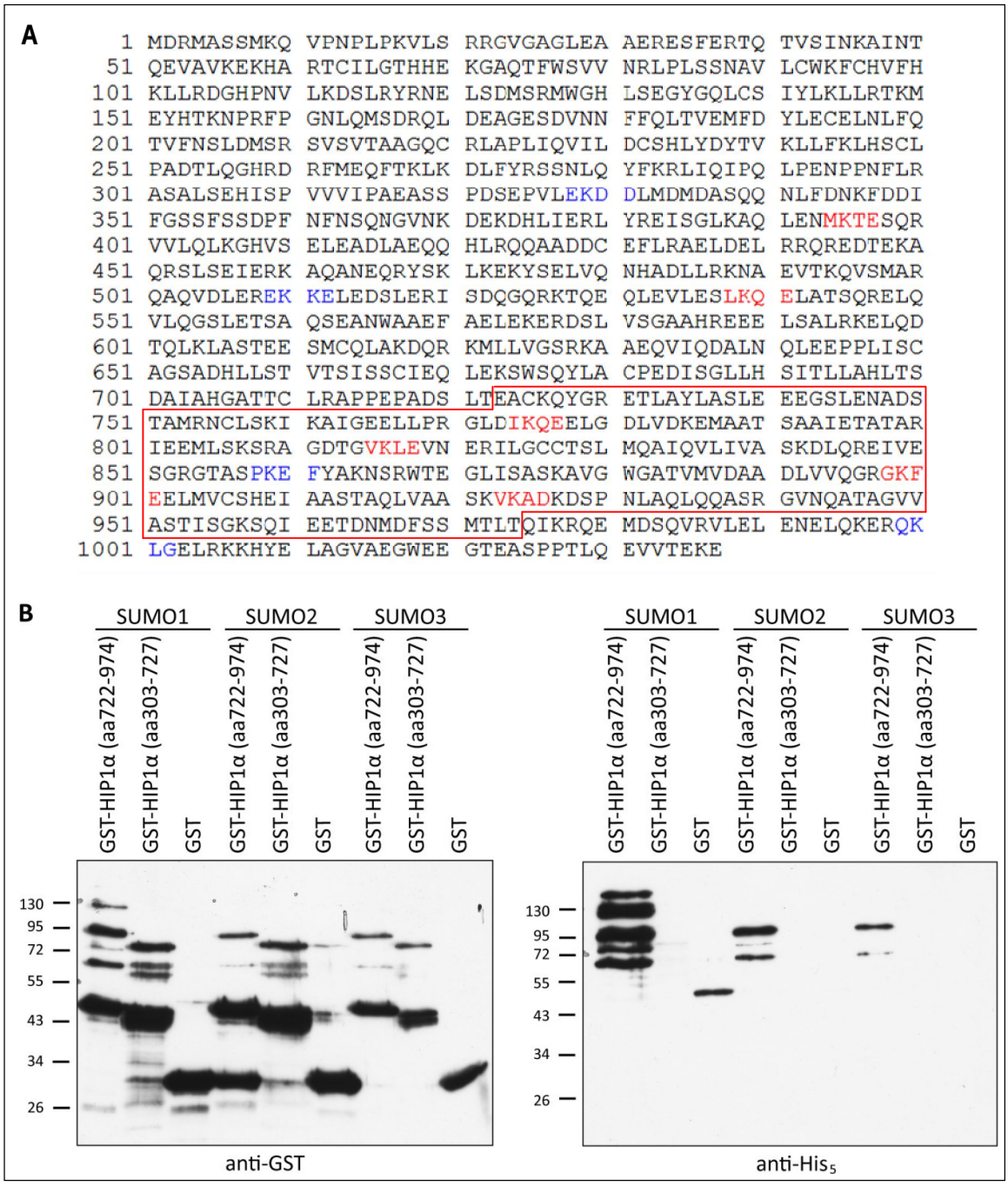


Figure 3.6: HIP1α is a SUMO substrate in the *E. coli* SUMOylation system

A) HIP1α harbors several possible SUMOylation sites. HIP1α sequence was subjected to SUMOplot analysis (Abcam; San Diego, USA), a SUMOylation site prediction program. Motifs marked in red have a high probability to be SUMOylated (> 50 %), motifs marked in blue a lower one (< 50 %). The domain which is predominantly SUMOylated in *E. coli* (refer to point B) is boxed in red.

B) Small scale purification of His_6-SUMOylated GST-HIP1α domains. GST-HIP1α domains (303-727) and (722-974) were subjected to *E. coli* SUMOylation without an additional E3 ligase and to subsequent GSH affinity purification. Eluted proteins were separated by SDS-PAGE detected with anti-GST or anti-His_5, respectively.

Together, these data demonstrated that the *E. coli* SUMOylation systems are functional tools for a range of substrates. Furthermore, it seems that substrate and SUMO variant specificity are maintained (compare SUMOylation of PML and Sp100 to NEMO and HIP1α). SUMOylation of the respective substrates likely occurs with some specificity at previously identified sites (e.g. Sp100 K297) or predictable sites (compare HIP1α domains).
The efficiently SUMOylated GST-fusions of PML (50-179), Sp100 (1-332) and HIP1α (722-974) have been subsequently purified in large scale. Purified proteins either contained the GST tag or it was cleaved off during purification.

3.2.1 PML & Sp100

The purification of SUMOylated PML (50-179) and Sp100 (1-332) has been established during this work and is described in section 2.2.4.4. As the method is the same for both proteins, the single steps are illustrated only for SUMO-3 modified PML (50-179) in Figure 3.7.
During purification, the proportion of SUMOylated forms of the substrate is massively enriched compared to the non-modified form; GST-PML (50-179) migrates at ~ 38 kDa, SUMOylated forms above. After GSH affinity purification strikingly only a small proportion of the GST-tagged substrate is SUMOylated (see Figure 3.7 A). This proportion is then enriched by Ni^{2+}-NTA affinity purification as it only binds the SUMOylated forms of the substrate (see Figure 3.7 B). PML as well as Sp100 dimerizes and therefore, a smaller proportion of non-conjugated substrate cannot be removed under native conditions and elute in the same fractions from gel filtration (see Figure 3.7 C; untagged PML (50-179) migrates at ~ 15 kDa, SUMOylated forms at ~ 30 kDa or slower).

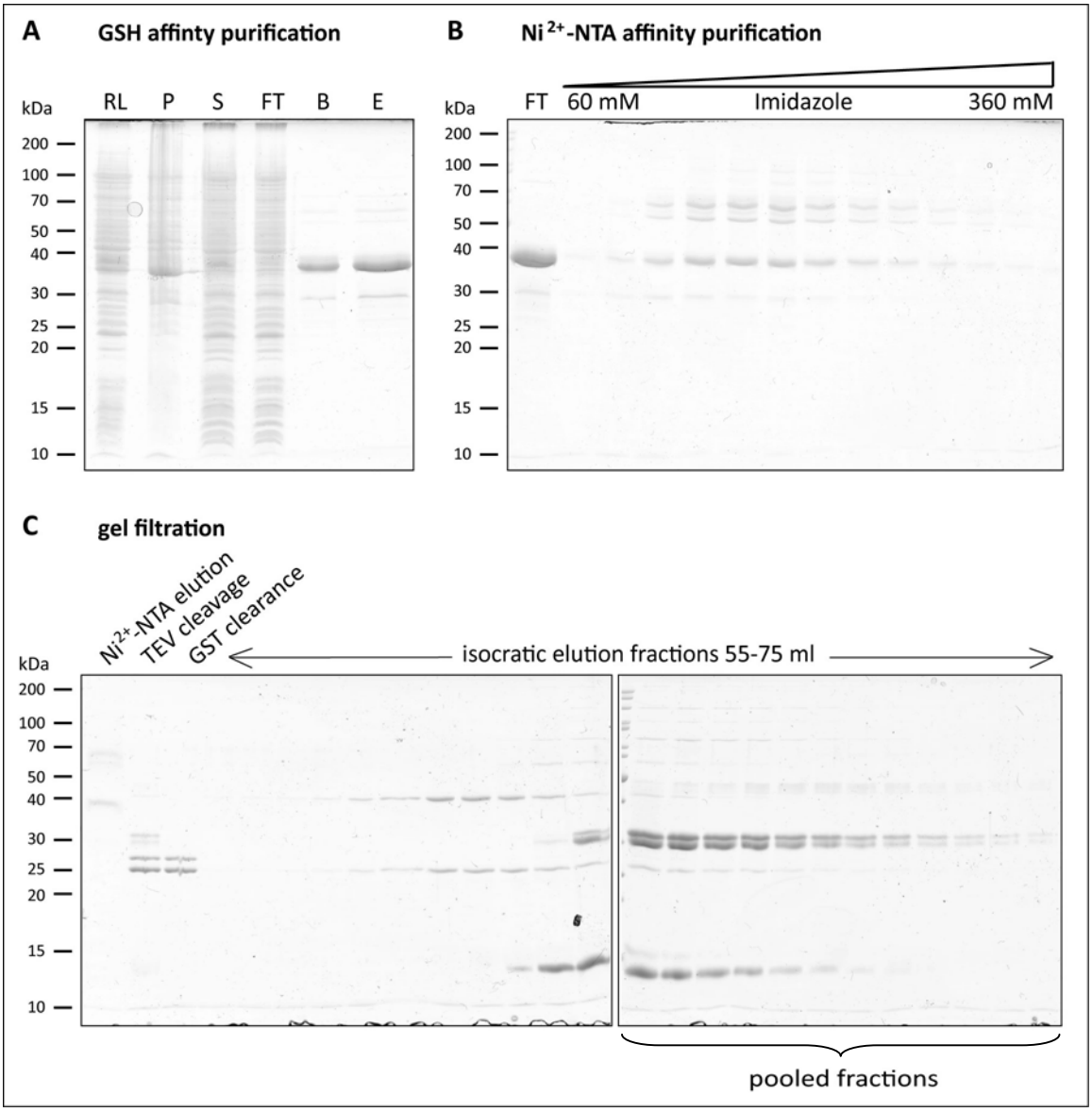


Figure 3.7: Purification steps for SUMOylated proteins
Purification steps are exemplified by showing the Coomassie-stained SDS-PA gels of the SUMO-3 modified PML (50-179) purification.
A) The raw *E. coli* lysate (RL) was cleared by centrifugation and the resulting supernatant (S) was applied on GSH-beads (B). After extensive washing, GST-tagged proteins were eluted with buffer containing GSH (E; P, pellet; FT, flow through).
B) Ni^{2+}-NTA affinity purification of eluted GST-tagged proteins. SUMOylated GST-tagged proteins were bound and eluted at Imidazole concentrations around 200 mM (FT, flow through). Elution fractions were pooled and subjected to o/n TEV cleavage.
C) TEV cleaved SUMOylated proteins (TEV cleavage) were applied on fresh GSH beads (GST clearance) to remove GST or residual GST-tagged proteins from the SUMOylated proteins. These are finally subjected to size exclusion chromatography on a HiLoad 16/60 Superdex 75 column and eluted in the respective fractions.

For *in vitro* studies, PML (50-179) as well as Sp100 (1-332) have been purified either non-conjugated, SUMO-1 modified or SUMO-3 modified. PML K65 and K160 mutants have also been subjected to large scale SUMOylation and purification. Usually, from 10 l expression cultures, 1-10 mg SUMOylated proteins have been obtained per purification. Purification of non-conjugated substrates has been conducted in a similar way, except

that the Ni^{2+}-NTA affinity purification was not necessary. Figure 3.8 summarizes the purified non-conjugated forms and SUMO-3 conjugated forms of the respective proteins and provides an impression of their migration in SDS-PA gels.

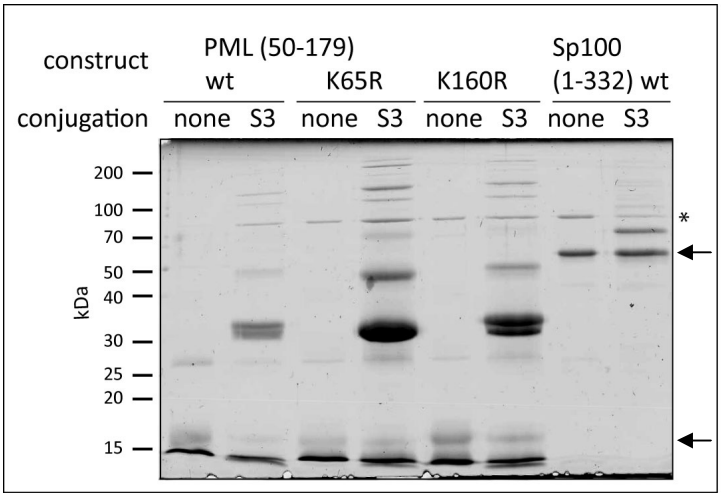


Figure 3.8: Migration of SUMOylated and non-conjugated proteins purified in large scale
Purified proteins were separated by SDS-PAGE and stained with Coomassie. Arrows indicate non-conjugated PML (50-179) or Sp100 (1-332), respectively. SUMOylated forms migrate at different sizes and likely represent either different SUMO attachment sites and/or SUMO chains (S3, SUMO-3). The asterisk marks a contaminating protein, meanwhile identified as bacterial DnaK (see section 2.2.4.6).

3.2.2 HIP1α

The purification of SUMO-1 modified HIP1α (722-974) basically followed the same protocol as described above for PML (50-179). Further studies have been performed by Ian Mills group in Cambridge. Figure 3.9 shows the purified non-conjugated and SUMOylated domain in comparison.

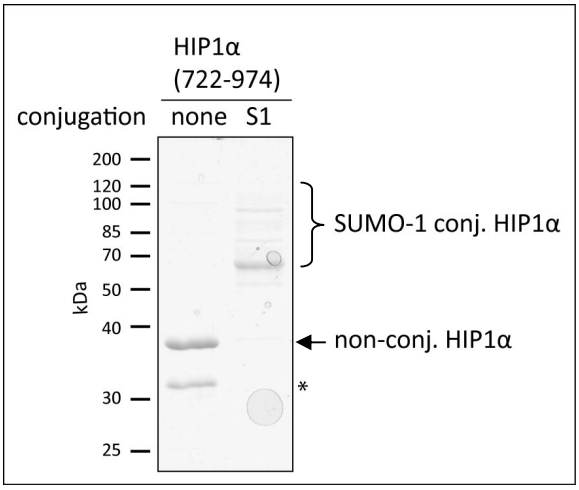


Figure 3.9: Comparison of purified HIP1α (722-974) non-conjugated and SUMO-1 modified.
This HIP1α domain is efficiently SUMO-1 (S1) modified when subjected to the *E. coli* SUMOylation system and SUMO-1 modified HIP1α (722-974) could be purified in large scale. Shown is a Coomassie stain after separating the purified proteins by SDS-PAGE. It is depicted here as an example of how efficient the SUMOylated forms of a protein can be separated by the consecutive affinity purifications applied. As this domain probably does not dimerize, the non-modified substrate has been completely removed from the purified SUMO-1 conjugated forms. The asterisk marks a prominent degradation band.

3.2.3 Analysis of different SUMO-E3 ligases on substrate SUMOylation

To improve the SUMOylation efficiency of PML (50-179) and Sp100 (1-332) the influence of several SUMO E3 ligases has been tested (see Figure 3.10 and 3.11).
All four tested SUMO E3 ligases promoted SUMO-1 modification of Sp100 (1-332) in comparable amounts (see Figure 3.10).

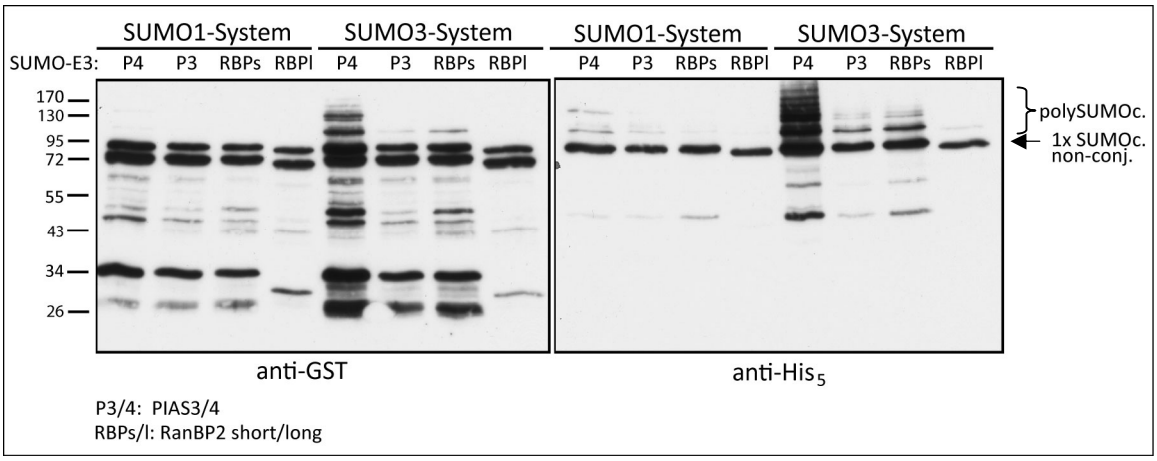


Figure 3.10: Impact of different SUMO E3 ligases on Sp100 (1-332) SUMOylation efficiency
GST-Sp100 (1-332) was subjected to *E. coli* SUMOylation with coexpression of different E3 enzymes (RanBP2short, aa 2633-2711; RanBP2long, aa 2553-2711) and subsequent GSH affinity purification. Proteins were separated by SDS-PAGE and detected with anti-GST or anti-His_5, respectively. SUMO conjugates (SUMOc.) are indicated.

For SUMO-3, only PIAS4 seems to generate poly-SUMO chains efficiently on the substrate (note the typical ladder in the respective lane). PIAS3 and the short RanBP2 promoted chain formation to a minor extend whereas it is nearly completely abrogated with the long RanBP2 form which only allows mono-SUMOylation of Sp100 (1-332). Sp100 (1-332) alone was also efficiently SUMOylated in *E. coli* (data not shown).

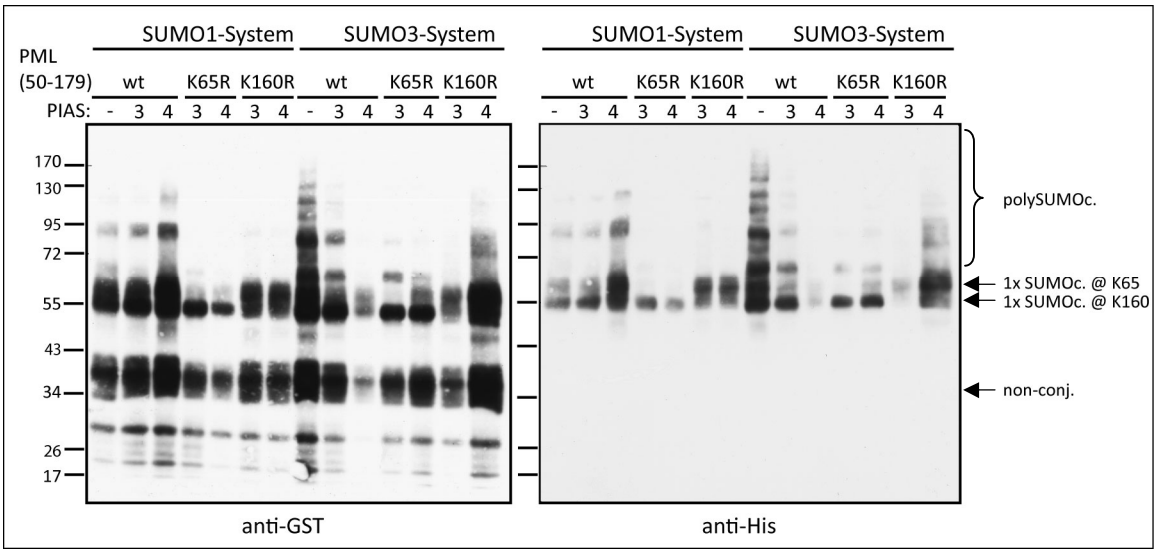


Figure 3.11: Impact of different SUMO E3 ligases on PML (50-179) SUMOylation efficiency
GST-PML (50-179) and its K→R mutants were subjected to *E. coli* SUMOylation with coexpression of PIAS3 or PIAS4 and subsequent GSH affinity purification. Proteins were separated by SDS-PAGE and detected with anti-GST or anti-His_5, respectively. SUMO conjugated forms (SUMOc.) are indicated at the right.

SUMO-1 modification of PML (50-179) was most efficient with PIAS4 coexpression. PML (50-179) SUMOylation also occurred without the coexpression of an additional E3 ligase in *E. coli*. In the case of SUMO-3 modification, it was even the most efficient strategy to form polymeric chains and indicated a SUMO E3 ligase activity for PML as neither PIAS3

nor PIAS4 were as efficiently SUMOylating PML (50-179). By using lysine mutants of PML (50-179), differences in migration through SDS-PAGE could be observed for the SUMO conjugated forms depending on the SUMO attachment site. SUMO attached to K160 resulted in protein species migrating faster than the ones where it is attached to K65 (compare running behavior of mono-SUMOylated PML (50-179) K65R and K160R mutants as indicated by arrows in Figure 3.11). A reduction of self-SUMOylation efficiency was not observed in PML (50-179) lysine mutants (data not shown).

Together, particularly the SUMO-3 conjugation was strongly influenced by the choice of the coexpressed SUMO E3 ligase and differed between substrates. In light of the following data shown, SUMO-3 modified PML (50-179) was purified several times, either when coexpressed with PIAS4 or without the coexpression of an additional ligase. SUMO-1 modified PML (50-179) was purified after coexression with PIAS4. SUMOylated Sp100 was generated by coexpression with PIAS4 and subsequent purification.

3.3 Establishment of an *in vitro* ubiquitylation assay

In vitro ubiquitylation requires purified active ubiquitin E1, E2 and E3 enzymes as well as a substrate and ubiquitin itself. For the special case of SUMO-dependent ubiquitylation, the substrate has to be SUMOylated. Furthermore, the appropriate E3 enzyme that recognizes SUMOylated proteins needs to be identified and then tested for its ability to act as a ULS (see section 3.3.2 and following ones).

3.3.1 E2 purification & activity test

To establish SUMO-dependent ubiquitylation *in vitro*, the appropriate E2 enzyme had to be purified. Yeast ULS proteins use the E2 members Ubc4 and Ubc5 that are homologous to the human UbcH5 family (Uzunova, 2007). Together with the fact that these proteins consists simply of the 'E2 core' and therefore accept a huge variety of ubiquitin E3 ligases (Ye and Rape, 2009), it was tempting to speculate that they might also act in conjunction with possible human ULS proteins. Therefore, UbcH5b was chosen and purified (as described in section 2.2.4.3.4).
Enzymatic activity was analyzed by thioester formation to purified ubiquitin (see Figure 3.12). Thioesters between purified untagged UbcH5b (~ 16 kDa) and ubiquitin (~ 8 kDa) have been formed in comparable efficiency to the purchased His_6-tagged UbcH5b (Biomol, Hamburg). Note that the thioester between the tagged UbcH5b and ubiquitin migrated slower than the one between the self-made untagged UbcH5b and ubiquitin. The use of untagged (and not His_6-tagged) UbcH5b for *in vitro* ubiquitylation studies avoided cross detection as the SUMOs in the SUMOylated putative ULS substrates were His_6-tagged and were supposed to be detected with anti-His_5.

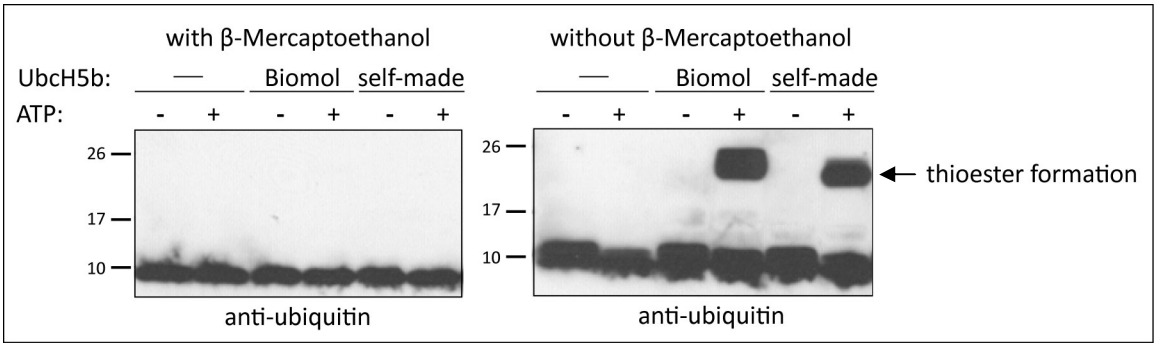


Figure 3.12: UbcH5b activity test
Activity of self-made UbcH5b was tested in comparison to the commercial available His_6-tagged UbcH5b (Biomol, Hamburg) by assessing thioester formation with ubiquitin. 0,2 µg/µl Ube1, 1 µg/µl UbcH5b and 3 µg/µl ubiquitin were incubated at RT for 5 min in 50 mM Tris pH 7.5, 50 mM NaCl and 10 mM $MgCl_2$ +/- 5 mM ATP. Reactions were divided and terminated by adding 2x SDS sample buffer either containing the reducing agent β-Mercaptoethanol or not. Proteins were separated by SDS-PAGE and detected with anti-ubiquitin.

3.3.2 Identification of putative ULS proteins for PML

As SUMOylated substrates accumulate predominantly at PML-NBs (see section 3.1), putative human ULS proteins could also localize to these structures. The nuclear protein database (http://npd.hgu.mrc.ac.uk/user/) provided a list of 76 proteins that had been localized to PML-NBs until 2007 (start of search).

Out of these, several candidate ULS proteins have been identified by complying with the two requirements for putative ULS proteins: domains that might confer ubiquitin ligase activity (such as HECT, RING or U-Box) and putative SIMs for substrate recognition.

PML itself has a RING domain and a SIM and although it has been published to promote SUMOylation in *S. cerevisiae* (Quimby, 2006), it was tested here for ubiquitin ligase activity. Also, during the initial phase of this work PML was not known to be a ULS target.

Furthermore, two other TRIM proteins with ubiquitin ligase activity, TRIM23/ARD1 (Vichi, 2005) and TRIM24/TIF1α (Allton, 2009) harbour putative SIMs. Additionally, the zinc finger protein ZNF198 and the RING finger protein RNF4 have been found to meet the defined requirements. ZNF198 can be SUMOylated, possesses at least two SIMs and localizes to PML-NBs (Hecker, 2006; Kunapuli, 2006), but a ubiquitin ligase activity has not been identified yet. RNF4 is a homologue to the *S. pombe* ULS proteins Rfp1 and Rfp2 and has been introduced in section 1.5.3. Figure 3.13 gives an overview of the candidate ULS proteins.

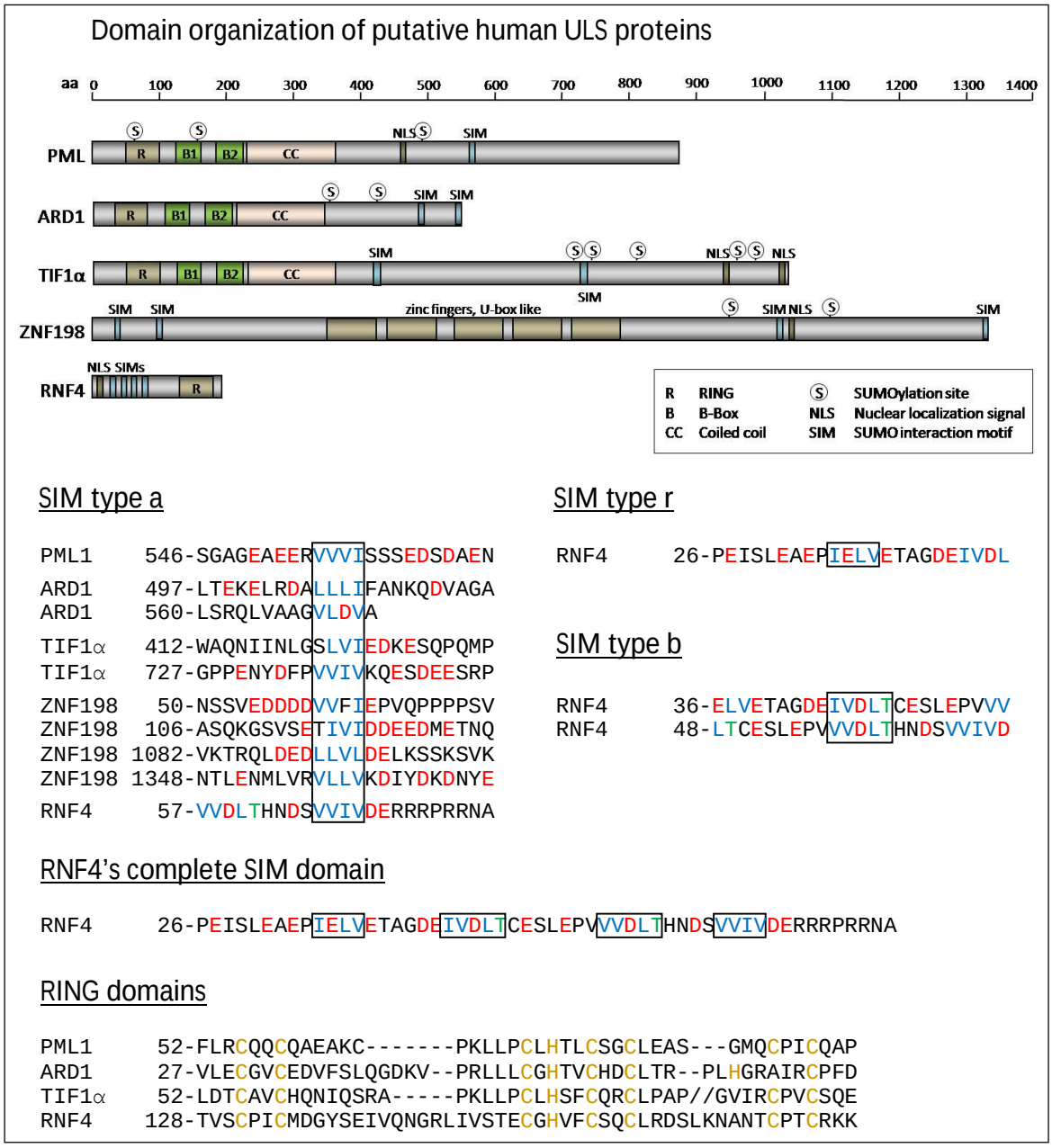


Figure 3.13: Domain organization and sequence homology of putative human ULS proteins
All identified possible human ULS proteins possess at least one SIM and a RING/U-Box like domain (see scheme). Diverse additional domains of ARD1, TIF1α and ZNF198 are not depicted here to focus on the requirements for ULS proteins. SIM types and sequences are given as well as the RING sequences (the RING of TIF1α has an extension compared to the others which is not depicted here due to space problems and is indicated by //). The zinc fingers of ZNF198 resemble in part a U-Box and are depicted in Appendix Figure A.1. SIM relevant hydrophobic amino acids are depicted in blue, acidic amino acids in red and threonine residues in green. The SIM cores are boxed. Zinc chelating residues in the RING domains are depicted in gold.

In light of the homology of RNF4 to the *S. pombe* ULS proteins Rfp1/Rfp2 and its ability to complement for yeast ULS deletion phenotypes (see section 1.5.3), made it the most promising among the candidates. Moreover, its domain organization shows that it more or less consists of a SIM 'domain with up to four SIMs, the NLS and the RING domain.

3.3.3 Activity test for putative ULS proteins

The possible human ULS proteins were cloned and expressed as GST fusion proteins in *E. coli* with the help of Anke Krause. Expression and purification of ARD1 was not successful and it was therefore not further analyzed.

To assess their potential as ubiquitin ligases, the candidate ULS proteins were subjected to a typical E3 assay in low salt conditions (2 mM $MgCl_2$; Joazeiro, 1999) in order to promote 'self-ubiquitylation' (which results in most cases in formation of ubiquitin chains, either free, or on E2 or on the respective E3 enzyme; see Figure 3.14).

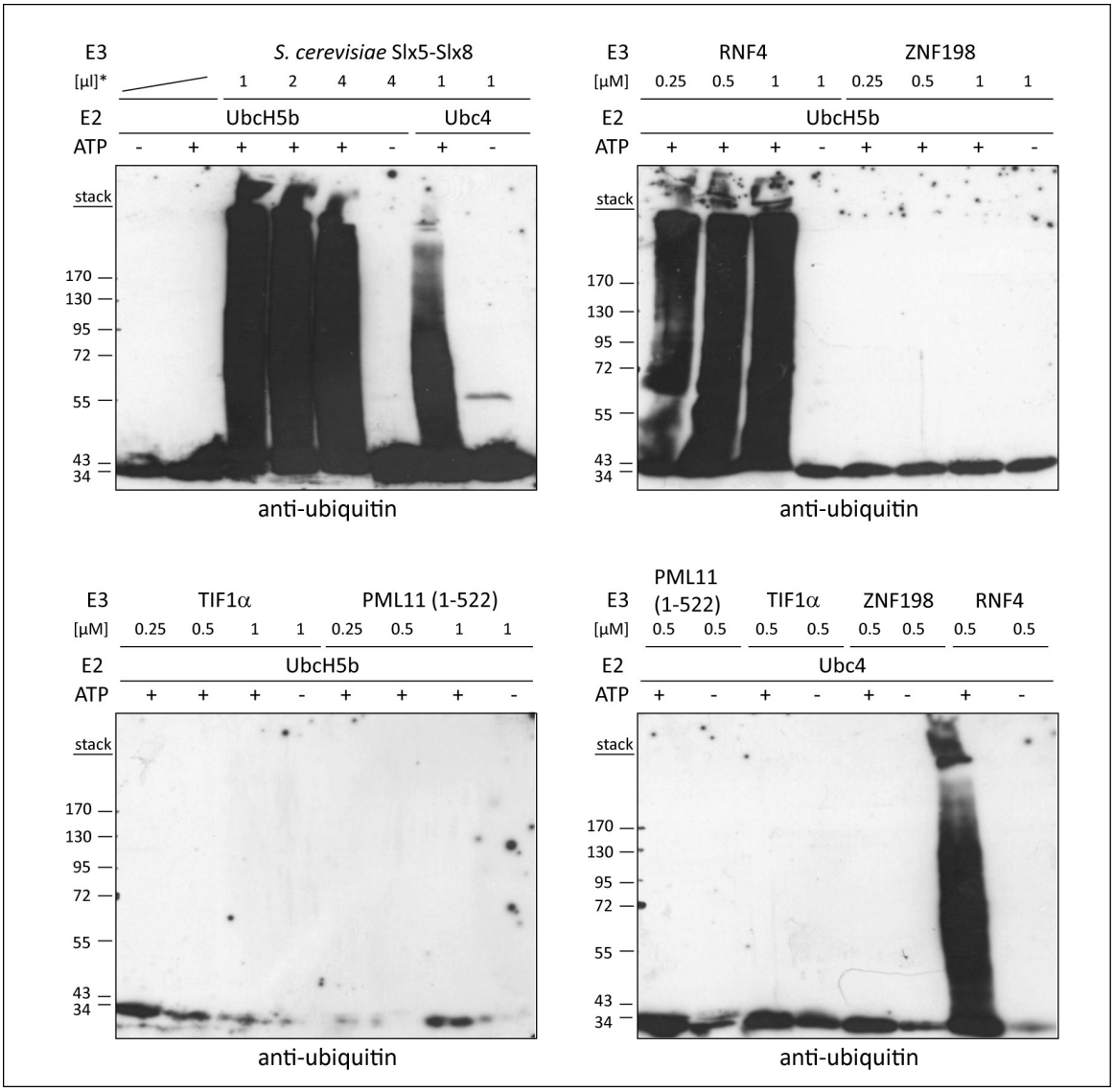


Figure 3.14: Ubiquitin ligase activity of potential human ULS proteins
Potential ULS proteins were analyzed for their self-ubiquitylation activity in comparison to the known *S. cerevisiae* ULS proteins Slx5-Slx8 (a kind gift of Maria Miteva and Jürgen Dohmen, Cologne; the asterisk indicates that the protein concentration is not further determined). Reactions have been carried out for 7 h at 30 °C using 7.5 nM Ube1, 150 nM UbcH5b or *S. cerevisiae* Ubc4, 5 µM ubiquitin and 0.25 µM, 0.5 µM or 1 µM of the ULS candidates in 50 mM Tris pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 10 U creatine phosphokinase, 1 mM creatine phosphate +/- 1 mM ATP. The reactions were terminated by adding 2 x SDS sample buffer. Proteins were separated by SDS-PAGE and detected with anti-ubiquitin.

Except for the active Slx5-Slx8 complex (analyzed as well for the purpose of comparison), only RNF4 exhibited ubiquitylation activity in this assay which was demonstrated by the increase of ubiquitin conjugates with increasing RNF4 concentrations and its strict ATP dependence (compare lanes 1-4 of the blot at upper right). Furthermore, both the *S. cerevisiae* Slx5-Slx8 complex and RNF4 could promote ubiquitylation in conjunction with either UbcH5b or yeast Ubc4 which underlines the conservation of RING-E2 interfaces across species borders.
As RNF4 seemed to fulfill all set requirements and displayed ubiquitylation activity, it was next tested for its ability to act as a ULS.

3.4 *In vitro* ubiquitylation of SUMOylated PML demonstrates a function of RNF4 as a mammalian ULS

3.4.1 ULS assay development

The first substrate chosen for assessing RNF4's capability as ULS enzyme was the in *E. coli* His_6-SUMOylated GST-PML (50-179) introduced in section 3.2. It has been shown previously that RNF4 physically interacts with PML in a SUMO-dependent manner (Häkli, 2005) and that SUMOylated PML species are stabilized during proteasome inhibition (Lallemand-Breitenbach, 2001).
First, GST-tagged PML (50-179) or its SUMOylated forms were subjected to RNF4-mediated ubiquitylation reactions (see Figure 3.15 A). The anti-GST blot suggested that higher SUMOylated species decrease when RNF4 is present. Unfortunately, this also happened without the addition of ATP, suggesting that some residual ATP or ADP might have been present in the purified protein fractions and was recycled with the help of the added ATP regenerating system (creatine phosphokinase and creatine phosphate). Nonetheless, RNF4 had some influence to the higher SUMOylated species of GST-PML (50-179) running at ~ 55 kDa that vanish (marked with a red circle), though slower migrating species could not be detected with anti-GST (no additional bands above 55 kDa). The appearance of such species in presence of RNF4 and ATP would strongly suggest an additional modification of the respective substrate with ubiquitin. This could be due to a detection problem: when the substrate is modified by both, ubiquitin and SUMO, it simply could disturb the antibody-antigen binding on the membrane. The same problem arose when the blot was subjected to immunodetection with anti-His_5 to detect the SUMOylated species (data not shown). By testing several variants, the first time that additional bands appeared in presence of RNF4 and ATP was an assay with PML (50-179) or its SUMOylated variants where the GST-tag was cleaved off. Instead, GST-tagged RNF4 was used in that reaction and substrates were detected with anti-His_5 (see Figure 3.15 B).

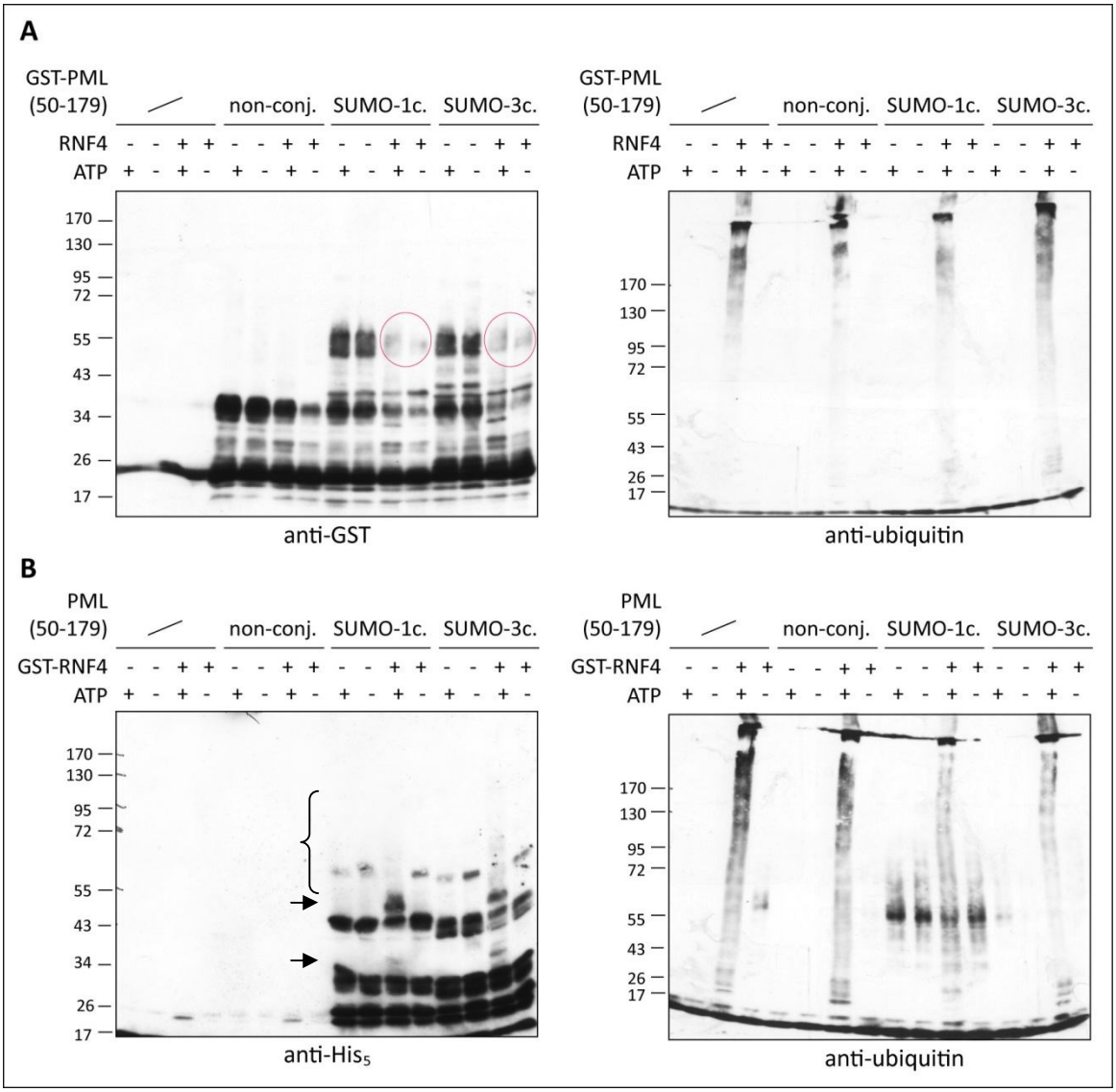


Figure 3.15: RNF4 dependent ubiquitylation of SUMOylated PML (50-179) *in vitro*
A) GST-tagged PML (50-179) or its His_6-SUMO conjugated forms (SUMO-1c. or SUMO-3c.) were subjected to a ubiquitylation reaction containing RNF4 or not. Reactions have been carried out for 16 h o/n at 30 °C in 50 mM Tris pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 10 U creatine phosphokinase, 1 mM creatine phosphate and protease inhibitors +/- 5 mM ATP containing 3 ng/µl Ube1, 3 ng/µl UbcH5b, 300 ng/µl ubiquitin, 100 ng/µl GST-PML (50-179) or its SUMOylated forms +/- 30 ng/µl RNF4. Reactions were terminated by adding 2 x SDS sample buffer. Proteins were separated by SDS-PAGE and detected with anti-GST or anti-ubiquitin. B) Untagged PML (50-179) or its His_6-SUMOylated forms (SUMO-1c. or SUMO-3c.) were subjected to a similar reaction as in A, containing each 100 ng/µl substrate and 30 ng/µl GST-RNF4. All other parameters were unchanged. Detection was carried out using antibodies recognizing His_5 or ubiquitin. The arrow indicates a probably mono-ubiquitylated form of SUMOylated PML (50-179), whereas the brace indicates a smear which might represent poly-ubiquitylated substrates.

The SUMOylated forms of PML (50-179) migrating at ~ 43 kDa seemed to decrease and instead, an additional band (indicated by an arrow) appeared slightly higher together with some faint smear above it (indicated by a brace). Assuming that these bands are ubiquitylated forms of SUMOylated PML (50-179) – either mono-ubiquitylated (arrow) or poly-ubiquitylated (brace), this strongly suggests that RNF4 is indeed a ULS protein, predominantly recognizing and ubiquitylating the slower migrating, higher SUMOylated

species of PML (50-179). Both SUMO-1 and SUMO-3 modified PML (50-179) served as substrate for ubiquitylation through RNF4. Of course, with the His_5-antibody, the non-conjugated PML (50-179) control could not be detected, so that a simple recognition of RNF4 and PML (50-179) with subsequent PML ubiquitylation could not be ruled out. An antibody efficiently recognizing PML itself in Western blots was not available at that time.
Also, any conclusions could be drawn from the anti-ubiquitin blot because 'self-ubiquitylation' was detected in all lanes where RNF4 and ATP had been added to the reaction. This uncontrolled formation of ubiquitin chains was so strong that there weren't any detectable changes between the provided PML substrates (Figure 3.15, left).

To increase specificity towards the SUMOylated substrate, several steps have been taken to render the reaction more stringent.
First, untagged RNF4 was purified again in a higher purity than the old batch which greatly reduced unspecific ubiquitylation (see protocol in section 2.2.4.3.1).
Second, the salt concentration was elevated; as unspecific ubiquitylation occurs in low salt conditions (see ubiquitin E3 activity test and Figure 3.16). Out of 33 mM, 50 mM, 67 mM and 100 mM KCl, ubiquitylation specificity was achieved at 67 mM KCl while keeping an adequate efficiency which was lost at higher salt concentrations (data are not shown for 33 mM, 50 mM and 100 mM KCl).

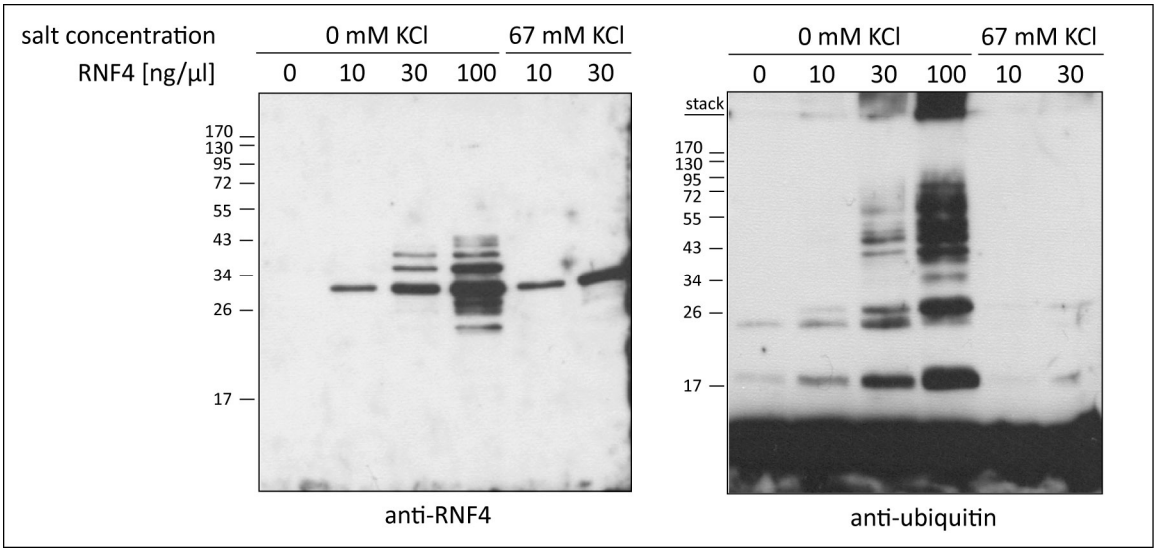


Figure 3.16: Comparison of RNF4-mediated (self-) ubiquitylation in dependence of salt concentration
Exemplarily, low salt conditions containing only 2 mM $MgCl_2$ and no additional KCl (0 mM KCl) are compared to the chosen assay conditions containing 67 mM KCl. Different RNF4 concentrations have been added as indicated to ubiquitylation reactions containing 3 ng/µl Ube1, 3 ng/µl UbcH5b and 300 ng/µl ubiquitin in 50 mM Tris, 2 mM $MgCl_2$, 1 mM DTT, protease inhibitors and 5 mM ATP +/- 67 mM KCl. Reactions were incubated at 30 °C and terminated after 7 h by adding 2 x SDS sample buffer. Proteins were separated by SDS-PAGE and detected with anti-RNF4 or anti-ubiquitin. At the RNF4 concentration used for substrate ubiquitylation (30 ng/µl), uncontrolled ubiquitylation was prevented in conditions with 67 mM KCl.

Third, in order to block unspecific activity, addition of bovine serum albumin (BSA) to the assay was tested and found to further increase specificity without changing the efficiency of ubiquitylation towards the SUMOylated PML (50-179).
Together, these improvements made the reaction specific towards the SUMOylated PML (50-179) (compare ubiquitin blots in Figure 3.17 and see Figure 3.19).

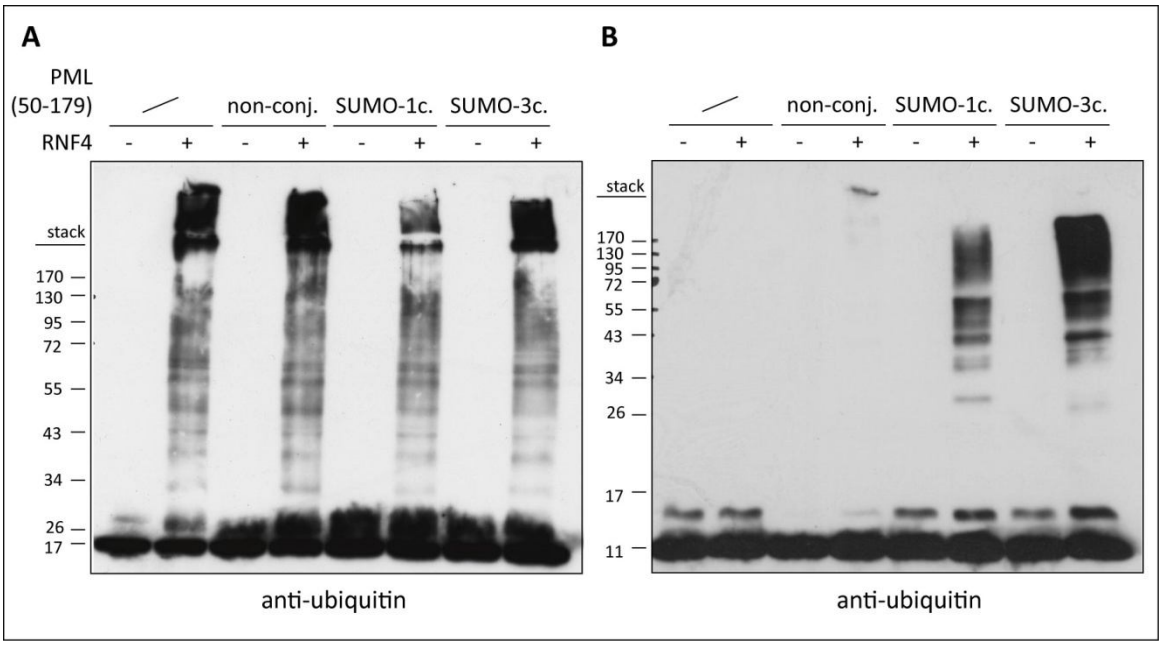


Figure 3.17: Improvement of assay conditions results in RNF4 specificity towards SUMO conjugates
100 ng/µl PML (50-179) or its His_6-SUMOylated forms (SUMO-1c. or SUMO-3c.) were subjected to *in vitro* ubiquitylation by RNF4 in 50 mM Tris pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, protease inhibitors and 5 mM ATP containing 3 ng/µl Ube1, 3 ng/µl UbcH5b, 300 ng/µl ubiquitin +/- 30 ng/µl RNF4 (A) or in 40 mM Tris pH 7.5, 67 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 200 ng/µl BSA, protease inhibitors and 5 mM ATP containing 3 ng/µl Ube1, 3 ng/µl UbcH5b, 300 ng/µl ubiquitin +/- 30 ng/µl RNF4 (B). Reactions were terminated by adding 2 x SDS sample buffer. Proteins have been separated by SDS-PAGE and detected with anti-ubiquitin.

Furthermore, under the chosen conditions, ULS activity of RNF4 seemed to be restricted to appropriate SUMOylated substrates: Ubiquitylation of a GST-SUMO fusion which occured under low salt conditions (Sun, 2007) was prevented (see Figure 3.18). Note that also GST alone was slightly ubiquitylated under low salt conditions.

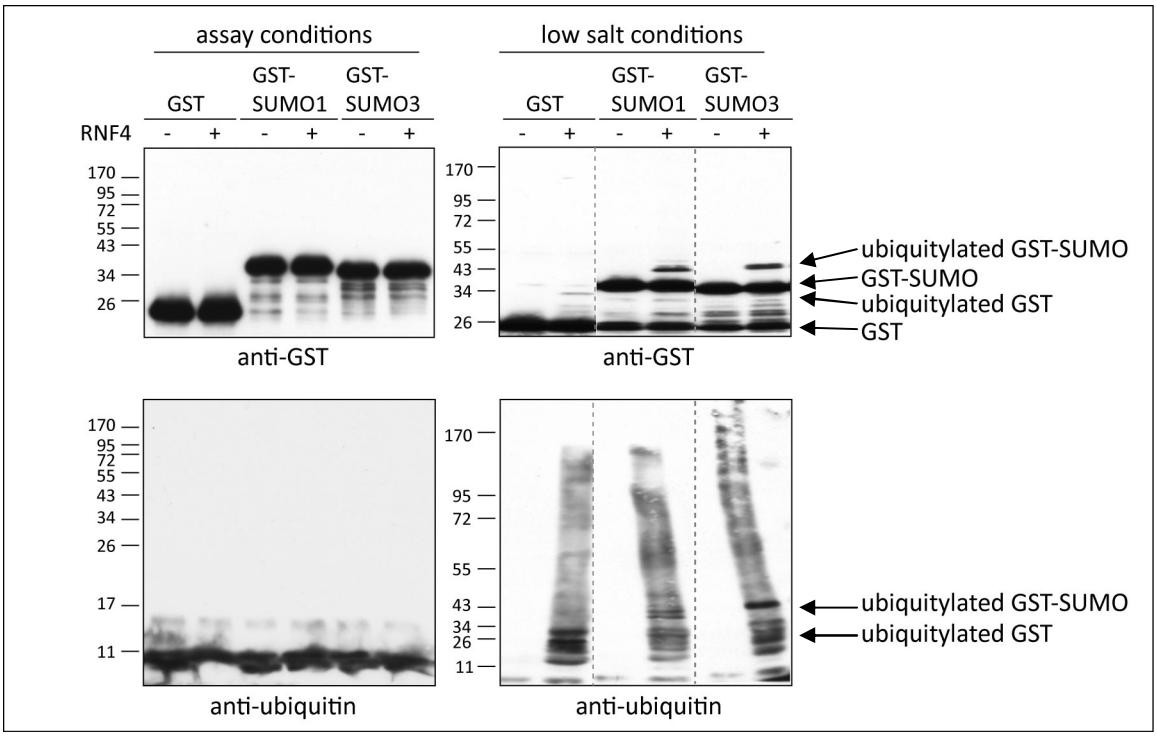


Figure 3.18: RNF4 *in vitro* ubiquitylates GST-SUMO fusion proteins only under low salt conditions
GST, GST-SUMO-1 or GST-SUMO3 linear fusions (each 100 ng/µl) were subjected to *in vitro* ubiquitylation by RNF4 either under low salt conditions as described in Figure 3.17 A or in the advanced assay conditions described in Figure 3.17 B. Proteins were separated by SDS-PAGE and detected with anti-GST or anti-ubiquitin. Ubiquitylated species are indicated.

3.4.2 RNF4 is a ULS and prefers SUMO3-modified PML as substrate

By subjecting PML (50-179) or its SUMO-conjugated forms to the more stringent *in vitro* conditions, it became apparent, that RNF4 indeed acts as a ubiquitin ligase for SUMO conjugates: Figure 3.19 A shows the RNF4-dependent ubiquitylation of SUMO-modified PML *in vitro* analyzed by SDS-PAGE and subsequent detection by anti-His_5 and anti-ubiquitin after Western blotting. Since unspecific ubiquitylation was prevented, the intensity of ubiquitin conjugates revealed a RNF4-dependent ubiquitylation of PML (50-179) modified with SUMO-1 and -3 while RNF4 mediated ubiquitylation of unmodified PML (50-179) was negligible.
Additionally, RNF4-dependent ubiquitylation of both types of SUMO-modified PML were detected by anti-His_5. Slower migrating bands are visible for both, His_6-SUMO-1 and His_6-SUMO-3. However, His_6-SUMO-3 modified PML (50-179) was ubiquitylated more efficiently which is also demonstrated by a stronger ubiquitin signal.
Note that SUMO-1 and SUMO-3 modified PML (50-179) forms migrated at comparable heights (input on the right, Figure 3.19 A).
In order to directly show that ubiquitin was attached to His_6-SUMOylated PML (50-179), a Ni^{2+}-NTA affinity isolation was performed after the *in vitro* ubiquitylation reaction (see Figure 3.19 B). His_6-tagged SUMOs were isolated from the reaction mixture under denaturing conditions such that only proteins covalently attached to could be extracted

as well. Detected ubiquitylated species are therefore most likely PML-SUMO-ubiquitin hybrid conjugates. This isolation also underlined the preference of RNF4 towards SUMO-3 modified PML (50-179) already observed in Figure 3.19 A. Although the anti-His_5 blot shows that comparable amounts of SUMO-1 and SUMO-3 conjugated PML (50-179) were isolated from RNF4 containing reactions, there were more ubiquitin conjugated species detected for SUMO-3 modified PML (50-179).

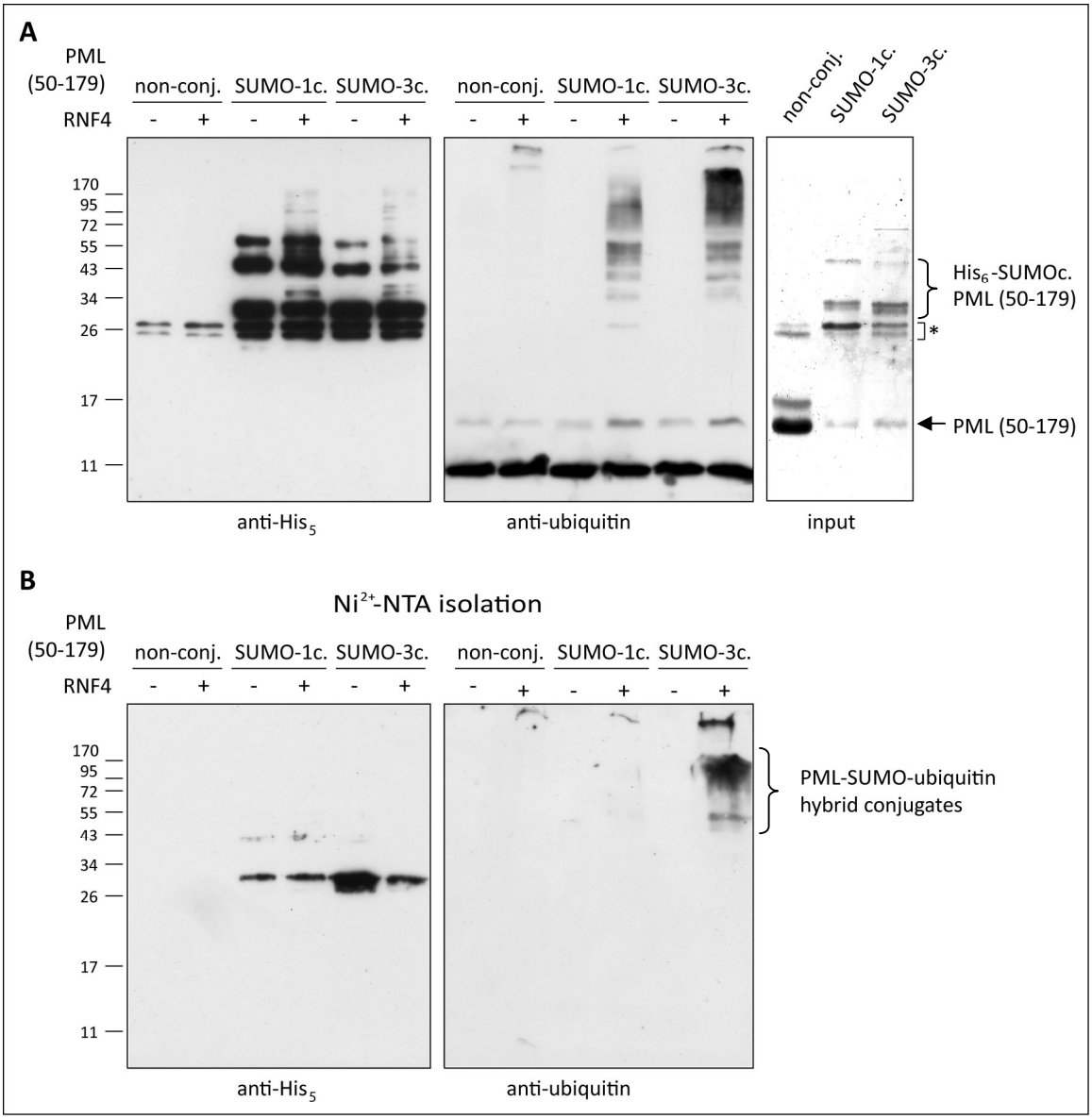


Figure 3.19: SUMO-3-modified PML is the preferential target of RNF4-dependent *in vitro* ubiquitylation
Unmodified (non-conj.) PML (50-179) or its His_6-SUMO conjugated forms (SUMO-1c. or SUMO-3c.) were subjected to *in vitro* ubiquitylation by RNF4 as described in Figure 3.17 B. Reactions were divided and further treated as described in A and B.
A) Reactions were terminated by adding 2 x SDS sample buffer, proteins were separated by SDS-PAGE and detected with anti-His_5 or anti-ubiquitin (left and middle). Input of purified PML (50-179) species is given in the right panel (Coomassie). Residual His_6-tagged TEV protease is marked with an asterisk.
B) Reactions were subjected to denaturing Ni^{2+}-NTA affinity purification as described in 2.2.4.8. Proteins were separated by SDS-PAGE and detected with anti-His_5 or anti-ubiquitin.

Together, these data demonstrated a highly specific ubiquitin ligase activity of RNF4 towards SUMOylated PML, suggesting that RNF4 is indeed a ULS protein. Furthermore, it confirmed with *in vitro* data that PML is a substrate for the ULS pathway in mammalian cells. The following experiments focused on SUMO-3 modified PML (50-179) due to the observed preference of RNF4 towards these forms.

3.4.3 The SUMOylation site of the PML substrate is not important for recognition by RNF4 *in vitro*

To circumvent the detection problems for PML itself, highly purified PML (50-179) was used to generate a specific rabbit antibody (see section 2.2.4.5). It became a very useful tool as it detected the unmodified and the modified PML (50-179) species so that they could be compared directly (see Figure 3.20).

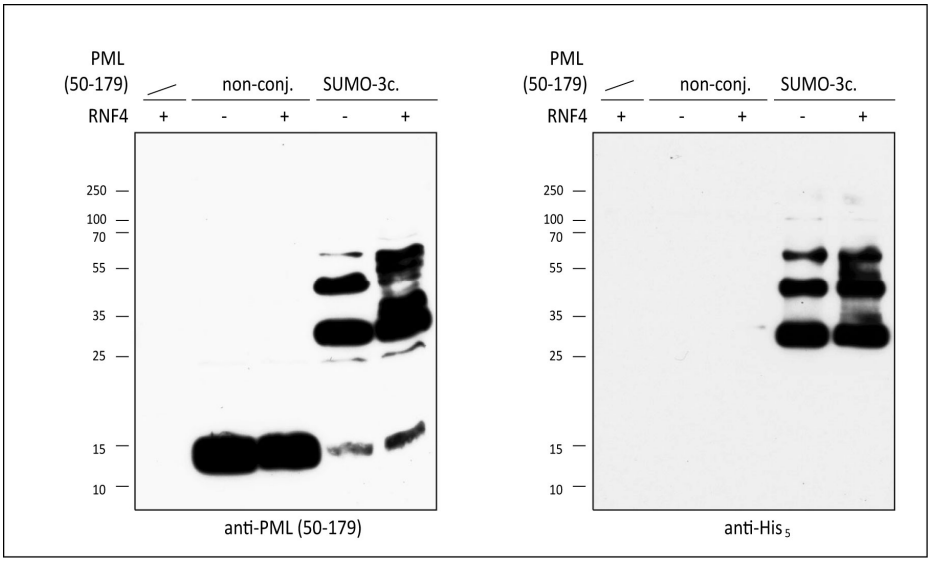


Figure 3.20: Newly generated antibody against PML (50-179) allowed comparison of PML species
Unmodified (non-conj.) PML (50-179) or its His_6-SUMO-3 conjugated form (SUMO-3c.) were subjected to *in vitro* ubiquitylation by RNF4 as described in Figure 3.17 B. Reactions were terminated by adding 2 x SDS sample buffer, proteins were separated by SDS-PAGE and detected with anti-His_5 or anti-PML (50-179). Rabbit-anti-PML (50-179) (serum 3004) specifically recognizes unmodified, SUMO-modified and ubiquitylated SUMO-modified PML. Anti-PML (50-179) detection sensitivity of the latter two is comparable to that of anti-His_5.

This antibody also recognized PML (50-179) lysine mutants K65R and K160R and was used to dissect the influence of the substrate SUMOylation site for RNF4-dependent ubiquitylation. In human cells, ATO stimulates the ubiquitylation of SUMOylated PML and the PML-RARα fusion protein. PML K160 seems to be required for this process (Lallemand-Breitenbach, 2001).
However, RNF4-dependent *in vitro* ubiquitylation of the PML (50-179) K160R mutant is still very efficient (see Figure 3.21). In fact, huge differences in ubiquitylation efficiency depending on the substrate SUMOylation site could not be observed. Although ubiquitylation of SUMOylated PML (50-179) wt was relatively inefficient in this assay

(compared for example with the one shown in Figure 3.20), the SUMOylated K65R and K160R mutants were well ubiquitylated.

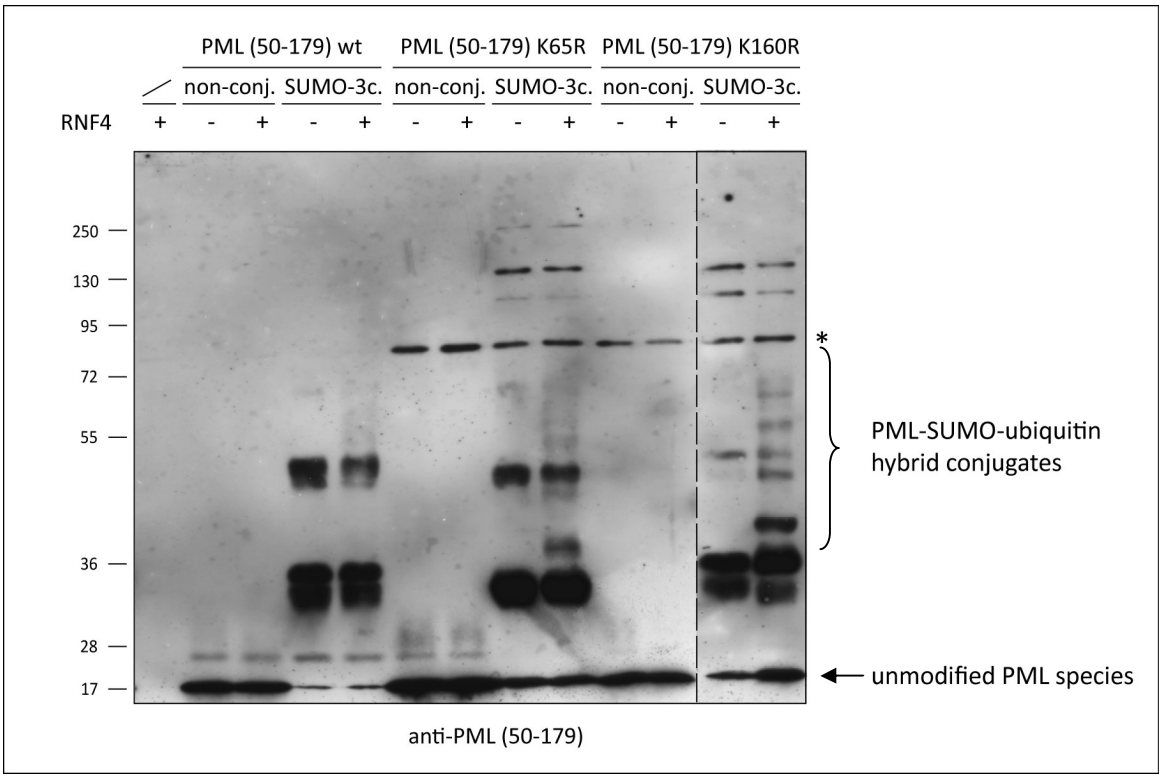


Figure 3.21: RNF4-dependent *in vitro* ubiquitylation of site-specific SUMOylated PML (50-179)
Unmodified (non-conj.) and His_6-SUMO-3 (SUMO-3c.) modified PML (50-179) wt, K65R or K160R were subjected to *in vitro* ubiquitylation by RNF4 as described in Figure 3.17 B. Reactions were terminated by adding 2 x SDS sample buffer, proteins were separated by SDS-PAGE and detected with anti-PML (50-179). The asterisk marks residual DnaK from *E. coli*.

3.4.4 RNF4 preferentially ubiquitylates the SUMO moiety of its substrate

The preceding experiment suggested that at least under *in vitro* conditions, RNF4 does not discriminate between SUMOs attached to different sites in the substrate protein. RNF4 thus seems to be directed to its substrate via the SUMO moieties. A question arising from this observation – apart from a general interest – was the following: are the ubiquitin molecules attached to lysines in the conjugated SUMOs or to lysines in the modified substrate PML?

A first attempt to address this question was the reversal of SUMOylation after subjecting His_6-SUMO-3 modified PML (50-179) to RNF4-dependent ubiquitylation. For that purpose, the catalytic domains of the SUMO proteases SENP1 and SENP6 were purified. Then, after subjecting unmodified or His_6-SUMO-3 modified PML (50-179) to RNF4-dependent ubiquitylation, reactions were either left untreated or incubated with SENP1, SENP6 or both catalytic domains. SDS-PAGE analysis followed by anti-PML (50-179), anti-His_5 and anti-ubiquitin detection (see Figure 3.22) revealed that the ubiquitin molecules are most likely attached to the SUMO moieties, not to PML (50-179).

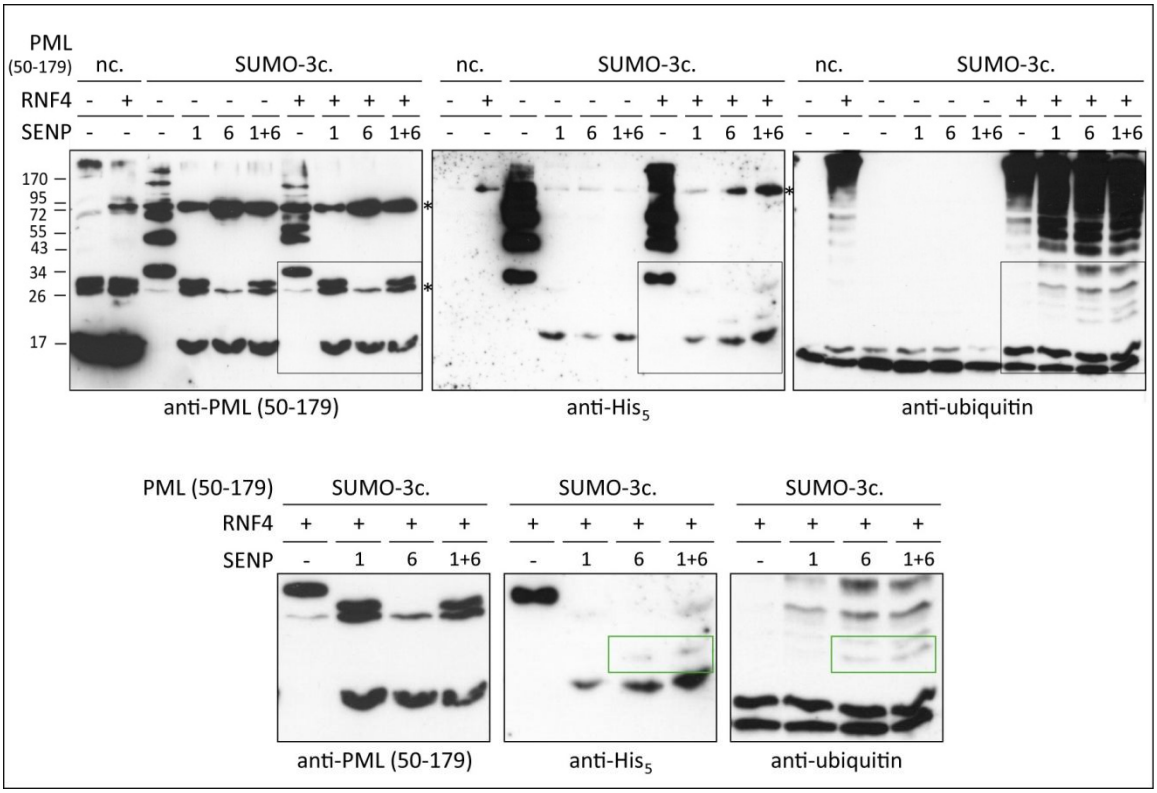


Figure 3.22: SENP treatment of *in vitro* ubiquitylated SUMO-modified PML

Unmodified (nc.) and His_6-SUMO-3 (SUMO-3c.) modified PML (50-179) were subjected to *in vitro* ubiquitylation by RNF4 as described Figure 3.17 B. Modified species have been produced without the coexpression of a SUMO E3 ligase. Purified catalytic domains of SENP1, SENP6 or both (each or together ~ 30 ng/µl, respectively) were added or not to the reactions as indicated.

Reactions were terminated by adding 2 x SDS sample buffer, proteins were separated by SDS-PAGE and detected with anti-PML (50-179), anti-His_5 or anti-ubiquitin. Asterisks on the right of the respective blots mark cross-reactive bands. Black boxed blot parts are enlarged beneath the total blot view. Green boxes highlight ubiquitylated His_6-SUMO-3.

The resulting blots show that both, SENP1 and SENP6 catalytic domains were active in deSUMOylating PML (50-179) (compare western blots decorated with anti-PML (50-179) and anti-His_5 in Figure 3.22). The anti-ubiquitin blot shows that the ubiquitylation reaction was not as specific as in the experiments before (compare RNF4-dependent ubiquitylated unmodified to modified PML). Reactions without substrate displayed a similar ubiquitylation pattern (data not shown). That problem occurred independently from salt or protein concentration and could be linked to the use of a new Tris batch (data not shown). A small fraction of unmodified PML (50-179) was also ubiquitylated by RNF4 (see faint band beneath 26 kDa). However, after ubiquitylation of SUMO-modified PML (50-179) and subsequent SENP treatment, additional bands migrating slower than the single moieties were only detected for His_6-SUMO-3, not for PML (50-179). Moreover, these bands were also recognized with anti-ubiquitin (green boxed bands in the enlarged blot pictures). Although a minor ubiquitylation of the PML moiety cannot be excluded due to the limits of detection, RNF4 obviously preferred to ubiquitylate the SUMO moiety of its substrate.
In order to identify the lysine residues for ubiquitin attachment, ubiquitylated His_6-SUMO-3 modified PML bands visible in a Coomassie stained SDS-PA gel were subjected to mass spectrometry (MS) analysis (see section 2.2.4.9, Table 3.1 and Figure 3.23).

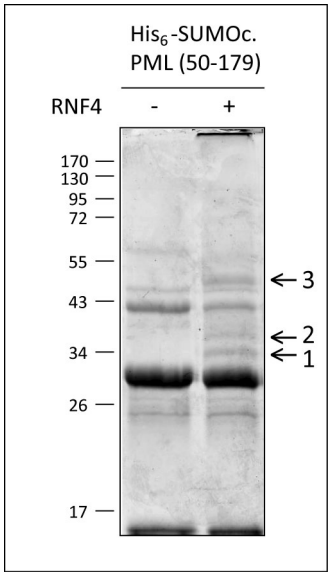


Figure 3.23: Coomassie stained SDS-PA gel for MS analysis to identify the ubiquitin attachment sites of *in vitro* ubiquitylated His_6-SUMO-3 modified PML (50-179)
His_6-SUMO-3 modified PML (50-179) was subjected to in vitro ubiquitylation by RNF4 and subsequently separated by SDS-PAGE.
Ubiquitylated bands visible after Coomassie-staining (indicated by arrows) were excised from the gel and analyzed by mass spectrometry (see Table 3.).

Table 3.1: Ubiquitin attachment sites of *in vitro* ubiquitylated His_6-SUMO-3 modified PML (50-179)

	SUMO-3	PML (50-179)	Ubiquitin
1	**K11**/K32/K41	none detected	K48
2	**K11**	none detected	K48
3	**K11**/K41	none detected	none detected

Summary of MS analysis; samples 1, 2, 3 correspond to the ubiquitylated bands excised from the Coomassie-stained gel depicted in Figure 3.23. Residues depicted in bold represent the modified lysine most abundantly detected. The sequence coverage of detected peptides ranged between 50-55 % for SUMO-3, 15 % for PML (50-179) and 97 % for ubiquitin.

MS analysis revealed that indeed the SUMO moiety of His_6-SUMO-3 modified PML is ubiquitylated by RNF4 while ubiquitylation of PML (50-179) was not detectable. Moreover, some detected peptides corresponded to ubiquitin-ubiquitin linkages at lysine 48, indicating that ubiquitin chains were attached to the SUMO molecules.

3.4.5 Sp100 - a second substrate of RNF4-dependent ubiquitylation?

As RNF4 seems to recognize SUMOylated proteins and to ubiquitylate the SUMO moiety, it should display its ULS function for any multi- or polySUMOylated protein. To test this hypothesis, unmodified and SUMO-3 modified Sp100 (1-332) were subjected to RNF4-dependent ubiquitylation (see Figure 3.24). SUMO-3 modification of Sp100 resulted in species that are most probably modified with SUMO chains as Sp100 possesses only one consensus SUMOylation site (compare also Figure 3.8 and Figure 3.10).

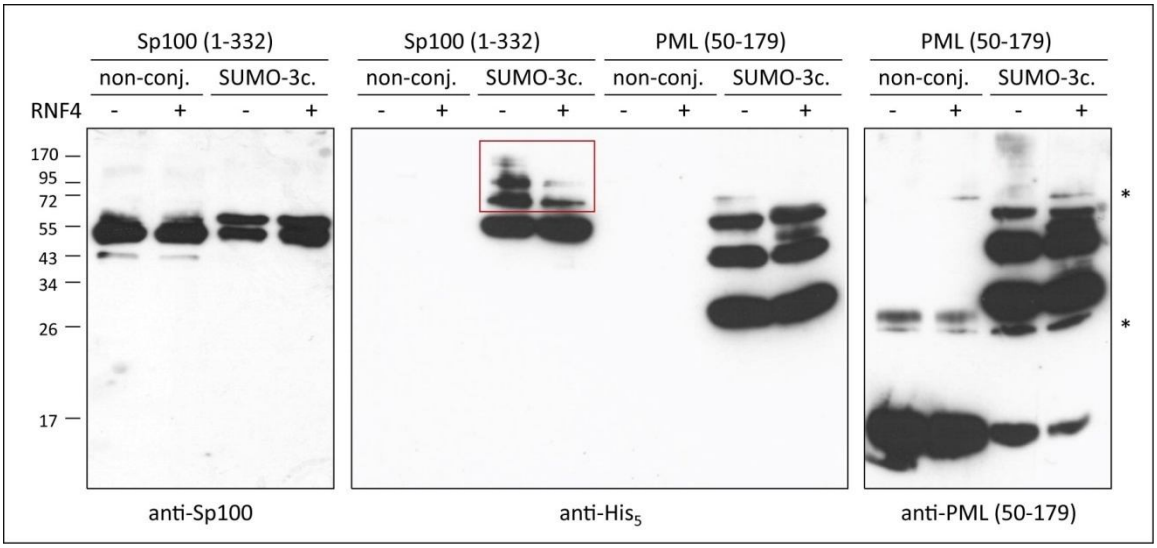


Figure 3.24: RNF4 dependent *in vitro* ubiquitylation of Sp100 (1-332) compared to PML (50-179)
Unmodified (non-conj.) and His_6-SUMO-3 (SUMO-3c.) modified Sp100 (1-332) or PML (50-179) were subjected to *in vitro* ubiquitylation by RNF4 as described in Figure 3.17 B. Reactions were terminated by adding 2 x SDS sample buffer, proteins were separated by SDS-PAGE and detected with anti-Sp100, anti-His_5 or anti-PML (50-179). Asterisks mark cross-reactive bands of anti-PML (50-179). Poly-SUMOylated Sp100 species are boxed in red.

Again, the ubiquitin blot was not very conclusive (data not shown) and the antibody recognizing Sp100 seemed to detect only unmodified and mono-SUMOylated Sp100 (left panel). Nonetheless, detection with the His_5-antibody showed that Sp100 species modified with a minimum of two SUMOs seemed to vanish upon RNF4-mediated ubiquitylation (red box in the middle panel). However, additionally appearing bands could not be detected. For comparison, RNF4-dependent ubiquitylation of unmodified and His_6-SUMO-3 modified PML (50-179) was performed in parallel (right side).
Thus, whether SUMOylated Sp100 could be a second target for RNF4-dependent ubiquitylation is - due to the detection limits - not conclusively clear.

3.5 The RNF4 SIMs bind preferentially to SUMO chains

In vitro ubiquitylation assays presented in the last section 3.4 show that the human RNF4 is a SUMO-dependent ubiquitin ligase. The next two sections are dealing with the binding properties of RNF4 to SUMOs.

The purified His_6-SUMO-1 and His_6-SUMO-3 modified PML (50-179) species displayed a similar running behavior in SDS-PAGE analysis (refer to Figure 3.19 A), suggesting that the respective PML (50-179) species were either modified on K65, K160 or on both sites. Beyond that, His_6-SUMO-3 chains on PML (50-179) do not seem to be present to a detectable extend at least when coexpressed with PIAS4 used for most assays shown except the one depicted in Figure 3.22. However, RNF4 still seemed to prefer His_6-SUMO-3 modified PML (50-179) for ubiquitylation (see Figure 3.19).

The analysis of His_6-SUMO-3 modified Sp100 (1-332) hints towards a chain recognizing mechanism by RNF4: only those species vanished that contained a minimum of two SUMOs in the His_6-SUMO-3 chain formed on Sp100 (1-332).

The RNF4 sequence contains up to four possible SIMs and thus it is likely that RNF4 binding to substrates is triggered by multiple SUMOs as for example provided by a SUMO chain. In order to investigate RNF4 binding to SUMO chains, only the part of RNF4 containing the SIMs (aa 1-105) was purified. Furthermore, SUMO chains were generated artificially. A special cloning strategy (see section 2.2.2.2) and subsequent protein purification resulted in linear SUMO chains lacking the first 11 amino acids (SUMO-2ΔN11 and SUMO-3ΔN11), mimicking a natural SUMO chain (Tatham, 2008). Likewise, for SUMO-1, the corresponding N-terminal extension including the first 15 amino acids was deleted to produce artificial SUMO-1 chains when several SUMO-1ΔN15 were cloned linearly.

As SUMO-2 and SUMO-3 are nearly identical and do not differ in their SIM binding region, binding studies to the RNF4 SIM domain were performed with SUMO-2 chains.

First, RNF4 binding to SUMO and SUMO chains was investigated by analytical gel filtrations on a Superdex 75 (10/300 GL) column (see Figures 3.25 and 3.26). For that purpose, 50 µM RNF4 (1-105) was incubated with 200 µM mono-SUMOs, 100 µM di-SUMOs or 50 µM tetra-SUMOs overnight. Assuming all presumed SIMs are capable of binding to SUMOs, these concentrations chosen should result in a 1:1 binding stoichiometry.

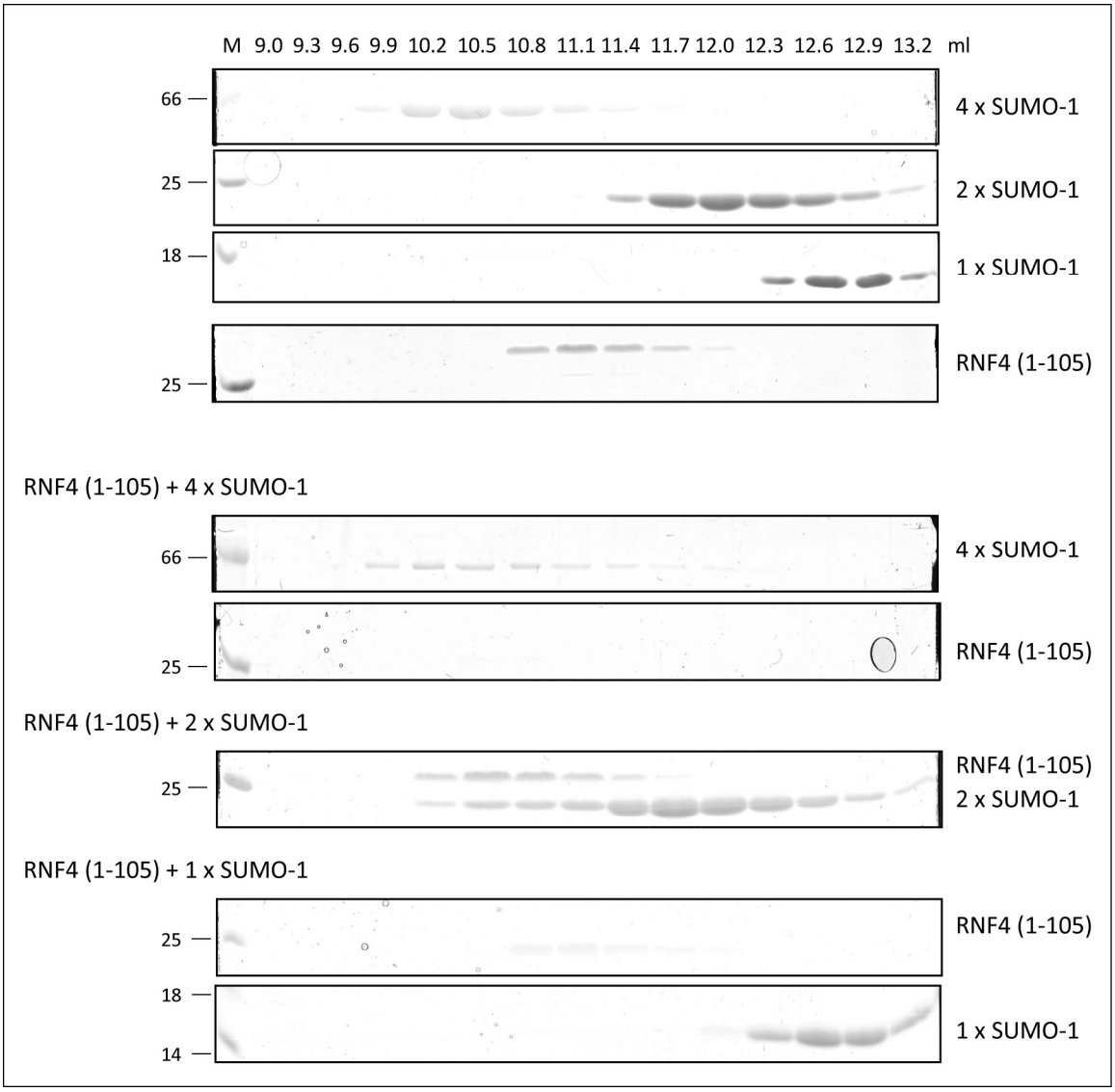


Figure 3.25: Gel filtration of RNF4 SIM domain and artificial SUMO-1 chain complexes
50 µM RNF4 (1-105) has been incubated with either 200 µM monoSUMO-1 (1 x SUMO-1), 100 µM diSUMO-1ΔN15 (2 x SUMO-1) or 50 µM tetra-SUMO-1ΔN15 (4 x SUMO-1) o/n in GST1 buffer + 5 % Glycerol. Proteins have been subjected to analytical gel filtrations performed at 0.5 ml/min in GST1 buffer + 5 % Glycerol and eluted protein fractions were separated by SDS-PAGE and stained with Coomassie. Fractions from 9.0 to 13.2 ml are depicted here after the respective runs indicated. The incubation of RNF4 (1-105) with tetra-SUMO-1ΔN15 represented a special case, as protein precipitated directly while mixing them. Overnight incubation led to a clear solution, but high-speed centrifugation (13000 g at 4 °C) resulted in a small protein containing pellet (examined with addition of Bradford solution). The supernatant was applied to analytical gel filtration.

For RNF4 (1-105) mixed with tetra-SUMO-1ΔN15, proteins precipitated upon mixing. Gel filtration of the remaining supernatant completely lacks RNF4 (1-105) in the respective elution fractions and tetra-SUMO-1ΔN15 is also reduced. It seems that under the chosen conditions, RNF4 (1-105) binding to artificial tetra-SUMO-1ΔN15 results rather in protein aggregation than in proper protein complex formation.

In contrast to that, binding of di-SUMO-1ΔN15 to RNF4 (1-105) could be observed since formed complexes were stable during gel filtration and eluted earlier than each component alone. The amount of di-SUMO-1 and RNF4 (1-105) in the elution fractions containing the complexes seemed to be the same as estimated from Coomassie staining which would point to a 1:1 stoichiometry. Mono-SUMO-1 did not bind efficiently to RNF4 (1-105) and complexes between the both could not be observed after analytical gel filtration.

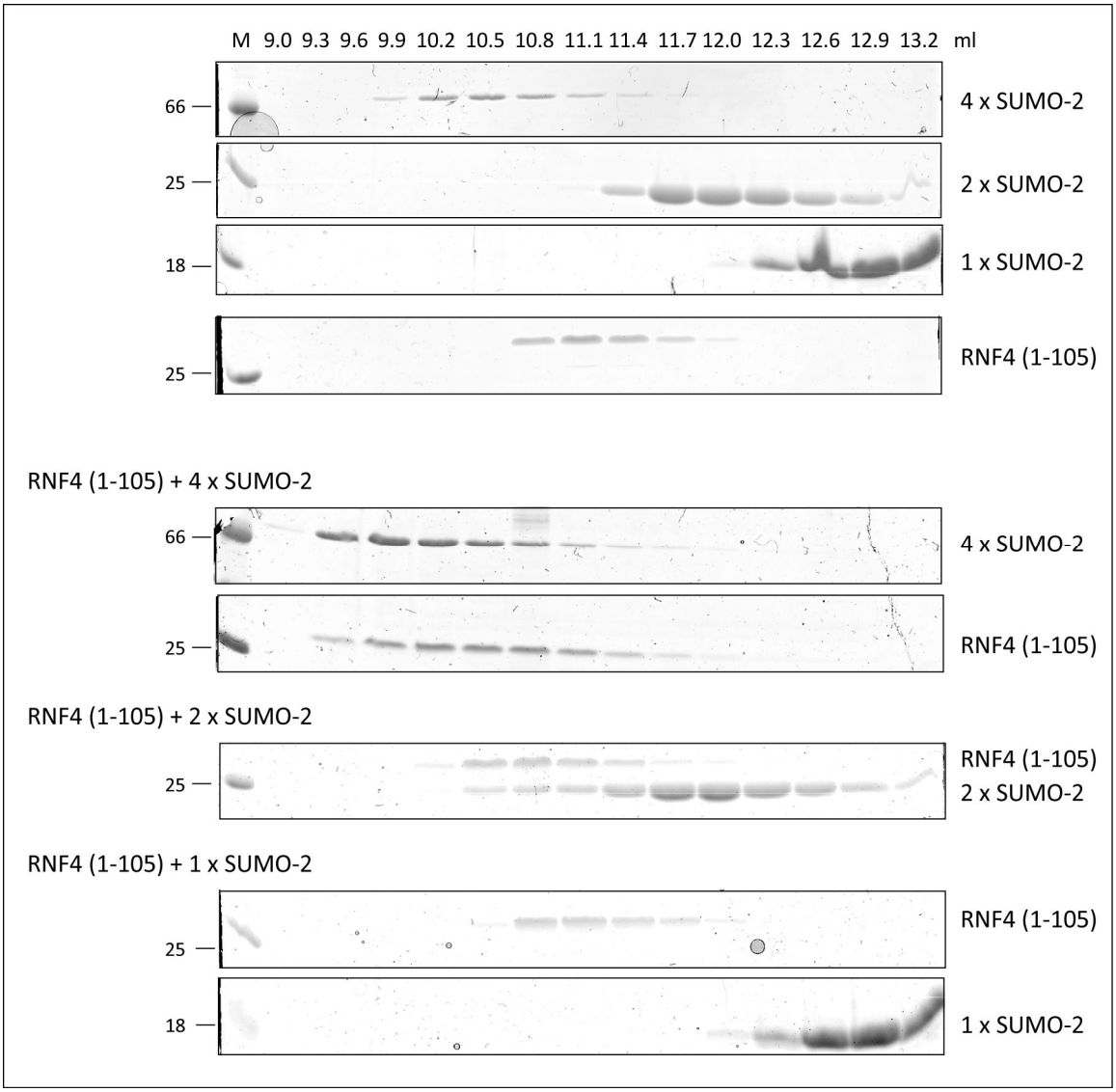


Figure 3.26: Gel filtration of RNF4 SIM domain and artificial SUMO-2 chain complexes
50 µM RNF4 (1-105) has been incubated with either 200 µM single SUMO-2 (1 x SUMO-2), 100 µM di-SUMO-2ΔN11 (2 x SUMO-2) or 50 µM tetra-SUMO-2ΔN11 (4 x SUMO-2) o/n in GST1 buffer + 5 % Glycerol. Proteins have been subjected to analytical gel filtrations performed at 0.5 ml/min in GST1 buffer + 5 % Glycerol and eluted protein fractions were separated by SDS-PAGE and stained with Coomassie. Fractions from 9.0 to 13.2 ml are depicted here after the respective runs indicated.

In the case of RNF4 (1-105) mixed with tetra-SUMO-2ΔN11, a proper complex formation between the two proteins could be observed since both elute earlier after size exclusion

chromatography than during their respective single runs. Basically the whole amount of both proteins seemed to be engaged in complexes, pointing to a 1:1 stoichiometry. Analytical gel filtration of RNF4 (1-105) mixed with di-SUMO-2ΔN11 resulted in a similar elution profile as observed for RNF4 (1-105) mixed with di-SUMO-1ΔN15. A complex formation of mono-SUMO-2 with RNF4 (1-105) could not be observed after size exclusion chromatography.
Together, these data suggested that RNF4 is capable to bind to SUMO chains containing at least two SUMOs. A difference between SUMO-1 and SUMO-2 during di-SUMO binding could not be observed. However, artificial tetra-SUMO-1 chains induced protein aggregation when mixed with the RNF4 SIM domain while tetra-SUMO-2 chains formed proper complexes with RNF4 (1-105).

In order to investigate these complex formations in a more detailed fashion including binding constants, Isothermal Titration Calorimetry (ITC) was performed. For this method it is crucial to know the exact protein concentration. Since the RNF4 (1-105) construct does not include any aromatic residue and other methods for the determination of the protein concentrations yielded variable results, the protein was unsuitable to be investigated by this method. Therefore, a longer construct was generated, RNF4 (1-125), which contains a tyrosine residue and for which the protein concentration could be determined by measuring the absorbance at 280 nm. ITC experiments were carried out by injecting artificial polySUMOs as ligands into the experimental cell containing the RNF4 (1-125) solution (see Figures 3.27, 3.28 and 3.29).

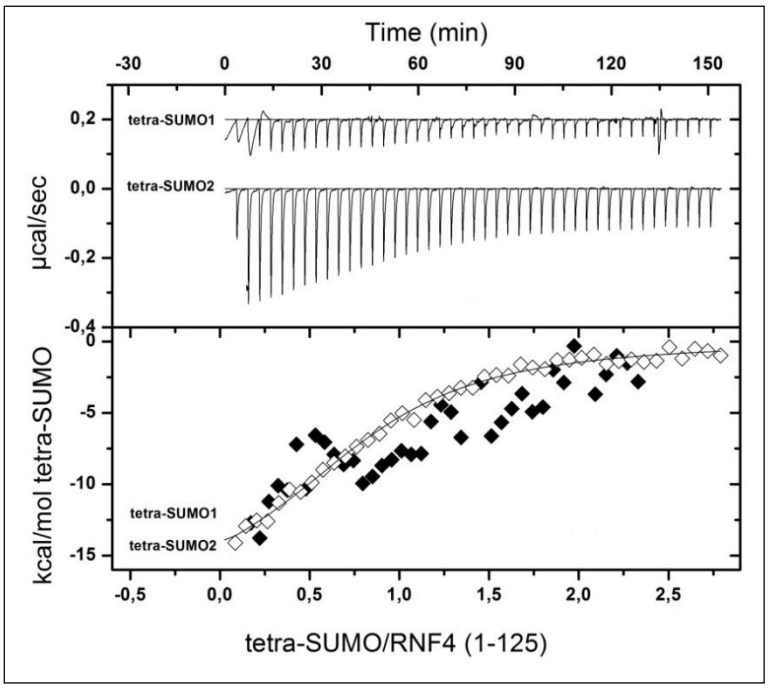


Figure 3.27: Interaction of artificial tetra-SUMO chains and RNF4 (1-125)
The interaction of tetra-SUMO chains and the SIM containing N-terminus of RNF4 was followed by isothermal titration calori-metry. The upper panel shows the heat of binding measured by stepwise injection of 60 µM tetra-SUMO-1ΔN15 into 3.3 µM RNF4 (1-125) and of 100 µM tetra-SUMO-2ΔN11 into 6.6 µM RNF4 (1-125) at 25 °C. The lower panel shows the integrated heating powers of the upper panel normalized to the concentrations of the artificial tetra-SUMOs. Fitting of a single binding side model yielded a K_D of 1.7 µM for tetra-SUMO2 and RNF4 (1-125).

For the interaction of tetra-SUMO-2ΔN11 and RNF4 (1-125), a one site binding model could be fitted with a K_D = 1.7 µM (see alsoTable 3.2). Due to the low available amounts of tetra-SUMO-1 ΔN15 to RNF4 (1-125) and several artefactual perturbations the binding could not be fitted. However, a trend for binding is indicative.

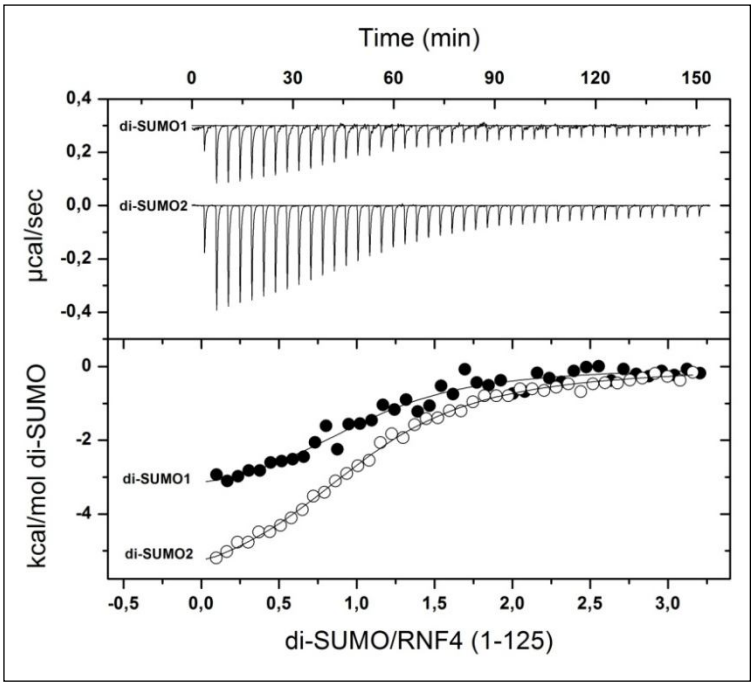


Figure 3.28: Interaction of artificial di-SUMO chains and RNF4 (1-125)
The interaction between di-SUMO-1ΔN15 or di-SUMO-2ΔN11 and the SIM containing N-terminus of RNF4 (aa 1-125) was followed by isothermal titration calorimetry. The upper panel shows the heat of binding measured by stepwise injection of 300 µM di-SUMO-1ΔN15 or di-SUMO-2ΔN11 into 20 µM RNF4 (1-125) at 25 °C. The lower panel shows the integrated heating powers of the upper panel normalized to the concentrations of the artificial di-SUMOs. Fitting of a single binding side model for the interaction to RNF4 yielded a K_D of 5.2 µM for di-SUMO-1 and of 3.9 µM for di-SUMO-2.

The interaction of both, di-SUMO-1ΔN15 or di-SUMO-2ΔN11 and RNF4 (1-125) were nearly as strong as the interaction of RNF4 (1-125) and tetra-SUMO-2ΔN11 with K_D values of 5.2 µM or 3.9 µM, respectively.

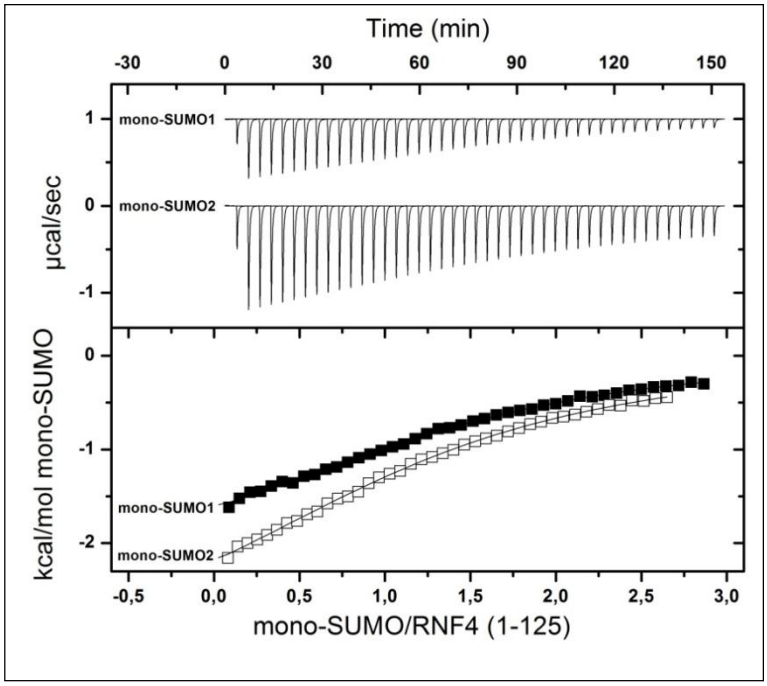


Figure 3.29: Interaction between mono-SUMOs and RNF4 (1-125)
The interaction between mono-SUMO-1ΔN15 or mono-SUMO-2ΔN11 and RNF4 (aa 1-125) was followed by isothermal titration calorimetry. The upper panel shows the heat of binding measured by stepwise injection of 1.8 mM mono-SUMO-1ΔN15 or di-SUMO-2ΔN11 into 120 µM RNF4 (1-125) at 25 °C. The lower panel shows the integrated heating powers of the upper panel normalized to the concentrations of the artificial mono-SUMOs.
Fitting of a single binding side model for the interaction to RNF4 yielded a K_D of 92 µM for mono-SUMO-1 and of 125 µM for mono-SUMO-2.

Mono-SUMOs were poor binding partners of RNF4 (1-125). When injected at a concentration of 300 µM into 20 µM RNF4 hardly any interaction was detectable (data not shown). However, when protein concentrations were increased to 1.8 mM mono-SUMOs and 120 µM RNF4 (1-125), interaction could be detected with K_D values of 92 µM in the case of mono-SUMO1ΔN15 and 125 µM for mono-SUMO2ΔN11.
Table 3.2 summarizes the binding constants obtained after one site modeling of the fitted binding curves.

Table 3.2: Binding constants of artificial (poly)SUMOs and RNF4 (1-125) SIMs

	K_D [µM]	N	ΔH [kcal/mol]	TΔS [kcal/mol]
mono-SUMO-1ΔN15	92	1.4	-2.3	3.2
mono-SUMO-2ΔN11	125	1.4	-3.4	1.9
di-SUMO-1ΔN15	5.2	1.00	-4.8	2.4
di-SUMO-2ΔN11	3.9	1.1	-6.1	1.3
tetra-SUMO-1ΔN15	N.D.	N.D.	N.D.	N.D.
tetra-SUMO-2ΔN11	1.7	0.8	-18.3	-10.4

K_D is the inverse of the association constant K_A obtained from the binding experiments performed at 25 °C. N represents the calculated stoichiometry. The values for ΔH and TΔS are given for the association reaction.

The comparison shows that the RNF4 SIM domain displayed a ~ 25 fold increase in binding affinity towards a di-SUMO chain and a ~ 50 to 70 fold increase towards the tetra-SUMO2ΔN11 compared to the binding affinities towards the mono-SUMOs.
Furthermore, it seems that for binding of the mono-SUMOs more than one binding site in RNF4 (1-125) is used although a fit for more than one site was not possible.

3.6 Mammalian SUMOs might discriminate between different SIM types

The observed preference of RNF4 for His_6-SUMO-3 modified PML (50-179) in *in vitro* ubiquitylation reactions may hint towards a SIM-type specific SUMO recognition mechanism. In contrast to this, no significant difference of the binding was observed by ITC measurements. The four putative SIMs in RNF4 belong to different types: SIM1 might be a SIMr, SIM2 and 3 are type b SIMs and SIM4 is a conventional SIMa (see Figure 3.13). In order to analyze the binding properties of different SIM types to different SUMO paralogs, several SIMs from different proteins were cloned as GST-fusions in their natural sequence environment of about 80-100 amino acids around it (see Figure 3.30). The RNF4 SIM domain (1-105) was used for comparison. Furthermore, the PML SIM has been shown to be phosphorylated at several serine residues next to the SIM core which

enhances SUMO binding (Stehmeier and Müller, 2009). In order to investigate the influence of a phosphorylated SIM in binding of SUMO paralogs, PML11 SIM mutants were generated which either mimic phosphorylated serines (S512-514, 517→D) or in which the respective serines were mutated to alanines (S512-514, 517→A).

RNF4 SIM domain (1-105):
1–MSTRKRRGGAINSRQAQKRTREATSTPEISLEAEPIELVETAGDEIVDLTCESLE
PVVVDLTHNDSVVIVDERRRPRRNARRLPQDHADSCVVSSDDEELSRDRD–105

SIM type a:

PML11 (419-522)wt:
419-DVSNT…GEAEERVVVISSSEDSDAENS–522

PML11 (419-522) S 512-514,517→A:
419-DVSNT…GEAEERVVVIAAAEDADAENS–522

PML11 (419-522) S 512-514,517→D:
419-DVSNT…GEAEERVVVIDDDEDDDAENS–522

Sp100 (274-332):
274–SPEA…NQASDIIVISSEDSE–332

Daxx SIM2 (625-740):
625–SGPP…TQCDPEEIIVLSDSD–740

SIM type b:

PIAS3 (394-470):
394–DEIQ…KKKVEVIDLTIESSSDEEDLPPTKKHCSV–470

PIAS4 (445-510):
445–GSTG…PGADVVDLTLDSSSSSEDEEEEEEEEEDEDEEGPR…LVPAC–510

Figure 3.30: SIMs chosen to study type-specific binding properties to SUMO paralogs
Shown are the SIM sequences of different proteins; GST-constructs of these SIMs used in the following experiment comprise the amino acids given in brackets. SIM relevant hydrophobic amino acids are depicted in blue, acidic amino acids in red and type b specific threonine residues in green.

The resulting proteins were subjected to GSH affinity purification and were left associated to the GSH beads to perform interaction studies with purified SUMO-1, SUMO-2 and SUMO-3 (see Figure 3.31).
SUMO-1 interacted with all tested SIM constructs. In the case of the PML SIM, the phosphomimic PML11 (419-522) S512-514,517 →D construct displayed indeed a stronger binding to SUMO-1 compared to its non-phosphorylated wt or phosphorylation defective S512-514,517 →A mutant form.

SUMO-2 and SUMO-3 only bound to the RNF4 (1-105) SIM domain, PIAS3 (394-470) and PIAS4 (445-510). In the experiment shown, binding of PIAS4 to SUMO-3 was not detectable, probably due to the low amount of SUMO-3 input. In other experiments, it bound equally well to SUMO-3 as to SUMO-2 (data not shown). Intriguingly, all three SIM constructs binding to SUMO-2 and SUMO-3 contain a type b SIM, in the case of RNF4 even two.

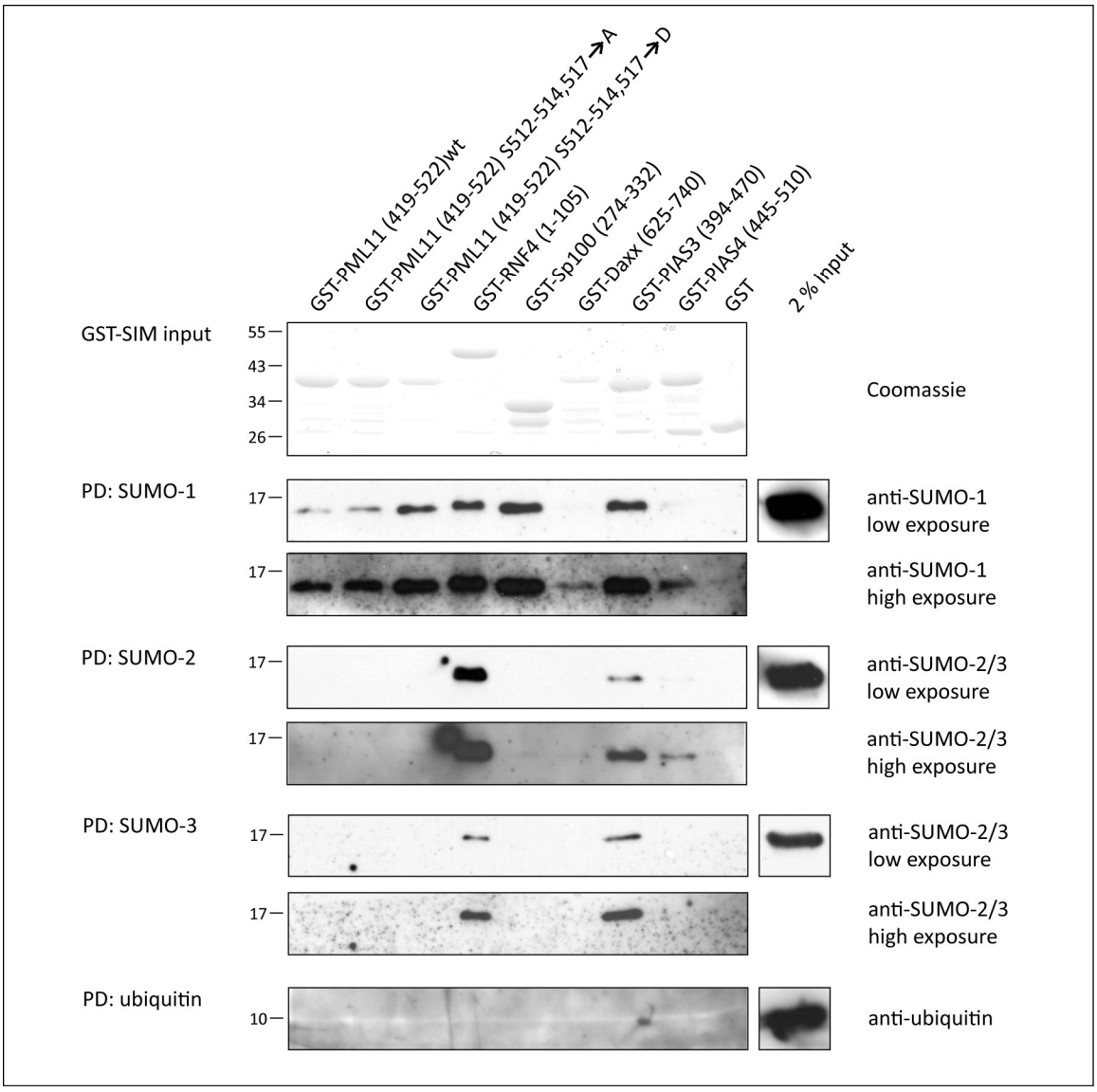


Figure 3.31: SUMO binding to different SIM types
Purified SUMO-1, SUMO-2 and SUMO-3 have been subjected to a pulldown (PD) assay with the GST-SIM constructs introduced in Figure 3.30 as bait. Purified ubiquitin served as negative control. Proteins were eluted in 2 x SDS sample buffer, separated by SDS-PAGE and detected by SUMO-1, SUMO-2/3 or ubiquitin antibodies, respectively. Input of GST-SIM constructs are displayed as Coomassie staining after SDS-PAGE.

Thus it seems that the binding properties of SIM type a are restricted to SUMO-1 whereas SIM type b is able to bind to SUMO-1 and to SUMO-2/3. In other words, SUMO-2 and SUMO-3 discriminate between type a and type b SIMs.

Whether type b SIMs have a higher affinity to SUMO-2/3 than to SUMO-1 - which would explain the preference of RNF4 for SUMO-3 conjugated PML (50-179) - could not be detected with this type of interaction study.
Therefore, Surface Plasmon Resonance (SPR) was performed to measure the interactions between PIAS3 (394-470) or RNF4 (1-105) SIMs and either SUMO-1 or SUMO-2 (the analysis focused on PIAS3 and RNF4 as they displayed the strongest binding to SUMO-2 and SUMO-3; see Figure 3.32).

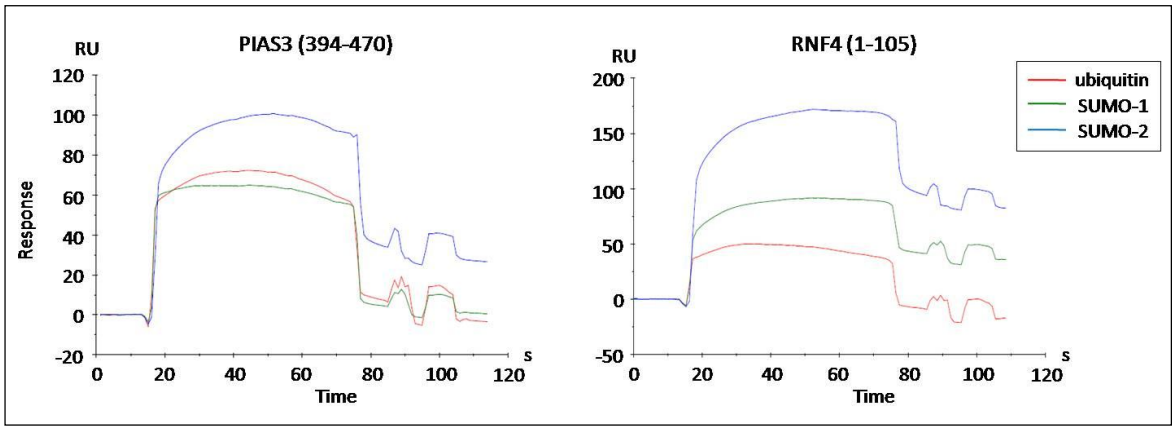


Figure 3.32: Surface plasmon resonance curves for the interaction of SUMOs to PIAS3 and RNF4 SIMs
SPR was performed at 25 °C on a Biacore T100. SUMO-1, SUMO-2 and ubiquitin (as a negative control) were coupled to a CM5 surface of a SPR biosensor chip (Biacore) and subsequently probed for the binding of ~ 25 µM PIAS3 (394-470) or RNF4 (1-105). RU, response units.

Though overall binding was weak, in both cases, binding to SUMO-2 was indeed stronger than binding to SUMO-1, suggesting that type b SIMs have a higher affinity for SUMO-2/3 (SUMO-3 does not differ from SUMO-2 in respect to the SIM binding sites). For PIAS3 (394-470), binding to SUMO-1 was not detectable with this method above the level of binding to the negative control ubiquitin. RNF4 (1-105) displayed binding to both, SUMO-1 and SUMO-2 while binding to SUMO-2 was approximately twice as strong as binding to SUMO-1 with respect to the relative response units remaining during the dissociation period.

3.7 Possible RNF4 substrates upon diverse cellular stresses

In the preceding sections of this work, it could be conclusively shown that RNF4 is a human ubiquitin ligase for SUMOylated PML *in vitro* and that RNF4 binds preferentially to SUMO-2/3 chains. Overall cellular SUMO-2/3 conjugation is upregulated upon diverse cellular stress stimuli like proteasomal inhibition, ATO treatment, unfolded protein stress or (slightly) upon osmotic stress (Saitoh and Hinchey, 2000; Weisshaar, 2008; Meyer-Teschendorf, 2010). Therefore, ULS substrates other than PML might also be regulated by RNF4 (or similar, yet to be identified ULS proteins) after these types of stress. These possible substrates should be either multi- or polySUMOylated upon the respective stress stimuli and further stabilized upon proteasomal inhibition.

As the RNF4 N-terminus comprises up to four SIMs, isolation of specifically multi- or polySUMOylated proteins from stress induced cells should be possible by making use of the GST-RNF4 (1-105) SIM domain construct. This construct should bind RNF4 substrates due to its SIMs but is not an active ubiquitin ligase as the RING domain is deleted. Recently, tandem-repeated ubiquitin-binding entities (TUBEs) that are based on the linear tandem-repeated fusion of several ubiquitin-associated (UBA) domains could be shown to recognize tetra-ubiquitin with a markedly higher affinity than single UBA domains (Hjerpe, 2009). This allowed poly-ubiquitylated proteins to be efficiently purified from cell extracts in native conditions. In order to identify further possible ULS substrates, an analogous method was established using the GST-RNF4 (1-105) SIM domain construct bound to GSH-beads as a bait to isolate multi- or polySUMOylated proteins binding to it (see section 2.2.4.13).
First, the method was tested for isolation of SUMOylated proteins with cell extracts from MG132-treated Hs27 primary human fibroblast cells (see Figure 3.33).

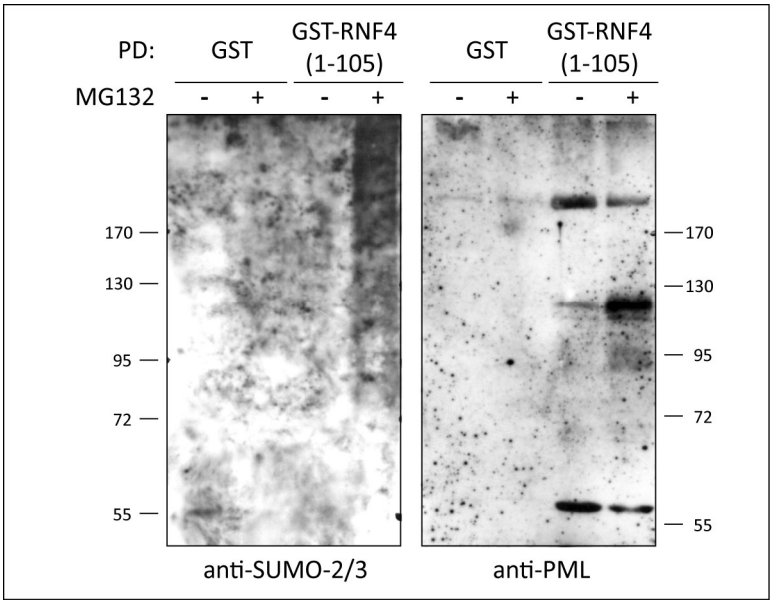


Figure 3.33: Isolation of multi-/ polySUMOylated proteins from MG132-treated cells
Hs27 cells were treated or not with 20 µM MG132 for 6 h and lysed in RIPA buffer. SUMO-conjugated proteins were isolated using purified GST-RNF4 (1-105) bound to GSH beads as a bait. Purified GST served as negative control. Bound proteins were eluted by adding 2 x SDS sample buffer, separated by SDS-PAGE and detected with anti-SUMO-2/3 or anti-PML (A301-167A, -168A), respectively (PD, pull down assay).

GST-RNF4 (1-105) could indeed be utilized to efficiently isolate SUMOylated proteins from the extracts of MG132-treated Hs27 cells, among them the already identified ULS substrate PML.
In order to identify further possible ULS substrates, this method was applied to isolate SUMOylated proteins after a range of cellular stresses, namely after proteasomal inhibition with MG132, ATO treatment, osmotic stress induced by the addition of sorbitol or after unfolded protein stress induced by the addition of the arginine analogue canavanine (see Figure 3.34). The samples were subjected to SDS-PAGE and several gel slices with a defined molecular weight range were excised to perform subsequent MS analysis (see section 2.2.4.13).

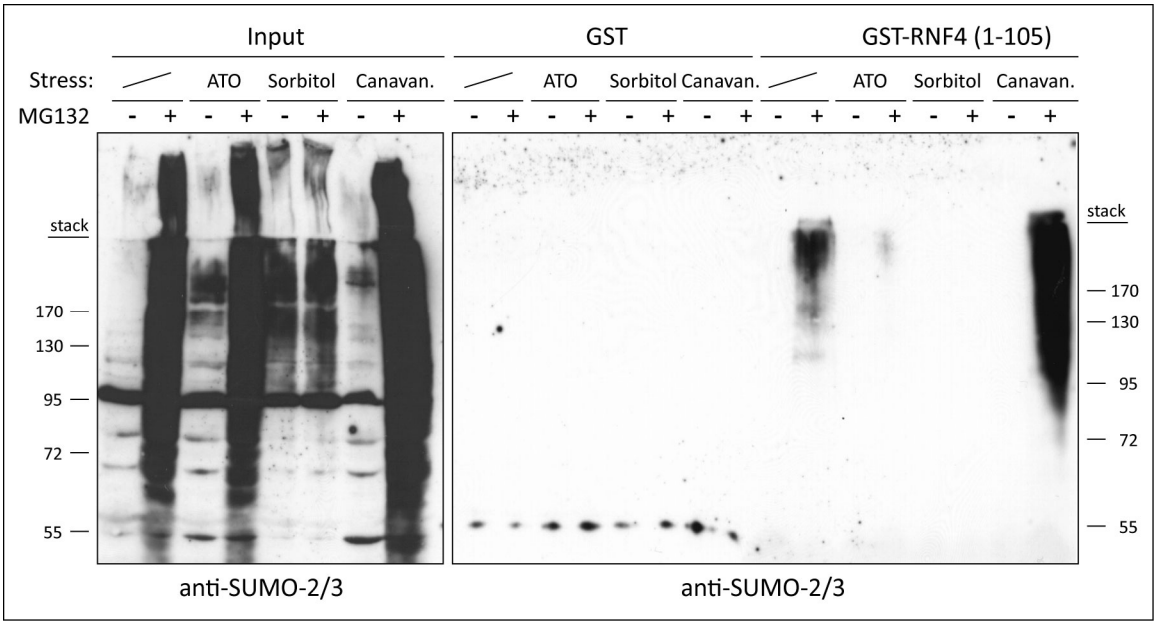


Figure 3.34: Isolation of multi-/polySUMOylated proteins from cells treated with different stress stimuli
Hs27 cells were either treated with DMSO (-) or 20 µM MG132 (+) for 6 h, with 3 µM ATO -/+ 20 µM MG132 for 6 h, with 1 M sorbitol -/+ 20 µM MG132 for 4 h or with 7.5 mM canavanine (Canavan.) -/+ 20 µM MG132 for 6 h (input at the left shows the respective cell lysates after SDS-PAGE and detection with anti-SUMO-2/3). Isolation of proteins binding to GST or GST-RNF4 (1-105) was performed as described in section 2.2.4.13. The majority of these isolated proteins was subjected to SDS-PAGE with subsequent Coomassie-staining and MS analysis (see Table 3.3 and Appendix Tables A.2-A.8). A small fraction of the isolated proteins was subjected to SDS-PAGE and detected with anti-SUMO-2/3 (right).

The resulting proteins were assessed for the presence of *bona-fide* SUMOylation sites using the SUMOplot SUMO site prediction program (accessible via the ExPASy proteomic tools server, http://www.expasy.ch/tools/) in order to increase the probability that the resulting proteins are regulated by the ULS pathway. Proteins that possess high probability SUMOylation sites are listed in black, those with low probability motifs in grey. Internal positive controls represented peptides found for PML, SUMO-2, SUMO-3 and ubiquitin. A complete list of proteins interacting with GST-RNF4 (1-105) is provided in Appendix tables A.2 – A.8.

The table below (Table 3.3) comprises a selection of interacting proteins that might be regulated by SUMO-dependent ubiquitylation. The proteins listed are either found in a higher molecular weight (M_W) range as their individual calculated M_W and/or show stabilization upon proteasomal inhibition. As the isolation of proteins from ATO- or sorbitol-treated cells was not very successful (see Figure 3.34), individual protein hit results from these isolations must be regarded as preliminary results.

MS analysis indicated that many signaling molecules are interacting with the RNF4 N-terminus.. For instance, IKK-1 and Nmi are found in all stressed samples and for some, a stabilization upon treatment with MG132 can be observed. Further, many other kinases and transcription factors could be isolated by GST-RNF4 (1-105).

Table 3.3: Selection of RNF4 (1-105) interacting proteins after diverse cellular stress stimuli

Possible RNF4 substrates after MG132 stress	Peptides	Accession	M_w [kDa]	M_w range [kDa]
5'-AMP-activated protein kinase subunit gamma-2	2	Q9UGJ0	63.0	170 - stack
Caspase-8 precursor (CASP-8)	2	Q14790	55.4	170 - stack
Centrosomal protein of 131 kDa (Cep131)	19	Q9UPN4	122.0	> 170 incl. stack
E3 SUMO-protein ligase PIAS2	3	O75928	68.2	90 - 120
F-box only protein 11 (Vitiligo-associated protein VIT-1)	2	Q86XK2	103.5	170 - stack
I kappa-B kinase alpha (IKK-alpha; IKK1)	7	O15111	84.6	stack
N-myc-interactor (Nmi) (N-myc and STAT interactor)	13	Q13287	35.0	> 170 incl. stack
Promyelocytic leukemia protein PML (TRIM 19)	12	P29590	97.5	> 170 incl. stack
Serine/threonine-protein kinase 36	5	Q9NRP7	143.9	stack
Serine/threonine-protein kinase SRPK2	3	P78362	77.5	90 - 120
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PP-1A)	2	P62136	37.5	stack
Transcriptional-regulating factor 1 (TReP-132)	5	Q96PN7	132.2	> 170 incl. stack
Possible RNF4 substrates after ATO stress	**Peptides**	**Accession**	**M_w [kDa]**	**M_w range [kDa]**
5'-AMP-activated protein kinase subunit gamma-2	4	Q9UGJ0	63.0	90 - 120
Chromobox protein homolog 8 (Polycomb 3 homolog)	10	Q9HC52	43.4	120 - 170
I kappa-B kinase alpha (IKK-alpha; IKK1)	12	O15111	84.6	70 - stack
Mitogen-activated protein kinase kinase kinase 6	2	O95382	112.4	stack
Myristoylated alanine-rich C-kinase substrate (MARCKS)	2	P29966	31.5	70 - 90
N-myc-interactor (Nmi) (N-myc and STAT interactor)	5	Q13287	35.0	> 70 incl. stack
Serine/threonine-protein kinase PLK2	8	Q9NYY3	78.2	stack
Wiskott-Aldrich syndrome protein family member 1	4	Q92558	61.6	90 - 120
Zinc finger and BTB domain-containing protein 43	11	O43298	52.6	90 - 170
Possible RNF4 substrates after ATO/MG132 stress	**Peptides**	**Accession**	**M_w [kDa]**	**M_w range [kDa]**
5'-AMP-activated protein kinase subunit gamma-2	8	Q9UGJ0	63.0	> 170 incl. stack
Centrosomal protein of 131 kDa (Cep131)	3	Q9UPN4	122.0	stack
COUP transcription factor 1 (COUP-TF1)	2	P10589	46.1	70 - 90
I kappa-B kinase alpha (IKK-alpha; IKK1)	11	O15111	84.6	90 - stack
N-myc-interactor (Nmi) (N-myc and STAT interactor)	2	Q13287	35.0	120 - 170
Serine/threonine-protein kinase PLK2	4	Q9NYY3	78.2	120 - 170

Possible RNF4 substrates after sorbitol stress	Peptides	Accession	M_w [kDa]	M_w range [kDa]
150 kDa oxygen-regulated protein precursor (Orp150) (Hypoxia up-regulated 1)	8	Q9Y4L1	111.3	170 - stack
5'-AMP-activated protein kinase subunit gamma-2	25	Q9UGJ0	63.0	50 - 90
Centrosomal protein of 131 kDa (Cep131 protein)	10	Q9UPN4	122.0	> 50 incl. stack
E3 SUMO-protein ligase PIAS2	2	O75928	68.2	120 - 170
Forkhead box protein D1 (Forkhead-related transcription factor 4) (FREAC-4)	4	P55854	11.6	stack
I kappa-B kinase alpha (IKK-alpha; IKK1)	39	O15111	84.6	> 50 incl. stack
Mitogen-activated protein kinase kinase kinase 8	2	P41279	52.9	170 - stack
N-myc-interactor (Nmi) (N-myc and STAT interactor)	8	Q13287	35.0	90 - stack
Serine/threonine-protein kinase PLK2	12	Q9NYY3	78.2	90 - 120
Transcription intermediary factor 1-alpha (TIF1-alpha) (TRIM 24)	2	O15164	116.8	170 - stack

Possible RNF4 substrates after sorbitol/MG132 stress	Peptides	Accession	M_w [kDa]	M_w range [kDa]
5'-AMP-activated protein kinase subunit gamma-2	32	Q9UGJ0	63.0	> 50 incl. stack
Centrosomal protein of 152 kDa (Cep152 protein)	4	O94986	147.3	170 - stack
Homeobox prospero-like protein PROX1 (PROX 1)	16	Q92786	83.2	> 90 incl. stack
I kappa-B kinase alpha (IKK-alpha; IKK1)	18	Q92786	83.2	> 90 incl. stack
N-myc-interactor (Nmi) (N-myc and STAT interactor)	13	Q13287	35.0	> 170 incl. stack
Retinoic acid receptor alpha (RAR-alpha)	2	P10276	50.7	70 - 90
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PP-1A)	2	P62136	37.5	stack
Transcription factor HES-5	3	Q5TA89	18.2	170 - stack

Possible RNF4 substrates after canavanine stress	Peptides	Accession	M_w [kDa]	M_w range [kDa]
150 kDa oxygen-regulated protein precursor (Orp150) (Hypoxia up-regulated 1)	9	Q9Y4L1	111.3	170 - stack
5'-AMP-activated protein kinase subunit gamma-2	16	Q9UGJ0	63.0	90 -120
Centromere protein H (CENP-H)	2	Q9H3R5	28.5	120 - 170
Creatine kinase M-type	2	P06732	43.1	170 - stack
DNA topoisomerase 2-beta	4	Q02880	183.2	stack
E3 SUMO-protein ligase PIAS2	10	O75928	68.2	> 170 incl. stack
ELAV-like protein 3 (Hu-antigen C)	9	Q14576	39.5	stack

Possible RNF4 substrates after canavanine stress	Peptides	Accession	M_w [kDa]	M_w range [kDa]
Homeobox prospero-like protein PROX1 (PROX 1)	5	Q92786	83.2	> 70 incl. stack
I kappa-B kinase alpha (IKK-alpha; IKK1)	27	O15111	84.6	> 170 incl. stack
Kelch repeat and BTB domain-containing protein 10	10	O60662	68.0	90 - 170
Myristoylated alanine-rich C-kinase substrate (MARCKS)	4	P29966	31.5	70 - 90
Serine/threonine-protein kinase 36	3	Q9NRP7	143.9	170 - stack
Serine/threonine-protein kinase PLK2	4	Q9NYY3	78.2	70 - 90
Serine/threonine-protein kinase SRPK2	2	P78362	77.5	90 - 120
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PP-1A)	13	P62136	37.5	50 - 70 and stack
Signal transducer and activator of transcription 2 (p113) (STAT2)	2	P52630	97.9	120 - 170
Transcriptional-regulating factor 1 (TReP-132)	3	Q96PN7	132.2	stack
WD repeat protein 13 (WDR13)	4	Q9H1Z4	53.7	stack
Possible RNF4 substrates after Canavan./MG132 stress	**Peptides**	**Accession**	**M_w [kDa]**	**M_w range [kDa]**
5'-AMP-activated protein kinase subunit gamma-2	16	Q9UGJ0	63.0	> 120 incl. stack
Centrosomal protein of 131 kDa (Cep131 protein)	5	Q9UPN4	122.0	120 - 170
DNA topoisomerase 2-beta	3	Q02880	183.2	stack
Dual specificity protein phosphatase 16 (DUS16) (MAP kinase phosphatase 7) (MKP-7)	2	Q9BY84	73.1	170 - stack
GABA(A) receptor-associated protein (GABARAP)	7	O95166	13.9	70 - 90
Homeobox prospero-like protein PROX1 (PROX 1)	12	Q92786	83.2	> 70 incl. stack
I kappa-B kinase alpha (IKK-alpha; IKK1)	50	O15111	84.6	> 50 incl. stack
Kelch repeat and BTB domain-containing protein 10	22	O60662	68.0	120 - stack
Mitogen-activated protein kinase 14 (MAP kinase p38)	3	Q16539	41.3	170 - stack
N-myc-interactor (Nmi) (N-myc and STAT interactor)	5	Q13287	35.0	170 - stack
Probable transcription factor PML (TRIM 19)	4	P29590	97.5	170 - stack
Serine/threonine-protein kinase 36	5	Q9NRP7	143.9	170 - stack
Serine/threonine-protein kinase PLK2	7	Q9NYY3	78.2	> 120 incl. stack
Serine/threonine-protein kinase TAO3	3	Q9H2K8	105.3	120 - 170
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PP-1A)	18	P62136	37.5	> 50 incl. stack
WD repeat protein 13 (WDR13)	8	Q9H1Z4	53.7	> 70 incl. stack

4. DISCUSSION

This work aimed to identify a human ubiquitin ligase targeting SUMO conjugates (ULS) for degradation. In order to demonstrate that possible candidates are ULS proteins, an *in vitro* ubiquitylation assay for SUMO conjugates has been developed. For that purpose, SUMOylated proteins were generated and purified as *in vitro* substrates from *E. coli*. While establishing an efficient method for modifying proteins with SUMO in *E. coli* and their purification, an intrinsic SUMO E3 activity was observed for PML (discussed in section 4.1).
SUMOylated PML was efficiently *in vitro* ubiquitylated by RNF4, a human RING finger protein which complements yeast ULS deletion phenotypes (discussed in section 4.3). Binding of RNF4 to its substrate and substrate ubiquitylation was strictly SUMO dependent such that RNF4 was indeed identified as a human ULS protein. Investigation of the SUMO binding properties of RNF4s SIM domain revealed that the interaction was specifically enhanced by the presence of SUMO chains of more than two SUMOs. Further, a SIM type specific binding mode was noticed for different SUMO paralogs (discussed in section 4.2). Finally, in an attempt to find other ULS regulated cellular proteins, an RNF4 SIM domain construct was used to isolate poly- or multi-SUMOylated proteins from cells subjected to diverse cell stresses (see outlook section 4.3.4).

4.1 PML might act as a SUMO-E3 ligase

PML is mainly known to promote PML nuclear body formation (Shen, 2006). However, besides this important function, a role for PML as SUMO E3 ligase was speculated due to a observed stimulation of SUMOylation in yeast. This activity was first detected while co-transformed with SUMO-1 and depended on an intact PML RING domain as the C72A mutant failed to self-SUMOylate efficiently (Quimby, 2006). Additionally, endogenous SUMOylation of Smt3 was also stimulated by PML transformation (Quimby, 2006; Weisshaar, 2008).
While testing several SUMO E3 ligases for efficient PML (50-179) SUMOylation in the *E. coli* SUMOylation system established during this work, it became apparent that PML (50-179) modification with SUMO-3 is most efficient without the coexpression of an additional SUMO E3 ligase (see Figure 3.11). Contrary, SUMO-1 modification was most efficient during coexpression of PIAS4. The simplest explanation for SUMOylation in absence of an additional E3 ligase would be that Ubc9 can directly bind to the SUMOylation consensus motifs in its substrate proteins (Sampson, 2001; Bernier-Villamor, 2002). However, another possibility could be that Ubc9~SUMO is recruited to the PML RING by forming an E2-E3 complex, thereby leading to SUMOylation of PML (50-179). An additionally expressed SUMO E3 ligase would then compete for

Ubc9~SUMO binding leading to a decrease of PML (50-179) SUMOylation. As this was observed in the case of SUMO-3 modification, PML may act as a SUMO E3 ligase, probably with a preference for SUMO-2/3.
This finding confirms the observations made in yeast (Quimby, 2006; Weisshaar, 2008) and is further supported by a study which showed that Ubc9 indeed interacts with the RING domain of PML (Duprez, 1999). This interaction depends on the structural integrity of the PML RING but not on an intact K65 SUMOylation site. In line with this study, an overall reduction of SUMOylation in *E. coli* of the single PML (50-179) lysine mutants K65R and K160R was not observed, suggesting that the intrinsic SUMO E3 ligase activity is neither dependent on single consensus motifs nor influenced by SUMO modification of the RING or the B1 domain in this short construct. However, in full length PML-L, these two sites have been shown to influence the SUMO conjugation of each other (Lallemand-Breitenbach, 2008). These contradicting results may reflect a regulatory mechanism which is either mediated by additional proteins in cells or by inter-/intramolecular interactions of full length PML. An accelerated self-SUMOylation via a SIM-mediated SUMOylation in *cis* (Lin, 2006; Knipscheer, 2008; Meulmeester, 2008; Cho, 2009) can be excluded as well in the case of the construct PML (50-179) which does not contain a SIM.
Other TRIM proteins have been already shown to display E3 ligase activity, in most cases mediating ubiquitylation. For instance, TRIM5α monoubiquitylates itself (Yamauchi, 2008), TRIM11 is implicated in the turnover of the neuroprotective Humanin (Niikura, 2003), TRIM18/MID1 targets the phosphatase 2A for degradation (Trockenbacher, 2001) and TRIM25/Efp has a dual role as ubiquitin and ISG15 ligase (Urano, 2002) (Zou and Zhang, 2006). For TRIM23/ARD1, in vitro polyubiquitylation has been shown (Vichi, 2005) and TRIM24/TIF1α has been shown to ubiquitylate p53 (Allton, 2009).
In the case of PML, a ubiquitin ligase activity has not been published yet and could neither be observed in the *in vitro* ULS candidate activity test (see Figure 3.14). Therefore, the observed stimulation of SUMOylation by PML in yeast and *E. coli* seems to be a specific process and not a matter of forced RING/E2 interactions.
Whether PML might exert SUMO E3 activity towards other proteins has to be further analyzed either *in vitro* or by using the *E. coli* SUMOylation system, which lacks other SUMO E3 ligases compared to the alternative systems of human cell cultures or trans-vivo systems in yeast. As negative control, a mutant defective in Ubc9 binding could be used analogous to the RING mutants already investigated to abolish E2 binding in the ubiquitin system (for instance the W408 or I383 mutants of c-Cbl; Joazeiro, 1999; Zheng, 2000).
As this work concentrated on the identification of human ULS proteins and the reconstitution of ULS activity *in vitro*, a final conclusion concerning the SUMO E3 ligase activity of PML as well as putative substrates remain to be elusive.

Of note, while performing the last corrections of this thesis, another group reported that the PML RING interacts to Ubc9 and that this interaction is enhanced by the replacement of the zinc atoms within the PML RING and B2 zinc fingers with arsenic after treatment with ATO. This replacement triggered a conformational change of the PML RBCC motif which enhanced PML oligomerization (Zhang, 2010). A modeled structure of the PML RING-Ubc9 binding interface is depicted in Figure 4.1. An increased Ubc9 binding to PML triggered by direct binding of arsenic to the PML RING also solves the mechanism by which ATO leads to enhanced SUMOylation of PML thereby inducing the SUMO-dependent ubiquitylation of PML mediated by RNF4.

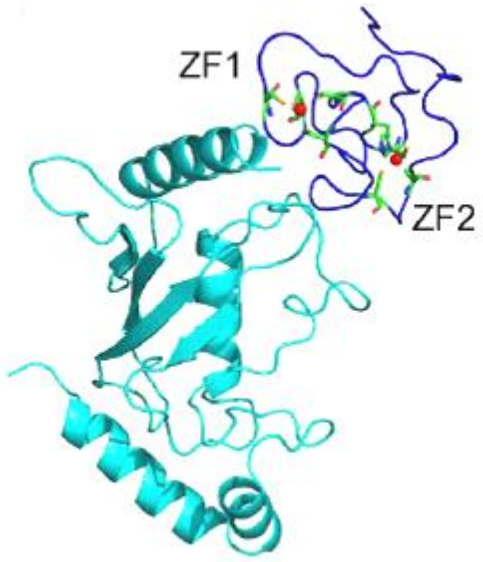


Figure 4.1: Predicted structural model of the Ubc9/PML RING complex
Computational simulation of a Ubc9/PML RING binding mode on the basis of the UbcH7/c-Cbl structure (PDB ID: 1FBV). UBC9 is shown in cyan; the PML RING is displayed as a blue cartoon structure. The two zinc ions in the PML RING are indicated by two red balls. The cysteines and histidine coordinated with zinc ions are shown as colored sticks. The model was taken from (Zhang, 2010).

4.2 Specificities in SUMO/SIM interactions

In section 3.6, several SIMs of different proteins have been analyzed for their ability to interact with different SUMO paralogs in comparison to the RNF4 SIM domain construct (1-105). Under the chosen conditions in an *in vitro* binding assay, all tested SIMs (type a or type b) were found to bind to SUMO-1 while only proteins containing type b SIMs also bound to SUMO-2 and SUMO-3 (see Figure 3.31). This suggests that SUMO-2 and SUMO-3 are able to discriminate between different SIM types with a strong preference for type b SIMs. Although this preliminary conclusion needs a more detailed investigation, several hints in the literature support this notion. Most type a SIMs investigated so far for direct interactions with SUMO were only analyzed for SUMO-1 binding, including the SIMs of PML and Daxx (Lin, 2006; Shen, 2006). In contrast, type b SIMs have been shown to directly interact with SUMO-1 as well as with SUMO-2/3, including SIMs of the PIAS proteins, MCAF1 and Usp25 (Song, 2004; Song, 2005; Meulmeester, 2008; Sekiyama, 2008).

NMR spectroscopy or X-ray crystallography structural studies are only available for the type b SIMs of PIAS2 bound to SUMO-1 and MCAF1 bound to SUMO-2 (see Figure 4.2) as well as for a type r SIM of RanBP2 bound to SUMO-1 (Song, 2005; Reverter and Lima,

2005; Sekiyama, 2008). These structures show that the type r SIM is indeed binding SUMO in a reversed orientation compared to the type b SIMs. The closely related type b SIMs of PIAS2 and MCAF1 display similar binding interfaces while interacting to SUMO-1 and SUMO-2, respectively. The highly conserved aspartate residue at position three of the SIM type b core contacts a lysine residue conserved in all human SUMO paralogs. This lysine residue (K32 in SUMO-3) is one of the *in vitro* ubiquitylation targets of RNF4 identified which emphasizes the idea that only the terminal SUMO moiety in the targeted SUMO chain is modified with ubiquitin as the K32 residues of other SUMO moieties might be masked by the SIMs of RNF4.

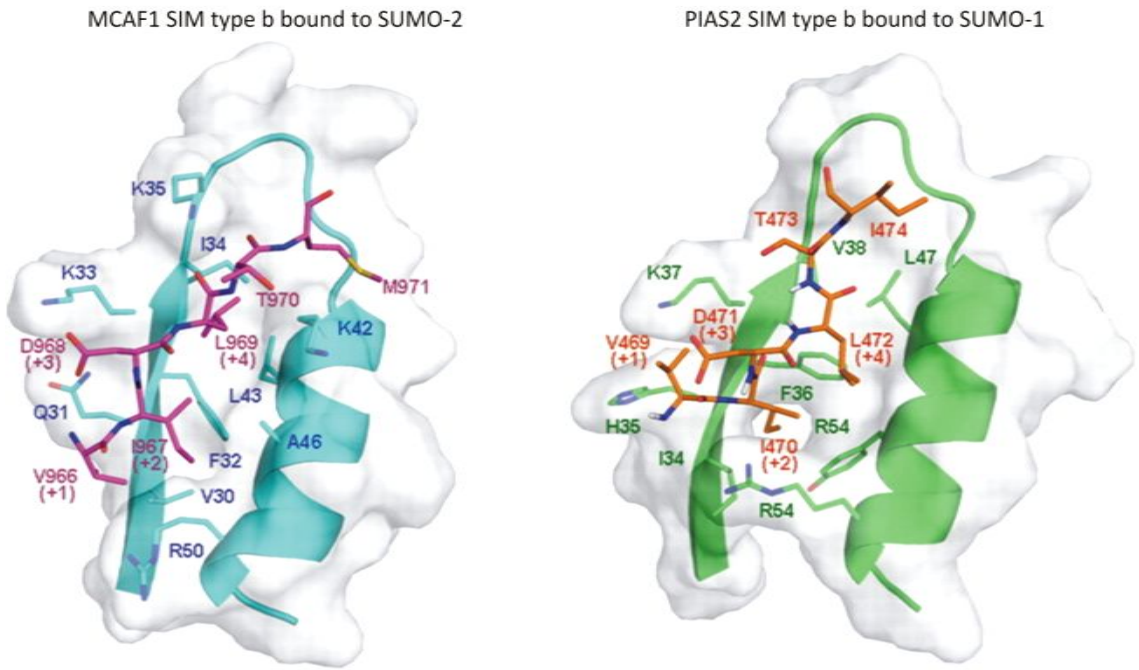


Figure 4.2: Molecular interfaces between type b SIMs and different SUMO paralogs
Molecular interfaces between the type b SIM of MCAF1 (magenta) and SUMO-2 (cyan) (PDB ID: 2RPQ) as well as between the type b SIM of PIAS2 (orange) and SUMO-1 (green) (PDB ID: 2ASQ). Amino acid residues that are involved in the interactions are shown in stick representation. The images were taken from (Sekiyama, 2008).

In an attempt to assess the binding properties towards different SUMO paralogs of the RNF4 (1-105) SIM domain construct in comparison to the type b SIM of PIAS3, a slight binding preference for SUMO-2 was observed in both cases (see Figure 3.32). This is supported by several studies in which type b SIMs seem to prefer binding to SUMO-2/3 over SUMO-1 (Song, 2004; Meulmeester, 2008; Sekiyama, 2008).
A SUMO E3 ligase encoded by Kaposi's sarcoma associated herpesvirus (KSHV), K-bZIP, harbors a type b SIM that seems to only recognize SUMO-2/3 and not SUMO-1 (Chang, 2010). This SIM displays the archetypical V-I-D-L-T sequence of most type b SIMs. The only obvious difference to other SUMO-1 binding SIMs is the lack of the acidic stretch behind the core motif. However, deletion of this stretch in Usp25 lowered the affinity to SUMOs, especially to SUMO-1, dramatically but binding was still detectable

(Meulmeester, 2008). Previously, it has already been suggested that the acidic stretch in human SIMs supports mainly SUMO-1 binding, while SUMO-2/3 binds both to SIMs with and without acidic regions (Hecker, 2006). This was based on the unusual type b SIM of TTRAP (I-V-D-V-W) that lacks an acidic stretch and the deletion mutants of acidic stretches of type a and b SIMs. This study is also the only one that detected a direct interaction of a type a SIM with SUMO-2 and SUMO-3 in an *in vitro* binding assay which is contrasting the results shown in Figure 3.31. However, this paradigm might be due to different experimental setups and in order to elucidate this question, more investigations concerning this matter are needed. Direct binding measurements by ITC might help to dissect different binding affinities for different SIM/SUMO combinations. Nonetheless, these biochemical studies need to be analyzed also in terms of functional consequences of these findings. One such example is the SUMOylation of Usp25 which depended on its type b SIM. This *in cis* E3-like SUMOylation results in SUMO-2 specific modification of Usp25 and in the inactivation of its hydrolase activity towards tetra-ubiquitin chains (Meulmeester, 2008). However, whether the paralog-specific attachment of SUMO-2 was important for that inactivation or only SUMO modification *per se* was not assessed. Further, investigation of SIM/SUMO paralog specificity in living cells might reveal tightly regulated processes similar to the differential signaling potential of ubiquitin chains.

4.3 RNF4 is a ubiquitin ligase for SUMO conjugates

The data presented in sections 3.4 to 3.6 demonstrate that RNF4 is a human SUMO-dependent ubiquitin ligase involved in the control of PML stability, suggesting that the ULS pathway is conserved to humans. While this work was in progress, two other studies have confirmed these results *in vitro* and in cells (Lallemand-Breitenbach, 2008; Tatham, 2008).

4.3.1 PML-NBs - a place for SUMO-dependent degradation in mammalian cells

Several studies pointed to the conservation of the ULS pathway in humans (Kosoy, 2007; Prudden, 2007; Sun, 2007; Uzunova, 2007; Weißhaar, 2008). This pathway includes an upregulation of SUMO conjugation upon diverse stress stimuli and seems to implicate SUMO chain formation (Saitoh and Hinchey, 2000; Zhou, 2004). Proteasomal inhibition in HeLa cells stabilized specifically SUMO-2/3 conjugates (Uzunova, 2007).

To investigate the human ULS pathway, HeLa cells transfected with myc-tagged SUMOs or their non-conjugatable ΔGG mutants have been treated with the proteasomal inhibitor MG132. Conjugates of all three SUMO paralogs accumulated at PML nuclear bodies (see section 3.1). Proteasome inhibition was already shown to induce the accumulation of SUMO-1 conjugates at PML-NBs (Bailey and O'Hare, 2005) and

downregulation of SENP6, the SUMO protease recognizing SUMO-2/3 chains, resulted in increased PML-NB size and number (Mukhopadhyay, 2006) which suggests that also SUMO-2/3 conjugates and therefore putative ULS substrates accumulate at PML nuclear bodies. Together with the fact that PML was probably regulated via the ULS pathway upon arsenic trioxide treatment (Lallemand-Breitenbach, 2001; Weißhaar, 2008), it was tempting to speculate that PML nuclear bodies might be a place for SUMO-dependent degradation in human cells.

Several PML nuclear body proteins could be identified as putative ULS proteins, among them RNF4 (see section 3.3.2) that could be verified as ULS (as discussed in the following sections). Further transient transfection studies in human cells could indeed show that ATO and RNF4 act synergistically in PML nuclear body dispersal, accompanied by a proteasomal degradation of SUMOylated PML species (Lallemand-Breitenbach, 2008; Weisshaar, 2008). Conversely, RNAi-mediated depletion of RNF4 abolished ATO induced turnover of PML and the dispersal of PML nuclear bodies (Lallemand-Breitenbach, 2008; Tatham, 2008). This SUMOylation-dependent turnover is also induced upon ATO treatment of APL cells which targets the oncogenic PML-RARα fusion protein for degradation and drives the leukemic cells to differentiation (Lallemand-Breitenbach, 2001; Lallemand-Breitenbach, 2008). However, ATO induced not only the recruitment of RNF4 but also of endogenous SUMOs, ubiquitin, the 20S core particle of the 26S proteasome as well as Sp100 and Daxx to PML nuclear bodies (Lallemand-Breitenbach, 2008). Recruitment of ubiquitin and proteasomal subunits has been observed previously during several cellular stimuli, including proteasome inhibition and IFN-γ stimulation (Fabunmi, 2001; Bailey and O'Hare, 2005).

Apart from ATO-induced degradation of PML, RNF4 seems to be generally involved in the overall stability of PML-NBs as they increase in size and number during RNAi-mediated RNF4 depletion (Lallemand-Breitenbach, 2008; Tatham, 2008).

Altogether, this indicates that the SUMO-dependent degradation pathway represents a specialized form of post-translational modification that is mediated at PML nuclear bodies.

4.3.2 RNF4 ubiquitylates specifically SUMOylated PML species

The ULS *in vitro* assays established in this work (see sections 3.4 to 3.6) clearly demonstrate that RNF4 is a specialized ubiquitin ligase that recognizes and modifies SUMOylated PML while unmodified PML is not ubiquitylated. The use of the purified in *E. coli* SUMOylated substrates and an efficient ubiquitylation reaction allowed the detection of the ubiquitylated species directly by western blotting (see for example Figures 3.19 and 3.20).

In contrast, the ubiquitylation of *in vitro* SUMO modified PML used in another study could only be detected as a smear after γ-counting of I^{125}-ubiquitin (Tatham, 2008). This study also demonstrates that RNF4 ubiquitylates only SUMO-modified PML and thus is a human ULS. *In vitro*, a preference of RNF4 for recognizing a site-specific SUMOylation on

PML could not be detected (compare ubiquitylation of PML mutants in Figure 3.21). The PML (50-179) K65R and K160R mutants are both ubiquitylated efficiently with a slight preference for the SUMOylation on K65 in the K160R mutant. Conversely, studies with full length PML-L in human cells showed that SUMOylation at K160 is important for ATO-induced degradation and suggested that RNF4 is recruited site-specifically to K160-SUMOylated PML (Lallemand-Breitenbach, 2001; Lallemand-Breitenbach, 2008). The latter study also dissected that mutation of K160 completely blocked SUMOylation of K65 and that mutation of K65 impairs SUMOylation of K160, pointing to a regulated interdependence of these SUMOylation sites in full-lentgh PML isoforms. The authors suggested that SUMOylation of K65 recruits Ubc9~SUMO thioesters in order to promote SUMO chain formation on K160 on the basis of the previously described mechanism (Tatham, 2003; Knipscheer, 2007). Thus, an impaired SUMO chain formation on K160 as well as the block of K65 SUMOylation could both result in the loss of RNF4 recruitment to PML and therefore to the observed resistance to ATO-induced PML degradation. The use of the efficiently SUMOylated PML (50-179) K65R and K160R mutants generated in *E. coli* already demonstrated that at least *in vitro*, RNF4 is recruited to either SUMOylated site of PML. Which of the discussed scenarios is indeed predominant *in vivo* remains to be discovered.

SENP1/6 treatment showed that RNF4 preferentially ubiquitylated the SUMO moieties of SUMOylated PML (50-179) (see Figure 3.22). Lysines 11, 32 and 41 of the SUMO-3 moiety were identified as attachment sites by mass spectrometry (see Table 3.1) while ubiquitylation of the PML (50-179) moiety was not detected. *In vitro* chain formation involving K42 in SUMO-2 or K41 in SUMO-3 has been observed previously to a minor extend but whether these lysines are used *in vivo* remains to be elusive (Jeram, 2010). MS analysis performed from *in vitro* SUMOylated and ubiquitylated PML 560 confirmed that the SUMO moiety is indeed ubiquitylated predominantly at these three lysines (Tatham, 2008). Ubiquitylation of lysines in a defined region of PML (predominantly lysines 380, 400, 401 and 476) have been detected as well. These lysine residues are not present in the short PML (50-179) construct applied in the *in vitro* assays performed in this work and at least lysine 401 could also be identified as ubiquitin acceptor site after isolation of cells treated with ATO (Lallemand-Breitenbach, 2008). Taken together, these data rather point to a mixed ubiquitylation of both the SUMO and the PML moiety of the substrate, as long as RNF4 is in proximity of appropriate lysine residues. Yet, there is no need of acceptor lysines for ubiquitin attachment in the modified substrate proteins as demonstrated by the ubiquitylation of SUMO modified PML (50-179).

A study in *S. cerevisiae* showed that the ULS heterodimer Slx5-Slx8 seems to predominantly ubiquitylate the N-terminus of the last Smt3 molecule in a SUMO chain while neither the other Smt3 molecules nor the tested *in vitro* substrate were significantly ubiquitylated (Mullen and Brill, 2008). Whether this is also the case for RNF4-mediated ubiquitylation remains to be elucidated. The closely located ubiquitin attachment sites K11, K15 and K19 are also the three acceptor lysines for

polySUMOylation in yeast. Attachment of ubiquitin to one of these residues might not only result in proteasomal degradation of the substrate but also in a termination of further SUMO chain formation due to sterical inhibition of the other attachment sites.
In contrast to Smt3, the detected ubiquitin acceptor lysines in SUMO-3 are more widespread over the sequence (K11, K32 and K41). Still, at least K32 and K41 are in close proximity to each other in the native SUMO-3 molecule (or the corresponding residues K33 and K42 in the SUMO-2 molecule) and both are part either of the β-sheet or the α-helix that are involved in SIM binding (see Figure 4.2; Hecker, 2006). Assuming that RNF4 binding masks K32 and K41 of chain-linked SUMOs and that K11 is used as SUMO attachment site within the chains, it is tempting to speculate that RNF4 indeed ubiquitylates the last SUMO moiety (see also section 4.2).
In the model presented in Figure 4.3, the RNF4-mediated ubiquitylation of SUMOylated PML is depicted schematically. For simplification, only the variant is depicted in which the ubiquitin chain is assembled on top of the SUMO chain.

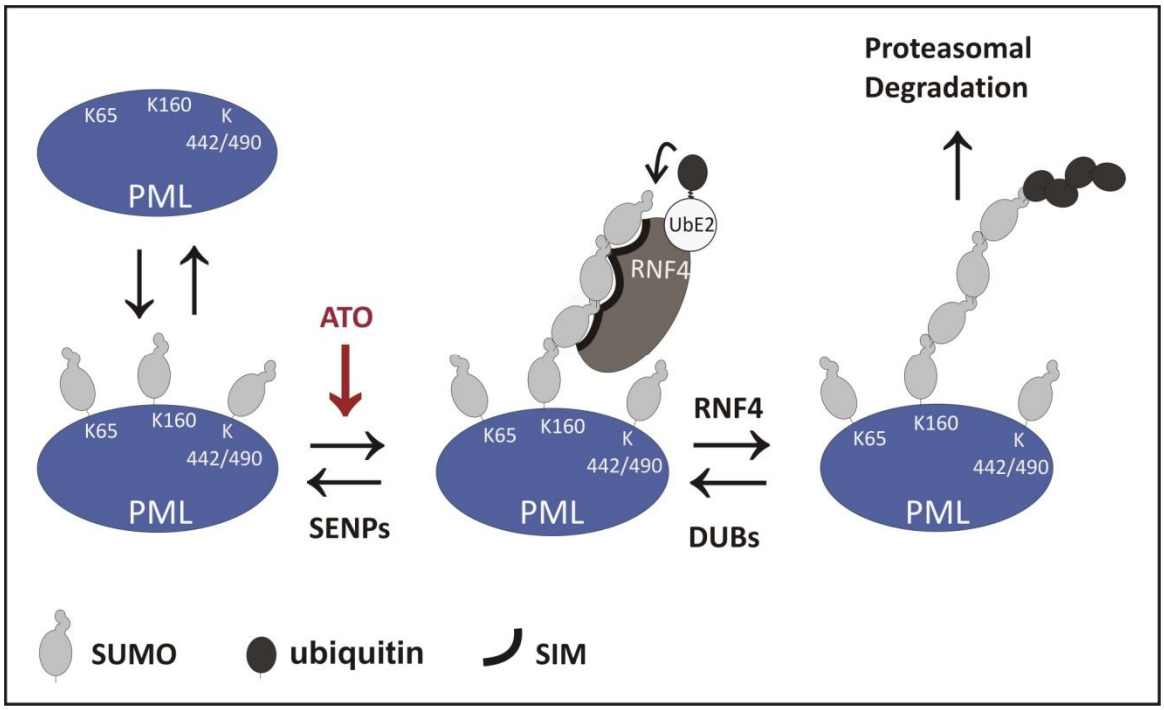


Figure 4.3: Model of RNF4-mediated ubiquitylation of SUMOylated PML
PML can be SUMOylated at three lysine residues, K65, K160 and K442/490 (left side). SUMOylation is a prerequisite for PML nuclear body formation (not depicted). Upon ATO treatment, RNF4-dependent PML turnover is accelerated due to an enhanced SUMO chain formation on PML, most probably at K160. RNF4 harbors three to four active SIMs which bind to the SUMO chain. The RING domain of RNF4 recruits the Ubiquitin E2 (UbE2) bound to an activated ubiquitin. Ubiquitin is then transferred to the K11 side chain of the terminal SUMO-2/3 moiety in the SUMO chain. Repeated cycles of ubiquitin attachment forms a K48-linked chain which serves as recognition signal for the proteasome and leads to the degradation of PML.

The ubiquitin chain targets the SUMO-ubiquitin-substrate hybrid conjugates to the proteasome. The mechanism of how these conjugates are unfolded at the proteasome and whether SUMO is recycled like ubiquitin by proteasome-associated SENPs or else degraded along with the substrate protein remains to be investigated. If it would be

degraded along with its substrate the proteasome had to be capable to cleave branched proteins which has not been shown yet.

4.3.3 RNF4 recruitment involves defined SUMO binding properties

The recruitment of RNF4 to SUMOylated proteins like PML is directed via the SIM domain of RNF4, the stretch of up to four SUMO interaction motifs in its N-terminus (refer to Figure 3.13 for protein sequence).
This was demonstrated by a range of experiments in this work as well as in the study of (Tatham, 2008). The most intriguing feature was the preference of RNF4 for SUMO chains: Both, gel filtration experiments with the N-terminal RNF4 SIM domain (shown in section 3.5) as well as binding assays with full length RNF4 performed by (Tatham, 2008) revealed that binding to chain-linked SUMOs is much stronger than binding to mono-SUMOs. While (Tatham, 2008) observed a preference of RNF4 for SUMO chains formed of at least three SUMOs, stable binding during gel filtration was already observed with di-SUMO chains. In order to elucidate the binding affinities of RNF4 for SUMO chains of different lengths, isothermal titration calorimetry has been performed. By applying this method, it could be strikingly shown that the biggest difference in binding affinity is achieved upon SUMO chain formation *per se*: The binding affinities of the RNF4 N-terminus (aa 1-125) to di-SUMOs are ~ 25-fold higher than those of RNF4 (1-125) to mono-SUMOs (compare Figures 3.28, 3.29 and see Table 3.2). A further extension of the SUMO chain from di- to tetra-SUMOs had no significant effect on the affinity (see Figures 3.27, 3.28 and Table 3.2). Moreover, the stoichiometry obtained for mono-SUMO binding indicates that there may be more than one SUMO binding site in the RNF4 SIM domain (see Table 3.2).
This binding mechanism resembles however the binding properties of some UBDs recognizing two ubiquitin molecules of a chain: Ubiquitin chain recognition is either conferred by the relative placement of two or more UBDs to each other and to the respective ubiquitin chain or by recognizing linkage-specific residues directly (like UBAN and NZF domains; (Raasi, 2005; Varadan, 2005; Husnjak, 2008; Schreiner, 2008; Komander, 2009c).
A similar conformational binding mode could be envisioned for RNF4 and its binding to SUMO chains: Obviously, as much as two SUMO molecules are sufficient to promote efficient binding of RNF4 SIMs. It might also be that the linkage to the lysine side chain is recognized by a sterical or another, yet unidentified mechanism. This is indicated by the striking difference in ubiquitylation efficiency of proper SUMOylated PML (50-179) and the artificial fusion construct of GST-SUMOs. Even the mono-SUMOylated species of PML are efficiently ubiquitylated while ubiquitylation of the GST-SUMO fusion is only mediated under unspecific low salt conditions (compare Figures 3.18 and 3.19). However, the artificial SUMO chains are also N-terminally linked to each other such that this theoretical excursion awaits a structural analysis of RNF4 bound to a SUMO chain for clarification.

Another aspect is the apparently regular arrangement of the SIMs in RNF4: They are arranged regularly within a stretch of 40 amino acids, each spaced by six, seven and four amino acids (see Figure 3.13), respectively. This arrangement seems to be perfect to bind to a tetrameric SUMO chain. Analysis of different single SIM mutants revealed however, that the two inner SIMs of this stretch, both of type b, are the most important ones for binding to tetra-SUMO-3 (Tatham, 2008). Mutation of the first SIM in the stretch (type r SIM) showed no effect on RNF4 binding properties while that of the fourth SIM had only minor effects (type a SIM). The mutation of the second RNF4 SIM displays the strongest effects on RNF4 binding properties (Tatham, 2008). This is somewhat puzzling when assumed that the two outermost SIMs are functional and could still bind to for instance the first and the last SUMO of a tetrameric chain. Moreover, because binding of two SUMOs seems to be sufficient for RNF4 recruitment (as discussed above), this mutant analysis also points to a mechanism where only the inner SIMs confer binding to SUMO chains and – at the same time – raises the possibility of a linkage-specific recognition.

On the other hand, the ITC measurements have been performed with the N-terminal half of RNF4 lacking the RING domain. Taking into account that RING domains usually form dimers or even oligomers, it is also a (if not the most) plausible explanation that RNF4 dimers would display an increased binding affinity to polymeric SUMO chains of more than two SUMOs. This scenario is similar to the binding of K48-linked tetra-ubiquitylated proteins to the UBDs of the 19S subunits Rpn10/S5a and Rpn13, both recognizing di-ubiquitin (Deveraux, 1994; Groll, 1997; Lam, 2002; Husnjak, 2008; Schreiner, 2008).

Such a coordinated binding mechanism might also explain the observations made in binding assays with full length RNF4 where only chains with a minimum of three SUMOs bound to RNF4 (Tatham, 2008). In order to address this question in more detail, this issue could be further investigated by ITC using full length RNF4 and the respective artificial polySUMOs.

ITC experiments also did not show huge differences in RNF4 (1-125) binding affinities regarding the SUMO paralogs investigated. An exception was the addition of tetra-SUMO-1ΔN15 which gave an odd binding behavior. Also, when mixing artificial tetra-SUMO-1ΔN15 chains with the shorter RNF4 N-terminal construct (1-105), the proteins aggregated and precipitated. Further, the *in vitro* ubiquitylation with full length RNF4 showed that also SUMO-1 modified PML (50-179) is recognized and ubiquitylated by RNF4. This indicates that at least *in vitro*, also mono-SUMOylation and multi-SUMOylation serves as a signal for RNF4 recruitment and subsequent ubiquitylation of SUMOylated PML species. It should be noted, however, that due to the dimerization of PML, the substrate of the in vitro reaction displays a multi-SUMOylation.

Nonetheless, RNF4 seems to preferentially ubiquitylate His_6-SUMO-3 modified PML (50-179) over His_6-SUMO-1 modified PML (50-179). This can only be explained by an overall intrinsic binding preference of RNF4 for SUMO-3 over SUMO-1, even in absence of a

SUMO chain. However, ITC experiments as well as other binding assays did not show any significant binding preference of the RNF4 SIM domain to one or the other SUMO paralog (see Table 3.2 and Figure 3.31). The only hint that RNF4 might prefer binding to SUMO-2/3 over SUMO-1 is given by Surface Plasmon Resonance: binding to SUMO-2 was two-fold stronger than binding to SUMO-1 (see Figure 3.31). A SUMO paralog-dependent SIM type preference might also play a role in the binding process (as discussed in section 4.2).

4.3.4 Outlook: Are ULS proteins master regulators of stress signaling?

Stress induction seems to be a key trigger for upregulated SUMO-2/3 modification and subsequent ubiquitylation in mammalian cells which can be observed after many stresses including heat, oxidative or osmotic stress, an induced unfolded protein response and treatment of cells with ethanol or ATO (Saitoh and Hinchey, 2000; Weisshaar, 2008; Meyer-Teschendorf, 2010).
In order to identify further proteins that are regulated via the ULS pathway, the binding properties of the RNF4 (1-105) SIM domain construct were used to isolate poly- (or multi-) SUMOylated proteins from cells after various stresses (see section 3.7). This method was at least partially successful, because peptides for PML, SUMO-2/3 and ubiquitin have been identified in isolated samples from MG132 treated cells and cells treated with a combination of canavanine and MG132 (see Appendix tables A.2 to A.8). Peptides of these proteins served as internal positive controls because PML is a known target of the ULS pathway and SUMO-2/3 and ubiquitin are prerequisites of it. Moreover, specifically SUMO-2/3 modified proteins could be detected after isolation with GST-RNF4 (1-105) (see Figures 3.33 and 3.34). In that respect, the RNF4 (1-105) construct could indeed be utilized for specifically isolating poly- or (multi-) SUMOylated proteins analogous to the TUBEs isolating ubiquitin chains (Hjerpe, 2009). TUBEs protect poly-ubiquitin-conjugated proteins both from proteasomal degradation and de-ubiquitylating activity in cell extracts (Hjerpe, 2009). Whether this kind of protection is also mediated by the RNF4 SIM domain construct is suggestive but remains to be shown.
Strikingly, many proteins involved in signaling were interacting with the RNF4 N-terminus. Some of them were found after each of the applied cellular stresses. For instance, IKK-1, part of the IκB kinase complex involved in the NFκB pathway (Solt and May, 2008), and the N-myc and STAT interactor Nmi, a scaffolding protein important for signal transduction of myc and STAT pathways (Shuai, 2000), were found in all stressed samples. In some samples, stabilization upon treatment with MG132 could be observed. Both molecules are usually localizing to the cytoplasm and if they are indeed ULS targets, they might rather be regulated by a ULS protein that resides in the cytoplasm. Alternatively, the stressed-induced (yet to be verified) SUMOylation of these proteins could induce their shuttling into the nucleus where they might be targets of RNF4-dependent ubiquitylation. To underline the latter hypothesis, a recent report showed

that IKKε shuttles into the nucleus upon genotoxic stress and localizes to PML-NBs where it is SUMOylated and phosphorylates target proteins (Renner, 2010).
Further, many other kinases and transcription factors could be isolated by GST-RNF4 (1-105), indicating that ULS proteins might be involved in many cellular pathways.
However, these results are only a first hint towards the identification of other ULS substrates. Data have to be validated by assessing the SUMO-dependent ubiquitylation of the individual proteins isolated, applying cellular and biochemical techniques.
It could well be that proteins upregulated by diverse stresses are not regulated by RNF4 but regulate RNF4: especially the high occurrence of isolated kinases might also reflect a phosphorylation induced regulation of RNF4 activity rather than a ULS regulation of kinases. Also, the isolation of the SUMO E3 ligase PIAS2 might reflect an indirect interaction that is mediated by a SUMO chain to which both, RNF4 (1-105) and PIAS2 bind to with their individual SIMs.
Still, some transcription factors were isolated, some of which showed a differential isolation pattern depending on the stress applied: Homeobox prospero-like protein PROX1, a negative transcriptional regulator involved in the control of energy homeostasis (Charest-Marcotte, 2010), has been predominantly found in samples of canavanine and canavanine/MG132 treated cells. This emphasizes the idea that upon different stress inductions, different signaling pathways and target genes are regulated via the ULS pathway, rendering ULS proteins like RNF4 as master regulators of stress signaling in human cells. That this might indeed be the case show examples of the transcription factors c-myb and BMAL1: C-myb has been shown to be extensively modified with SUMO-2/3 after heat stress (Sramko, 2006) but a connection to the ULS pathway remains to be shown. Yet, the circadian activated heterodimeric transcription factor BMAL1/CLOCK is a first example where the simultaneous modification of SUMO-2/3 and ubiquitin has been related to a function: BMAL1 (in humans 'aryl hydrocarbon receptor nuclear translocator-like protein 1' - ARNTL1) is subject to rhythmic conjugation with poly-SUMO-2/3, peaking at times of maximum circadian mediated transcription. Interestingly, SUMO-2/3 modification of BMAL1 is accompanied by a translocation of BMAL1 to PML-NBs while it simultaneously promotes BMAL1 transactivation and ubiquitin-dependent degradation (Lee, 2008). Thus, BMAL1 seems to be tightly regulated in its activation, probably ensuring a defined circadian transcription profile. This renders BMAL1 as another putative ULS substrate though a direct link to a ULS protein, for instance RNF4, has not been shown yet.
A true second RNF4 substrate has been reported recently. The inner kinetochore complex protein CENP-I has been found to be regulated by RNF4 in SENP6-depleted cells in order to promote proper assembly of the mitotic spindle and the metaphase plate (Mukhopadhyay, 2010). A binding partner of CENP-I, CENP-H could be identified by MS analysis as a RNF4 (1-105) interactor in canavanine treated cells (see Table 3.3).
Other SUMOylated proteins like Sp100 are probably not targets of RNF4, at least during the stress conditions applied. Sp100 was not identified in any sample isolated with GST-

RNF4 (1-105). Moreover, SUMOylated Sp100 seemed to be a poor *in vitro* substrate for RNF4 in comparison to PML (see section 3.4.5).

Interestingly, a second human protein, the Von Hippel-Lindau factor (VHL) was shown to display ULS activity, in this case towards the α subunit of the hypoxia inducible transcription factor (HIF1α) (Cheng, 2007). VHL is part of a ubiquitin ligase complex that is responsible for the specific ubiquitylation of prolyl-hydroxylated HIF1α under physiological oxygen levels. However, in SENP1-depleted mouse embryonic fibroblasts, VHL seem to target SUMOylated HIF1α for ubiquitylation and subsequent degradation. This finding is contrasting other reports that observed a SUMO-dependent stabilization of HIF1α (Bae, 2004; Carbia-Nagashima, 2007; Berta, 2007; Cai, 2010). These obviously contradicting observations might be solved by assuming a similar regulatory mechanism underlying the regulation of BMAL1, namely a consecutive cycle of SUMOylation and deSUMOylation and/or ubiquitylation in order to promote and deactivate a certain cellular trigger or stress signal, in this case during oxygen shortage.

In yeast, ULS proteins have been implicated in DNA damage control and chromosome maintenance, although the respective targets are still missing (Uzunova, 2007; Xie, 2007; Mullen and Brill, 2008; Wang and Prelich, 2009). The first *in vivo* target identified for the yeast ULS Slx5-Slx8 was Mot1, a transcriptional regulator (Wang and Prelich, 2009). This study also suggested a role for ULS proteins in the quality control system as an increased degradation of a Mot1 mutant protein as well as of wt Mot1 after canavanine treatment was observed. This parallels the finding in mammalian cells, where canavanine is a potent inducer of SUMO-2/3 conjugation (Meyer-Teschendorf, 2010). Furthermore, there are hints that also mating type switch is regulated by Slx5-SxI8, targeting the alpha-1 transcriptional activator for degradation (Nixon, 2010).

Taken together, ULS proteins seem to be involved in many cellular processes, ensuring the fine-tuned balance of SUMOylated proteins. Upon stress induction, the balance between SUMOylation and deSUMOylation is obviously shifted towards SUMOylation. ULS proteins target hyperSUMOylated proteins for degradation, indicating that it is important for the cell to eliminate these proteins rapidly. This might be explained by the assumption that SUMO chains are toxic by-products for the cell (Geoffroy and Hay, 2009). However, the fact that SUMO chains are assembled *in vivo* points towards a functional requirement for them and the example of BMAL1 illustrated that this is indeed the case.

APPENDIX

Oligonucleotides

PCR and mutation primer used to generate expression constructs
(numbers are according to the Oligonucleotide database of AG Praefcke):

17:
0017 - mUbc9-3'-XhoI
PCR Primer for XhoI-site insertion in MCS 2 of pRSF
CCGCTCGAGTTATGAGGGGGCAAACTTCTTCGCTTG

49:
0049 - hSP100 5' (aa 1) BamHI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
CGGGATCCATGGCAGGTGGGGGCGGCGAC

55:
0055 - hSP100 5' (aa 274) BamHI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
CGGGATCCCCAGAGGCAGAGCTACACAACCATGG

61:
0061 - hSP100 3' (aa 332) STOP EcoRI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
GGAATTCATTCAGAGTCCTCACTGCTGATGACTATTATG

62:
0062 - hSP100 3' (aa 480) STOP EcoRI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T- end of ORF
GGAATTCTAATCTTCTTTACCTGACCCTCTTCTTAGGG

65:
0065 - hPML11 5' (aa 1) BamHI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
CGGGATCCATGGAGCCTGCACCCGCC

66:
0066 - hPML11 5' (aa 50) BamHI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
CGGGATCCGAGGAGTTCCAGTTTCTGCGCTGCC

79:
0079 - hPML11 5' K65R
Mutation primer for aa change Sumoylation site Lysine 65 to Arginine
CAGGCGGAAGCCAGGTGCCCGAAGCTG

80:
0080 - hPML11 3' K65R
Mutation primer for aa change Sumoylation site Lysine 65 to Arginine reverse
CAGCTTCGGGCACCTGGCTTCCGCCTG

81:
0081 - hPML11 5' K160R
Mutation primer for aa change Sumoylation site Lysine 160 to Arginine
CAGTGGTTCCTCAGGCACGAGGCCCGG

82:
0082 - hPML11 3' K160R
Mutation primer for aa change Sumoylation site Lysine 160 to Arginine reverse
CCGGGCCTCGTGCCTGAGGAACCACTG

97:
0097 - hSAE2 5' (aa 4) Bgl II
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
GAAGATCTGTCGCGGGGGCTGCCCCGGG

98:
0098 - hSAE2 3' (aa 641) XhoI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
CCGCTCGAGGCGGCCGCTCAATCTAATGCTATGAC

99:
0099 - hSAE1 5' (aa 1) BamHI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
CGGGATCCATGGTGGAGAAGGAGGAGGCTGGCGG

117:
0117 - hPML11 5' (aa 419) BamHI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
(+ additional GS from BamHI)
CGGGATCCGATGTCTCCAATACAACGACAGCCCAGAAGAGG

118:
0118 - hPML11 3' (aa 503) STOP EcoRI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
GGAATTCACTCCCCGGCGCCACTGGCCACGTGG

119:
0119 - hPML11 3' (aa 522) STOP EcoRI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
GGAATTCACGAGTTTTCGGCATCTGAGTCTTCCGAGCTG

131:
0131 - hPIAS4 5' (aa1) -NdeI
NdeI primer for insertion into pETDUET-TEV-N v B 2.MCS (E3 enzyme)
GGAATTCCATATGGCGGCGGAGCTGGTGG

132:
0132 - hPIAS4 3' (aa 510) STOP-BglII
STOP-BglII 3 ' primer for insertion into pETDUET-TEV-N v B 2.MCS (E3 enzyme)
GAAGATCTCAGCAGGCCGGCACCAGG

137:
0137 - hTIF1-1 5' (aa 1) NcoI
PCR primer for insertion into pETDuet-GST-TevN and pGex-4TN
(ATG from Nco-site, 2. codon starts with G!!)
CATGCCATGGAGGTGGCGGTGGAGAAGGC

138:
0138 - hTIF1-1 3' (aa 1050) STOP NotI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
ATAGTTTAGCGGCCGCTATTTAAGCAACTGGCGTTCTTCAATGCTTTTGAGGCG

220:
0220 - hDaxx 3' (aa 740) EcoRI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
GGAATTCTAATCAGAGTCTGAGAGCACG

222:
0222 - hDaxx 5' (aa 625) BamHI
PCR primer for insertion into pETDuet-GST-Tev and pGex-4T
CGGGATCCTCTGGTCCCCCCTGCAAAAAATCTCG

234:
0234 - hPIAS4 5' (aa1) -NcoI
NcoI primer for insertion into pGEX TN
CATGCCATGGCGGCGGAGCTGGTGG

255:
0255 - hPIAS4 3' (aa 510) STOP-BamHI
STOP-BamHI 3 ' primer for insertion into pETDUET-TEV-N v B 2.MCS (E3 enzyme)
CGGGATCCTCAGCAGGCCGGCACCAGGC

343:
0343 - pGEX-TN-hSENP1 5' cat domain
5' - Mutation primer for generation of SENP1's catalytical domain only (deletion of N-terminus, aa1-414)
GGCGGATCCGAATTCATGGATAGTGAAGATGAATTTCCTG

344:
0344 - pGEX-TN-hSENP1 3' cat domain
3' - Mutation primer for generation of SENP1's catalytical domain only
(deletion of N-terminus, aa1-414)
CAGGAAATTCATCTTCACTATCCATGAATTCGGATCCGCC

417:
0417 - 3´SENP6 PCR primer (aa1112)
3' SENP6 PCR primer (aa1112)
CCGCTCGAGTCAATCTGAGATACTATTGACAC

419:
0419 - 5´SENP6-dN (SUSP1-Cat, aa625-1112)
5' SENP6-dN (aa625-1112) PCR primer (bp1900)
CGGGATCCATGAGAAGCAAACAAGAATTTCAG

431:
0431 - 5´ZNF198 PCR primer BamHI
PCR primer with BamHI-cut
CGGGATCCATGGACACAAGTTCAGTGGGAGGA

432:
0432 - 3´ZNF198 PCR primer NotI
PCR primer with NotI-cut
ATAGTTTAGCGGCCGCTTAGTCTGTGTCTTCATCC

447:
0447 - 5´RNF4 BamHI
PCR Primer for insertion into pETDuet-GST-Tev and pGEX (!without methionine!)
CGGGATCCACAAGAAAGCGTCGTGGTGGAGC

448:
0448 - 3´RNF4 EcoRI
PCR Primer for insertion into pETDuet-GST-Tev and pGEX
TCGAATTCATATATAAATGGGGTGGTACCG

471:
0471 - 5´Ubiquitin EcoRI
CCTGGTATACGCTAACAGGTCAAAGAATTCATGCAGATCTTCG

472:
0472 - 3´Ubiquitin XhoI
CCGCTCGAGTCAACCACCTCTCAGACGCAGGACCAGG

614:
0614 - 3' PML-RB1 with STOP (aa 179)
EcoRI-PCR-Primer for insertion in pET-Duet und pGEX
GGAATTCAGTCCAGGAACTCACGCACCGACTGGTTGCGC

620:
0620 - 3'-RNF4 (1-105)
3' EcoRI-Primer for cloning of RNF4 aa1-105
GGAATTCAGTCCCTGTCCCTGGACAACTCCTCATCGTC

622:
0622 - 5' - PIAS4 SIM BamHI-Primer
for cloning of PIAS4 aa 445 - 510
CGGGATCCACGGGTGGCGGCGGCC

623:
0623 - 3' - PIAS4 SIM EcoRI-Primer
for cloning of PIAS4 aa 445 - 510
GGAATTCAGCAGGCCGGCACCAGGCC

624:
0624 - 3' - PIAS3 SIM EcoRI-Primer
for cloning of PIAS3 aa 394 - 470
GGAATTCAAGGTAGGGCCGGGATGGCAGC

625:
0625 - 5' - SUMO2-dN11 BamHI-Primer
for cloning of polySUMO2
CGGGATCCACTGAGAACAACGATCATATTAATTTGAAGGTGGCG

626:
0626 - 3' - SUMO2-dN11 Bgl II-Primer
for cloning of polySUMO2
GAAGATCTACCTCCCGTCTGCTGTTGGAACACATC

627:
0627 - 5' - SUMO3-dN11 BamHI-Primer
for cloning of polySUMO3
CGGGATCCACAGAGAATGACCACATCAACCTGAAGGTGG

628:
0628 - 3' - SUMO3-dN11 Bgl II-Primer
for cloning of polySUMO3
GAAGATCTACCTCCCGTCTGCTGCTGGAACACG

629:
0629 - 5' - SUMO1-dN15 BamHI-Primer
for cloning of polySUMO1
CGGGATCCAAGAAGGAAGGTGAATATATTAAACTCAAAGTCATTGGAC

630:
0630 - 3' - SUMO1-dN15 Bgl II-Primer
for cloning of polySUMO1
GAAGATCTACCCCCCGTTTGTTCCTGATAAACTTCAATCACATC

635:
0635 - 5' mutagenesis S517A in PML
Mutagenesis primer for S517A
GATCAGCAGCTCGGAAGACGCAGATGCCGAAAACTCG

636:
0636 - 3' mutagenesis S517A in PML
Mutagenesis primer for S517A
CGAGTTTTCGGCATCTGCGTCTTCCGAGCTGCTGATC

734:
734 - 5'- PML 11 SSS 512-514 DDD
Mutagenesis primer for SSS 512-514 DDD SIM mutation
GGAACGCGTTGTGGTGATCGACGACGATGAAGACTCAGATGCCG

735:
735 - 3'- PML 11 SSS 512-514 DDD
Mutagenesis primer for SSS 512-514 DDD SIM mutation
CGGCATCTGAGTCTTCATCGTCGTCGATCACCACAACGCGTTCC

736:
736 - 5'- PML 11 S 517 D
Mutagenesis primer for S 517 D SIM mutation
GGTGATCGACGACGATGAAGACGATGATGCCGAAAACTCGTG

737:
737 - 3'- PML 11 S 517 D
Mutagenesis primer for S 517 D SIM mutation
CACGAGTTTTCGGCATCATCGTCTTCATCGTCGTCGATCACC

738:
738 - 5'- PML 11 SSS 512-514 AAA
Mutagenesis primer for SSS 512-514 AAA SIM mutation into PML S517A
GGAACGCGTTGTGGTGATCGCCGCCGCGGAAGACGCAGATGCCG

739:
739 - 3'- PML 11 SSS 512-514 AAA
Mutagenesis primer for SSS 512-514 AAA SIM mutation into PML S517A
CGGCATCTGCGTCTTCCGCGGCGGCGATCACCACAACGCGTTCC

782:
782-3'- EcoRI-Primer for cloning of RNF4 aa1-125
GGAATTCACCTGAGGCCTGTAGCGCCC

ZNF198 zinc fingers (present in one 'domain' from aa 330-795)

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KVTCANCKKPLQKGQTAYQRKGSAHLF--CSTTCLSSFSHKPA----PKKLCVMCKKDITTMKGTIVAQVDSSESFQEFCSTSCLSL
KSRCTICGKLTEIRHEVSFKNMTHKL---CSDHCFNRYRMANG---LIMNCCEQCGEYLPS-KGAGNNVLVIDGQQKRFCCQSCVSE
LTTCTGCRTQCRFFDMTQCIGPNGYMEPYCSTACMNSHKTKYAKSQSLGIICHFCKRNSLP----QYQATMPDGKLYNFCNSSCVAK
QLKCNYCKNSFCSKPEILEWENKVHQF--CSKTCSDDYKKLHC----IVTYCEYCQEEKT-----LHETVNFSGVKRPFCSEGCKLL
GLRCVTCNYCSQLCKKGATKELDGVVRDFCSEDCCKKFQDWYY----KAARCDCCKSQGT-----LKERVQWRGEMKHFCDQHCLLR
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Figure A.1: Zinc finger alignment of ZNF198
Zinc chelating residues in the RING domains are depicted in gold.

In the follwoing Tables A.2-A.8, the results of mass spectrometry results are summarized after isolation of cellular proteins binding to GST-RNF4 (1-105). Protein hits with more than two peptides found/protein and an ion score of > 20 have been assessed for possible SUMOylation sites by using a prediction program. Proteins with high probability motifs are listed in black, proteins with a low probability of being SUMOylated are listed in grey.

Table A.1: Ubiquitin-like modification systems

Ubl human (S.c.)	E1	E2	E3	Protease	Function	References
Ubiquitin	Ube1, Ube1L2	38, refer to Table A 1	600-1000	Many; 5 subgroups	Protein degradation, signalling, receptor endocytosis, etc.	(Komander, 2009a; Komander, 2009b; Ye, 2009)
SUMO (Smt3)	SAE1/SAE2 (AOS1/UBA2)	Ube2I (Ubc9)	Many; PIAS family, RanBP2, Pc2/CBX4, HDAC7	SENP1,2,3,5,6,7	Protein localization and interaction, DNA repair, signal for protein ubiquitylation	(Xu , 2009; Bergink and Jentsch, 2009; Garcia-Dominguez and Reyes, 2009; Uzunova , 2007)
NEDD8 (Rub1)	NAE1/NAE2 (APPBP1/UBA3)	Ube2M (Ubc12), Ube2F	Dcn1, Mdm2, c-Cbl, FBXO11	UCH-L3, NED1P/SENP8, CSN5	Regulation of Cullin-based ubiquitin ligases, inhibition of p53/p73 transcriptional activity, stabilization of ribosomal proteins	(Rabut and Peter, 2008; Huang , 2009)
ISG15	Ube1L	Ube2L6 (UbcH8), Ube2E1 (UbcH6)	Herc5, EFP (TRIM25), Hhari	UBP43/USP18 USP2, USP5, USP13, USP14	Innate antiviral response	(Sadler and Williams, 2008)
FAT10	Ube1L2 (UBA6)	unknown	unknown	unknown	proteolysis	(Groettrup , 2008)
GABARAPs, MAP-LC3s (Atg8)	Atg7	Atg3	ATG12-Atg5/ Atg16 multimeric complex?	Atg4	Modifies lipids (such as PE) to form and elongate autophagosomes.	(Geng and Klionsky, 2008)
Atg12	Atg7	Atg10	none	none	Modifies Atg5 to recruit Atg16. The complex is involved in autophagic vesicle elongation; possible E3 ligase for Atg8	(Geng and Klionsky, 2008)
Urm1 (Urm1p)	UBA4		Ncs6p		Sulfur carrier for the modification of tRNAs; regulates translation fidelity	(Pedrioli , 2008)
FUBI	unknown	unknown	unknown	unknown	unknown	(Michiels , 1993)
Ufm1	UBE1DC1/UBA5	Ufc1	Ufl1	UfSP1 and UfSP2	ER stress	(Komatsu , 2004; Azfer , 2006; Kang , 2007; Tatsumi , 2009)
Ubl5 (HUB1)	unknown/none	unknown/none	unknown/none	unknown/none	pre-mRNA splicing	(Wilkinson , 2004; Yashiroda and Tanaka, 2004)
Mycobacterial Pup	Dop	none	PafA	unknown	Proteasomal degradation, resistance against NO host defence	(Burns and Darwin, 2010)

Table A.2: Summary of mass spectrometry analysis of isolated proteins interacting with GST-RNF4 (1-105) from MG132 treated cells

RNF4 (1-105) interacting proteins after MG132 treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
F-box/LRR-repeat protein 19 (F-box and leucine-rich repeat protein 19)	2	26,68	Q6PCT2	73,7	70 - 90
Forkhead box protein D1 (Forkhead-related protein FKHL8) (Forkhead-related transcription factor 4) (FREAC-4)	3	23,74	Q16676	46,1	50 - 70
Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (GAPDH)	2	45,50	P04406	36,0	stack
Histone H2A type 1-H	2	40,08	Q96KK5	13,9	stack
Hydroxyacid oxidase 2 (EC 1.1.3.15) (HAOX2) ((S)-2-hydroxy-acid oxidase, peroxisomal)	4	30,22	Q9NYQ3	38,8	stack
Inhibitor of nuclear factor kappa-B kinase subunit alpha (EC 2.7.11.10) (I kappa-B kinase alpha) (IkBKA) (IKK-alpha) (IKK-A) (IkappaB kinase) (I-kappa-B kinase 1) (IKK1)	7	23,48	O15111	84,6	stack
Junction plakoglobin (Desmoplakin-3) (Desmoplakin III)	9	70,23	P14923	81,6	stack
Leucine-rich repeat LGI family member 2 precursor (Leucine-rich glioma-inactivated protein 2) (LGI1-like protein 2)	6	25,81	Q8N0V4	62,3	170 - stack
Lysosome-associated membrane glycoprotein 1 precursor (LAMP-1) (CD107a antigen)	2	52,24	P11279	44,7	90 - 120
Lysyl oxidase homolog 2 precursor (EC 1.4.3.-) (Lysyl oxidase-like protein 2) (Lysyl oxidase-related protein 2) (Lysyl oxidase-related protein WS9-14) (LOXL2)	3	29,88	Q9Y4K0	86,7	90 - 120
MMS19-like protein (hMMS19) (MET18 homolog)	4	25,26	Q96T76	113,2	170 - stack
N-myc-interactor (Nmi) (N-myc and STAT interactor)	13	24,50	Q13287	35,0	> 170 incl. stack
Periodic tryptophan protein 1 homolog	5	40,20	Q13610	55,8	70 - 90
PML (Tripartite motif-containing protein 19) (RING finger protein 71)	12	20,76	P29590	97,5	> 170 incl. stack
Protein FAM126A (Down-regulated by CTNNB1 protein A)	3	20,14	Q9BYI3	57,6	70 - 90
Protein S100-A8 (S100 calcium-binding protein A8) (Calgranulin-A) (Migration inhibitory factor-related protein 8) (MRP-8)	2	20,12	P05109	10,8	stack
Putative DNA helicase INO80 complex homolog 1 (EC 3.6.1.-) (hINO80)	2	22,66	Q9ULG1	176,6	stack
Reticulon-4 (Neurite outgrowth inhibitor) (Nogo protein) (Foocen) (Neuroendocrine-specific protein) (NSP) (Neuroendocrine-specific protein C homolog) (RTN-x)	2	21,12	Q9NQC3	129,9	170 - stack

RNF4 (1-105) interacting proteins after MG132 treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
RNA-binding protein 7 (RNA-binding motif protein 7)	2	21,26	Q9Y580	30,5	50 - 70
Semaphorin-3D precursor	2	20,80	O95025	89,6	170 - stack
Serine/threonine-protein kinase 36 (EC 2.7.11.1) (Fused homolog)	5	22,64	Q9NRP7	143,9	stack
Serine/threonine-protein kinase SRPK2 (EC 2.7.11.1) (Serine/arginine-rich protein-specific kinase 2) (SR-protein-specific kinase 2) (SFRS protein kinase 2)	3	34,33	P78362	77,5	90 - 120
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (EC 3.1.3.16) (PP-1A)	2	26,31	P62136	37,5	stack
Single Ig IL-1-related receptor (Single Ig IL-1R-related molecule) (Single immunoglobulin domain-containing IL1R-related protein) (Toll/interleukin-1 receptor 8) (TIR8)	3	22,67	Q6IA17	45,7	70 - 90
Small ubiquitin-related modifier 2 precursor (SUMO-2)	8	160,15	P61957	10,9	90 - stack
Small ubiquitin-related modifier 3 precursor (SUMO-3)	16	170,67	P55854	11,6	stack
Splicing factor U2AF 65 kDa subunit (U2 auxiliary factor 65 kDa subunit) (U2 snRNP auxiliary factor large subunit) (hU2AF(65))	5	71,95	P26368	53,5	50 - 70
Thyroid receptor-interacting protein 13 (TRIP-13) (HPV16 E1 protein-binding protein) (16E1-BP)	11	23,17	Q15645	48,5	stack
Transcriptional-regulating factor 1 (Transcriptional-regulating protein 132) (Zinc finger transcription factor TReP-132)	5	23,05	Q96PN7	132,2	> 170 incl. stack
Transmembrane channel-like protein 6 (Epidermodysplasia verruciformis protein 1) (Protein LAK-4)	3	23,64	Q7Z403	90,0	90 - 120
Tripeptidyl-peptidase 2 (EC 3.4.14.10) (Tripeptidyl-peptidase II) (TPP-II) (Tripeptidyl aminopeptidase)	3	29,05	P29144	138,3	170 - stack
Ubiquitin	43	46,23	P62988	8,6	> 50 incl. stack
Uncharacterized protein KIAA0179 - Homo sapiens (Human)	2	29,40	Q14684	84,4	90 - 120
Vimentin	6	32,18	O95741	53,6	> 170 incl. stack
Zinc finger protein 41	2	21,58	P51814	93,7	stack

Table A.3: Summary of mass spectrometry analysis of isolated proteins interacting with GST-RNF4 (1-105) from ATO treated cells

RNF4 (1-105) interacting proteins after ATO treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
5'-AMP-activated protein kinase subunit gamma-2 (AMPK gamma-2 chain) (AMPK gamma2) (H91620p)	4	25,44	Q9UGJ0	63,0	90 - 120
6-phosphofructokinase type C (EC 2.7.1.11) (Phosphofructokinase 1) (Phosphohexokinase) (Phosphofructo-1-kinase isozyme C) (PFK-C) (6-phosphofructokinase, platelet type)	10	133,99	Q01813	85,5	70 - 90
6-phosphofructokinase, liver type (EC 2.7.1.11) (Phosphofructokinase 1) (Phosphohexokinase) (Phosphofructo-1-kinase isozyme B) (PFK-B)	10	64,34	P17858	85,0	70 - 90
A/G-specific adenine DNA glycosylase (EC 3.2.2.-) (MutY homolog) (hMYH)	2	22,23	Q9UIF7	60,0	120 - 170
Alpha-internexin (Alpha-Inx) (66 kDa neurofilament protein) (Neurofilament-66) (NF-66)	5	89,61	Q16352	55,4	50 - 70
ATP-dependent RNA helicase DDX24 (EC 3.6.1.-) (DEAD box protein 24)	2	35,16	Q9GZR7	96,3	120 - 170
BTB/POZ domain-containing protein KCTD10	3	22,22	Q9H3F6	35,4	50 - 70
Chromobox protein homolog 8 (Polycomb 3 homolog) (Pc3) (hPc3) (Rectachrome 1)	10	53,98	Q9HC52	43,4	120 - 170
CTP synthase 1 (EC 6.3.4.2) (UTP--ammonia ligase 1) (CTP synthetase 1)	2	26,88	P17812	66,6	70 - 90
D(3) dopamine receptor	3	23,44	P35462	44,2	120 - 170
EH domain-containing protein 2	3	49,27	Q9NZN4	61,1	70 - 90
ER degradation-enhancing alpha-mannosidase-like 3	5	39,47	Q9BZQ6	100,2	120 - 170
Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 1 (EC 2.6.1.16) (Hexosephosphate aminotransferase 1) (D-fructose-6-phosphate amidotransferase 1) (GFAT 1) (GFAT1)	9	64,95	Q06210	78,8	70 - 90
Histone deacetylase complex subunit SAP30 (Sin3-associated polypeptide, 30 kDa) (Sin3 corepressor complex subunit SAP30)	2	21,79	O75446	23,3	70 - 90
Inhibitor of nuclear factor kappa-B kinase subunit alpha (EC 2.7.11.10) (I kappa-B kinase alpha) (IkBKA) (IKK-alpha) (IKK-A) (IkappaB kinase) (I-kappa-B kinase 1) (IKK1) (Conserved helix-loop-helix ubiquitous kinase) (Nuclear factor NF-kappa-B inhibitor kinase alpha) (NFKBIKA)	12	23,93	O15111	84,6	70 - stack
Insulin-like growth factor 2 mRNA-binding protein 2 (IGF-II mRNA-binding protein 2) (IMP-2) (Hepatocellular carcinoma autoantigen p62)	4	27,96	Q9Y6M1	61,8	70 - 90

RNF4 (1-105) interacting proteins after ATO treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
Mitogen-activated protein kinase kinase kinase 6 (EC 2.7.11.25)	2	24,73	O95382	112,4	stack
Myristoylated alanine-rich C-kinase substrate (MARCKS) (Protein kinase C substrate, 80 kDa protein, light chain) (PKCSL) (80K-L protein)	2	59,88	P29966	31,5	70 - 90
Neurosecretory protein VGF precursor	4	23,68	O15240	67,2	> 70 incl. stack
N-myc-interactor (Nmi) (N-myc and STAT interactor)	5	24,67	Q13287	35,0	> 70 incl. stack
Peripherin	15	361,89	P41219	53,6	50 - 70
Phosphate carrier protein, mitochondrial precursor (PTP) (Solute carrier family 25 member 3)	5	41,38	Q00325	40,1	120 -170
Potassium channel subfamily K member 12 (Tandem pore domain halothane-inhibited potassium channel 2) (THIK-2)	6	26,98	Q9HB15	46,9	stack
Protein disulfide-isomerase precursor (EC 5.3.4.1) (PDI) (Prolyl 4-hydroxylase subunit beta) (Cellular thyroid hormone-binding protein) (p55)	5	35,39	P07237	57,1	120 - 170
Protein FAM53B	8	25,28	Q14153	45,8	120 - 170
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (EC 3.6.3.8) (Calcium pump 2) (SERCA2)	8	105,26	P16615	114,7	120 - 170
Selenium-binding protein 1	2	23,91	Q13228	52,3	stack
Serine/threonine-protein kinase PLK2 (EC 2.7.11.21) (Polo-like kinase 1) (PLK-2) (Serine/threonine-protein kinase SNK) (Serum-inducible kinase)	8	20,89	Q9NYY3	78,2	stack
Signal transducer and activator of transcription 1-alpha/beta (Transcription factor ISGF-3 components p91/p84) (STAT1)	2	47,18	P42224	87,3	70 - 90
Src substrate cortactin (Amplaxin) (Oncogene EMS1)	2	32,96	Q14247	61,6	70 - 90
Ubiquitin	15	40,91	P62988	8,6	> 90 incl. stack
Wiskott-Aldrich syndrome protein family member 1 (WASP-family protein member 1) (Protein WAVE-1) (Verprolin homology domain-containing protein 1)	4	23,16	Q92558	61,6	90 - 120
Zinc finger and BTB domain-containing protein 43 (Zinc finger protein 297B) (ZnF-x) (Zinc finger and BTB domain-containing protein 22B)	11	22,81	O43298	52,6	90 - 170
Zinc finger protein 639 (Zinc finger protein ZASC1) (Zinc finger protein ANC_2H01)	7	20,61	Q9UID6	56,0	50 - 70

Table A.4: Summary of mass spectrometry analysis of isolated proteins interacting with GST-RNF4 (1-105) from ATO and MG132 treated cells

RNF4 (1- 105) interacting proteins after ATO and MG132 treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
5'-AMP-activated protein kinase subunit gamma-2 (AMPK gamma-2 chain) (AMPK gamma2) (H91620p)	8	24,96	Q9UGJ0	63,0	> 170 incl. stack
Centrosomal protein of 131 kDa (Cep131 protein)	3	23,20	Q9UPN4	122,0	stack
6-phosphofructokinase type C (EC 2.7.1.11) (Phosphofructokinase 1) (Phosphohexokinase) (Phosphofructo-1-kinase isozyme C) (PFK-C) (6-phosphofructokinase, platelet type)	6	103,88	Q01813	85,5	70 - 90
6-phosphofructokinase, liver type (EC 2.7.1.11) (Phosphofructokinase 1) (Phosphohexokinase) (Phosphofructo-1-kinase isozyme B) (PFK-B)	5	45,11	P17858	85,0	70 - 90
ADP/ATP translocase 3 (Adenine nucleotide translocator 2) (ANT 3) (ADP,ATP carrier protein 3) (Solute carrier family 25 member 6) (ADP,ATP carrier protein, isoform T2)	3	26,63	P12236	32,8	120 - 170
Alpha-1B-glycoprotein precursor (Alpha-1-B glycoprotein)	2	23,71	P04217	54,2	70 - 90
COUP transcription factor 1 (COUP-TF1) (COUP-TF I) (V-ERBA-related protein EAR-3)	2	21,42	P10589	46,1	70 - 90
Genetic suppressor element 1	3	24,36	Q14687	136,1	170 - stack
Inhibitor of nuclear factor kappa-B kinase subunit alpha (I kappa-B kinase alpha) (IkBKA) (IKK-alpha) (IKK-A) (IkappaB kinase) (I-kappa-B kinase 1) (IKK1)	11	23,17	O15111	84,6	90 - stack
Kelch repeat and BTB domain-containing protein 10 (Kelch-related protein 1) (Kel-like protein 23) (Sarcosin)	4	35,66	O60662	68,0	170 - stack
Leucine-rich repeat transmembrane neuronal protein 2 precursor (Leucine-rich repeat neuronal 2 protein)	5	22,40	O43300	59,0	70 - 90
Neurosecretory protein VGF precursor	4	26,42	O15240	67,2	70 - 90
N-myc-interactor (Nmi) (N-myc and STAT interactor) - Homo sapiens (Human)	2	20,47	Q13287	35,0	120 - 170
Peptidyl-prolyl cis-trans isomerase C (EC 5.2.1.8) (PPIase) (Rotamase) (Cyclophilin C)	3	25,28	P45877	22,7	70 - 90
Periodic tryptophan protein 1 homolog (Keratinocyte protein IEF SSP 9502)	6	38,20	Q13610	55,8	70 - 90
Rab-like protein 2A	4	21,77	Q9UBK7	26,1	70 - 90

RNF4 (1- 105) interacting proteins after ATO and MG132 treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
Serine/threonine-protein kinase PLK2 (EC 2.7.11.21) (Polo-like kinase 1) (PLK-2) (Serine/threonine-protein kinase SNK) (Serum-inducible kinase)	4	20,68	Q9NYY3	78,2	120 - 170
Ubiquitin	19	35,44	P62988	8,6	50 - stack

Table A.5: Summary of mass spectrometry analysis of isolated proteins interacting with GST-RNF4 (1-105) from Sorbitol treated cells

RNF4 (1-105) interacting proteins after Sorbitol treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
150 kDa oxygen-regulated protein precursor (Orp150) (Hypoxia up-regulated 1)	8	23,27	Q9Y4L1	111,3	170 - stack
5'-AMP-activated protein kinase subunit gamma-2 (AMPK gamma-2 chain) (AMPK gamma2) (H91620p)	25	22,99	Q9UGJ0	63,0	50 - 90
Active breakpoint cluster region-related protein	4	22,29	Q12979	97,6	90 - 120
ADP/ATP translocase 3 (Adenine nucleotide translocator 2) (ANT 3) (ADP,ATP carrier protein 3) (Solute carrier family 25 member 6) (ADP,ATP carrier protein, isoform T2)	4	50,30	P12236	32,8	170 - stack
Ankyrin repeat domain-containing protein 26	21	24,68	Q9UPS8	196,2	> 50 incl. stack
ATP-dependent RNA helicase DDX18 (EC 3.6.1.-) (DEAD box protein 18) (Myc-regulated DEAD box protein) (MrDb)	2	31,39	Q9NVP1	75,4	70 - 90
Centaurin-delta 1 (Cnt-d1) (Arf-GAP, Rho-GAP, ankyrin repeat and pleckstrin homology domain-containing protein 2) (PARX protein)	3	26,26	Q8WZ64	193,3	stack
Centrosomal protein of 131 kDa (Cep131 protein)	10	28,91	Q9UPN4	122,0	> 50 incl. stack
Charged multivesicular body protein 5 (Chromatin-modifying protein 5) (Vacuolar protein sorting 60) (Vps60) (hVps60) (SNF7 domain-containing protein 2)	4	24,42	Q9NZZ3	24,6	70 - 90
E3 SUMO-protein ligase PIAS2 (Protein inhibitor of activated STAT2) (Protein inhibitor of activated STAT x) (Msx-interacting zinc finger protein) (Miz1) (DAB2-interacting protein) (DIP) (Androgen receptor-interacting protein 3) (ARIP3) (PIAS-NY protein)	2	20,60	O75928	68,2	120 - 170
ER degradation-enhancing alpha-mannosidase-like 3 (EDEM3)	4	25,08	Q9BZQ6	100,2	90 - 120
Ferritin heavy polypeptide-like 17	8	24,05	Q9BXU8	21,1	70 - 90
Forkhead box protein D1 (Forkhead-related protein FKHL8) (Forkhead-related transcription factor 4) (FREAC-4)	4	24,10	Q16676	46,1	90 - 120
Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin)	2	20,84	P68871	16,0	stack
Inhibitor of nuclear factor kappa-B kinase subunit alpha (EC 2.7.11.10) (I kappa-B kinase alpha) (IkBKA) (IKK-alpha) (IKK-A) (IkappaB kinase) (I-kappa-B kinase 1) (IKK1) (Conserved helix-loop-helix ubiquitous kinase) (Nuclear factor NF-kappa-B inhibitor kinase alpha) (NFKBIKA)	39	22,85	O15111	84,6	> 50 incl. stack
Inorganic pyrophosphatase 2, mitochondrial precursor (EC 3.6.1.1) (PPase 2) (Pyrophosphatase SID6-306)	4	24,08	Q9H2U2	37,9	50 - 70

RNF4 (1-105) interacting proteins after Sorbitol treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
Inositol polyphosphate 1-phosphatase (EC 3.1.3.57) (IPPase) (IPP)	3	26,24	P49441	44,0	70 - 90
Kelch repeat and BTB domain-containing protein 10 (Kelch-related protein 1) (Kel-like protein 23) (Sarcosin)	6	21,61	O60662	68,0	120 - 170
Leucine-rich repeat transmembrane neuronal protein 2 precursor (Leucine-rich repeat neuronal 2 protein)	6	24,04	O43300	59,0	50 - 70
Leucine-rich repeat-containing protein 33 precursor	3	23,07	Q86YC3	76,3	70 - 90
Mitogen-activated protein kinase kinase kinase 5 (EC 2.7.11.25) (MAPK/ERK kinase kinase 5) (MEK kinase 5) (MEKK 5) (Apoptosis signal-regulating kinase 1) (ASK-1)	7	26,69	Q99683	154,4	90 - 120
Mitogen-activated protein kinase kinase kinase 8 (EC 2.7.11.25) (COT proto-oncogene serine/threonine-protein kinase) (C-COT) (Cancer Osaka thyroid oncogene)	2	20,91	P41279	52,9	170 - stack
NADP-dependent malic enzyme (EC 1.1.1.40) (NADP-ME) (Malic enzyme 1)	3	22,07	P48163	64,1	170 - stack
Neurosecretory protein VGF precursor	21	20,62	O15240	67,2	50 - 120
N-myc-interactor (Nmi) (N-myc and STAT interactor)	8	24,16	Q13287	35,0	90 - stack
Olfactory receptor 13H1	5	22,01	Q8NG92	34,5	stack
Protein S100-A7 (S100 calcium-binding protein A7) (Psoriasin)	2	20,21	P31151	11,5	stack
Protein Z-dependent protease inhibitor precursor (PZ-dependent protease inhibitor) (PZI) (Serpin A10)	2	33,52	Q9UK55	50,7	stack
Putative DNA helicase INO80 complex homolog 1 (EC 3.6.1.-) (hINO80)	2	24,34	Q9ULG1	176,6	stack
Replication factor C subunit 1 (Replication factor C large subunit) (RF-C 140 kDa subunit) (Activator 1 140 kDa subunit) (Activator 1 large subunit) (A1 140 kDa subunit) (DNA-binding protein PO-GA)	2	35,35	P35251	128,2	120 - 170
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (EC 3.6.3.8) (Calcium pump 2) (SERCA2) (SR Ca(2+)-ATPase 2) (Calcium-transporting ATPase sarcoplasmic reticulum type, slow twitch skeletal muscle isoform) (Endoplasmic reticulum class 1/2 Ca(2+) ATPase)	3	30,40	P16615	114,7	120 - 170
Serine/threonine-protein kinase PLK2 (EC 2.7.11.21) (Polo-like kinase 1) (PLK-2) (Serine/threonine-protein kinase SNK) (Serum-inducible kinase)	12	20,56	Q9NYY3	78,2	90 - 120
Transcription intermediary factor 1-alpha (TIF1-alpha) (Tripartite motif-containing protein 24)	2	24,53	O15164	116,8	170 - stack

RNF4 (1-105) interacting proteins after Sorbitol treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
Ubiquitin	16	45,50	P62988	8,6	70 - stack
Uncharacterized protein KIAA1143	2	20,18	Q96AT1	17,5	90 - 120
WD repeat protein 13	2	26,17	Q9H1Z4	53,7	50 - 70
Zinc finger protein 140	28	21,87	P52738	52,9	stack
Zinc finger protein 639 (Zinc finger protein ZASC1) (Zinc finger protein ANC_2H01)	9	21,33	Q9UID6	56,0	50 - 70

Table A.6: Summary of mass spectrometry analysis of isolated proteins interacting with GST-RNF4 (1-105) from Sorbitol and MG132 treated cells

RNF4 (1-105) interacting proteins after Sorbitol and MG132 treatment	*Peptides*	*Score*	*Accession*	M_w *[kDa]*	M_w *range [kDa]*
5'-AMP-activated protein kinase subunit gamma-2 (AMPK gamma-2 chain) (AMPK gamma2) (H91620p)	32	22,22	Q9UGJ0	63,0	> 50 incl. stack
6-phosphofructokinase type C (EC 2.7.1.11) (Phosphofructokinase 1) (Phosphohexokinase) (Phosphofructo-1-kinase isozyme C) (PFK-C) (6-phosphofructokinase, platelet type)	5	38,46	Q01813	85,5	70 - 90
ADP/ATP translocase 3 (Adenine nucleotide translocator 2) (ANT 3) (ADP,ATP carrier protein 3) (Solute carrier family 25 member 6) (ADP,ATP carrier protein, isoform T2)	5	50,30	P12236	32,8	170 - stack
Ankyrin repeat domain-containing protein 26	15	28,93	Q9UPS8	196,2	> 50 incl. stack
Ankyrin repeat domain-containing protein 47	7	23,53	Q6NY19	88,4	170 - stack
ATP-dependent RNA helicase DDX18 (EC 3.6.1.-) (DEAD box protein 18) (Myc-regulated DEAD box protein) (MrDb)	5	66,83	Q9NVP1	75,4	70 - 90
ATP-dependent RNA helicase DDX3Y (EC 3.6.1.-) (DEAD box protein 3, Y-chromosomal)	3	59,73	O15523	73,1	70 - 90
Beta-1,3-galactosyltransferase 5 (EC 2.4.1.-) (Beta-1,3-GalTase 5) (Beta3Gal-T5) (b3Gal-T5) (UDP-galactose:beta-N-acetylglucosamine beta-1,3-galactosyltransferase 5) (UDP-Gal:beta-GlcNAc beta-1,3-galactosyltransferase 5) (Beta-3-Gx-T5)	3	22,13	Q9Y2C3	36,2	stack
CDK5 and ABL1 enzyme substrate 2 (Interactor with CDK3 2) (Ik3-2)	2	25,36	Q9BTV7	52,2	70 - 90
Centrosomal protein of 152 kDa (Cep152 protein)	4	21,91	O94986	147,3	170 - stack
Cerebellin-4 precursor (Cerebellin-like glycoprotein 1)	2	21,49	Q9NTU7	21,8	120 - 170
Co-chaperone protein HscB, mitochondrial precursor (Hsc20)	2	21,32	Q8IWL3	27,4	50 - 70
Cohesin subunit SA-1 (Stromal antigen 1) (SCC3 homolog 1)	3	21,73	Q8WVM7	144,4	170 - stack
DNA repair protein REV1 (EC 2.7.7.-) (Rev1-like terminal deoxycytidyl transferase) (Alpha integrin-binding protein 80) (AIBP80)	2	28,44	Q9UBZ9	138,2	120 - 170
F-box/LRR-repeat protein 12 (F-box and leucine-rich repeat protein 12) (F-box protein FBL12)	3	22,83	Q9NXK8	37,0	170 - stack
Glucagon receptor precursor (GL-R)	3	24,67	P47871	54,0	> 70 incl. stack
Group IIE secretory phospholipase A2 precursor (EC 3.1.1.4) (Phosphatidylcholine 2-acylhydrolase GIIE) (GIIE sPLA2) (sPLA(2)-IIE)	2	23,72	Q9NZK7	16,0	120 - 170
Homeobox prospero-like protein PROX1 (PROX 1)	16	29,97	Q92786	83,2	> 90 incl. stack

RNF4 (1-105) interacting proteins after Sorbitol and MG132 treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
Inhibitor of nuclear factor kappa-B kinase subunit alpha (EC 2.7.11.10) (I kappa-B kinase alpha) (IkBKA) (IKK-alpha) (IKK-A) (IkappaB kinase) (I-kappa-B kinase 1) (IKK1) (Conserved helix-loop-helix ubiquitous kinase) (Nuclear factor NF-kappa-B inhibitor kinase alpha) (NFKBIKA)	18	21,11	O15111	84,6	> 50 incl. stack
Kelch repeat and BTB domain-containing protein 10 (Kelch-related protein 1) (Kel-like protein 23) (Sarcosin)	4	24,25	O60662	68,0	170 - stack
Long-chain specific acyl-CoA dehydrogenase, mitochondrial precursor (EC 1.3.99.13) (LCAD)	8	23,17	P28330	47,6	50 - 120
Low affinity immunoglobulin epsilon Fc receptor (Lymphocyte IgE receptor) (Fc-epsilon-RII) (BLAST-2) (Immunoglobulin E-binding factor) (CD23 antigen) [Contains: Low affinity immunoglobulin epsilon Fc receptor membrane-bound form; Low affinity immunoglobulin epsilon Fc receptor soluble form]	3	20,04	P06734	36,4	170 - stack
N(2),N(2)-dimethylguanosine tRNA methyltransferase (EC 2.1.1.32) (tRNA(guanine-26,N(2)-N(2)) methyltransferase) (tRNA 2,2-dimethylguanosine-26 methyltransferase) (tRNA(m(2,2)G26)dimethyltransferase)	7	25,23	Q9NXH9	72,2	50 - 120
NAD-dependent deacetylase sirtuin-3, mitochondrial precursor (EC 3.5.1.-) (SIR2-like protein 3) (hSIRT3)	3	22,57	Q9NTG7	43,5	stack
NADP-dependent malic enzyme (EC 1.1.1.40) (NADP-ME) (Malic enzyme 1)	7	20,13	P48163	64,1	90 - stack
Neurosecretory protein VGF precursor	25	20,90	O15240	67,2	> 50 incl. stack
N-myc-interactor (Nmi) (N-myc and STAT interactor)	13	20,04	Q13287	35,0	> 170 incl. stack
Nucleoprotein TPR	7	33,64	P12270	265,4	170 - stack
Pantothenate kinase 4 (EC 2.7.1.33) (Pantothenic acid kinase 4) (hPanK4)	3	24,96	Q9NVE7	85,9	70 - 90
Peptidyl-prolyl cis-trans isomerase C (EC 5.2.1.8) (PPIase) (Rotamase) (Cyclophilin C)	3	28,42	P45877	22,7	70 - 90
Poly(ADP-ribose) glycohydrolase ARH3 (EC 3.2.1.143) ([Protein ADP-ribosylarginine] hydrolase-like protein 2)	5	21,29	Q9NX46	38,9	> 170 incl. stack
Protein disulfide-isomerase A4 precursor (EC 5.3.4.1) (Protein ERp-72) (ERp72)	2	21,23	P13667	72,9	50 - 70
Protein S100-A8 (S100 calcium-binding protein A8) (Calgranulin-A) (Migration inhibitory factor-related protein 8) (MRP-8) (Cystic fibrosis antigen) (CFAG) (P8) (Leukocyte L1 complex light chain) (Calprotectin L1L subunit) (Urinary stone protein band A)	5	21,49	P05109	10,8	stack
Putative DNA helicase INO80 complex homolog 1 (EC 3.6.1.-) (hINO80)	2	22,92	Q9ULG1	176,6	stack

RNF4 (1-105) interacting proteins after Sorbitol and MG132 treatment	*Peptides*	*Score*	*Accession*	M_w *[kDa]*	M_w *range [kDa]*
Retinoic acid receptor alpha (RAR-alpha)	2	22,12	P10276	50,7	70 - 90
Selenium-binding protein 1	2	39,22	Q13228	52,3	stack
Serine/threonine-protein kinase PLK2 (EC 2.7.11.21) (Polo-like kinase 1) (PLK-2) (Serine/threonine-protein kinase SNK) (Serum-inducible kinase) [	6	20,01	Q9NYY3	78,2	70 - 90
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (EC 3.1.3.16) (PP-1A)	2	27,09	P62136	37,5	stack
Serpin B8 (Cytoplasmic antiproteinase 2) (CAP-2) (CAP2) (Protease inhibitor 8)	2	20,74	P50452	42,8	stack
SH2B adapter protein 2 (SH2 and PH domain-containing adapter protein APS) (Adapter protein with pleckstrin homology and Src homology 2 domains)	2	22,56	O14492	67,7	70 - 90
SPARC-related modular calcium-binding protein 1 precursor (Secreted modular calcium-binding protein 1) (SMOC-1)	3	23,41	Q9H4F8	48,1	70 -90
Transcription factor HES-5 (Hairy and enhancer of split 5)	3	28,47	Q5TA89	18,2	170 - stack
Ubiquitin	12	51,31	P62988	8,6	90 - stack
Zinc finger protein 425	2	20,85	Q6IV72	87,7	stack
Zinc finger protein 639 (Zinc finger protein ZASC1) (Zinc finger protein ANC_2H01)	20	22,33	Q9UID6	56,0	50 - 170

Table A.7: Summary of mass spectrometry analysis of isolated proteins interacting with GST-RNF4 (1-105) from canavanine treated cells

RNF4 (1-105) interacting proteins after canavanine treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
150 kDa oxygen-regulated protein precursor (Orp150) (Hypoxia up-regulated 1) (OXRP)	9	23,49	Q9Y4L1	111,3	170 - stack
4F2 cell-surface antigen heavy chain (4F2hc) (Lymphocyte activation antigen 4F2 large subunit) (4F2 heavy chain antigen) (CD98 antigen(4F2)	2	64,39	P08195	57,9	70 - 90
5'-AMP-activated protein kinase subunit gamma-2 (AMPK gamma-2 chain) (AMPK gamma2) (H91620p) (AAKG2)	16	20,29	Q9UGJ0	63,0	90 -120
ADP/ATP translocase 3 (Adenine nucleotide translocator 2) (ANT 3) (ADP,ATP carrier protein 3) (Solute carrier family 25 member 6) (ADP,ATP carrier protein, isoform T2) (ADT3)	3	27,57	P12236	32,8	170 - stack
Alpha-1B-glycoprotein precursor (Alpha-1-B glycoprotein) (A1BG)	4	41,63	P04217	54,2	stack
ATP synthase coupling factor 6, mitochondrial precursor (EC 3.6.3.14) (ATPase subunit F6) (ATP5J)	2	20,04	P18859	12,6	50 - 70
ATP-binding cassette sub-family F member 1 (ATP-binding cassette 50) (TNF-alpha-stimulated ABC protein) (ABCF1)	5	63,15	Q8NE71	95,9	90 - 120
ATP-binding cassette sub-family G member 8 (Sterolin-2) (ABCG8)	2	24,50	Q9H221	75,6	70 - 90
Centromere protein H (CENP-H) (Interphase centromere complex protein 35)	2	32,14	Q9H3R5	28,5	120 - 170
Citron Rho-interacting kinase (EC 2.7.11.1) (CRIK) (Rho-interacting, serine/threonine-protein kinase 21) (CTRO)	7	33,70	O14578	231,3	170 - stack
Creatine kinase M-type (EC 2.7.3.2) (Creatine kinase M chain) (M-CK) (KCRM)	2	75,36	P06732	43,1	170 - stack
DNA topoisomerase 2-beta (EC 5.99.1.3) (DNA topoisomerase II, beta isozyme)	4	21,47	Q02880	183,2	stack
Double-strand break repair protein MRE11A (MRE11 homolog 1) (MRE11 meiotic recombination 11 homolog A) (MRE11)	2	21,84	P49959	80,5	70 - 90
E3 SUMO-protein ligase PIAS2 (Protein inhibitor of activated STAT2)	10	22,63	O75928	68,2	> 170 incl. stack
ELAV-like protein 3 (Hu-antigen C) (HuC) (Paraneoplastic cerebellar degeneration-associated antigen) (ELAV3)	9	20,99	Q14576	39,5	stack
ELKS/RAB6-interacting/CAST family member 1 (RAB6-interacting protein 2) (ERC protein 1) (RB6I2)	6	28,86	Q8IUD2	128,0	170 - stack
Exportin-4 (Exp4) (XPO4)	6	22,47	Q9C0E2	130,1	170 - stack
Gamma-aminobutyric acid receptor-associated protein (GABA(A) receptor-associated protein) (MM46) (GBRAP)	4	22,60	O95166	13,9	70 - 90

RNF4 (1-105) interacting proteins after canavanine treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
Glucocorticoid receptor DNA-binding factor 1 (Glucocorticoid receptor repression factor 1) (GRF-1) (Rho GAP p190A) (p190-A) (GRLF1)	6	24,34	Q9NRY4	172,1	170 - stack
Homeobox prospero-like protein PROX1 (PROX 1)	5	22,39	Q92786	83,2	> 70 incl. stack
Inhibitor of nuclear factor kappa-B kinase subunit alpha (EC 2.7.11.10) (I kappa-B kinase alpha) (IkBKA) (IKK-alpha) (IKK-A) (IkappaB kinase) (I-kappa-B kinase 1) (IKK1)	27	22,48	O15111	84,6	> 170 incl. stack
Interleukin-17 receptor A precursor (IL-17 receptor) (I17RA)	2	21,73	Q96F46	96,1	stack
Islet cell autoantigen 1-like protein (Amyotrophic lateral sclerosis 2 chromosomal region candidate gene 15 protein) (ICA1L)	6	21,55	Q8NDH6	54,4	> 120 incl. stack
Kelch repeat and BTB domain-containing protein 10 (Kelch-related protein 1) (Kel-like protein 23) (Sarcosin) (KBTBA)	10	25,84	O60662	68,0	90 - 170
KRR1 small subunit processome component homolog (HIV-1 Rev-binding protein 2) (Rev-interacting protein 1) (Rip-1)	2	25,47	Q13601	43,6	90 - 120
Low affinity immunoglobulin epsilon Fc receptor (Lymphocyte IgE receptor) (Fc-epsilon-RII) (BLAST-2) (Immunoglobulin E-binding factor) (CD23 antigen) (FCER2)	11	55,35	P06734	36,4	170 - stack
Myb-binding protein 1A (MBB1A)	4	65,09	Q9BQG0	148,8	120 - 170
Myristoylated alanine-rich C-kinase substrate (MARCKS) (Protein kinase C substrate, 80 kDa protein, light chain) (PKCSL) (80K-L protein) (MARCS)	4	56,71	P29966	31,5	70 - 90
N(2),N(2)-dimethylguanosine tRNA methyltransferase (EC 2.1.1.32) (tRNA(guanine-26,N(2)-N(2)) methyltransferase) (tRNA 2,2-dimethylguanosine-26 methyltransferase) (tRNA(m(2,2)G26)dimethyltransferase) (TRM1)	10	31,36	Q9NXH9	72,2	90 incl. stack
Nuclear pore complex protein Nup98-Nup96 precursor (NUP98)	7	79,83	P52948	18,8	170 - stack
Nucleoprotein TPR	17	40,889	P12270	265,4	120 - stack
Oxysterol-binding protein-related protein 6 (OSBP-related protein 6) (ORP-6) (OSBL6)	4	21,31	Q9BZF3	106,2	90 - 120
Periodic tryptophan protein 1 homolog (Keratinocyte protein IEF SSP 9502) (PWP1)	6	87,83	Q13610	55,8	70 - 90
Protein inscuteable homolog (INSC)	2	22,79	Q1MX18	63,4	170 - stack
Protein polybromo-1 (hPB1) (Polybromo-1D) (BRG1-associated factor 180) (BAF180)	4	22,32	Q86U86	192,8	stack

RNF4 (1-105) interacting proteins after canavanine treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
Protein S100-A8 (S100 calcium-binding protein A8) (Calgranulin-A) (Migration inhibitory factor-related protein 8) (MRP-8)	4	32,66	P05109	10,8	> 170 incl. stack
Protein S100-A9 (S100 calcium-binding protein A9) (Calgranulin-B) (Migration inhibitory factor-related protein 14) (MRP-14)	2	20,19	P06702	13,2	stack
Protein transport protein Sec24B (SEC24-related protein B) (SC24B)	4	35,58	O95487	137,7	120 - 170
Putative ATP-dependent RNA helicase DHX30 (EC 3.6.1.-) (DEAH box protein 30)	2	25,46	Q7L2E3	133,9	120 - 170
Putative ATP-dependent RNA helicase DHX57 (EC 3.6.1.-) (DEAH box protein 57	4	44,13	Q6P158	155,5	120 -170
Replication factor C subunit 1 (Replication factor C large subunit) (RF-C 140 kDa subunit) (Activator 1 140 kDa subunit) (Activator 1 large subunit) (A1 140 kDa subunit) (DNA-binding protein PO-GA) (RFC1)	4	42,15	P35251	128,2	120 - 170
Selenium-binding protein 1 (SBP1)	2	27,66	Q13228	52,3	stack
Serine/threonine-protein kinase 36 (EC 2.7.11.1) (Fused homolog) – (STK36)	3	22,73	Q9NRP7	143,9	170 - stack
Serine/threonine-protein kinase PLK2 (EC 2.7.11.21) (Polo-like kinase 1) (PLK-2)	4	21,76	Q9NYY3	78,2	70 - 90
Serine/threonine-protein kinase SRPK2 (EC 2.7.11.1) (Serine/arginine-rich protein-specific kinase 2) (SR-protein-specific kinase 2) (SFRS protein kinase 2)	2	25,96	P78362	77,5	90 - 120
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PP-1A)	13	22,62	P62136	37,5	50 - 70 and stack
Signal transducer and activator of transcription 1-alpha/beta (Transcription factor ISGF-3 components p91/p84) (STAT1)	7	141,82	P42224	87,3	70 - 90
Signal transducer and activator of transcription 2 (p113) (STAT2)	2	32,14	P52630	97,9	120 - 170
Small inducible cytokine B16 precursor (Transmembrane chemokine CXCL16) (SR-PSOX) (Scavenger receptor for phosphatidylserine and oxidized low density lipoprotein) (SCYBG)	9	24,13	Q9H2A7	27,6	120 - stack
Sodium channel protein type 11 subunit alpha (Sodium channel protein type XI subunit alpha) (Voltage-gated sodium channel subunit alpha Nav1.9) (Sensory neuron sodium channel 2) (Peripheral nerve sodium channel 5) (hNaN) (SC11A)	4	26,49	Q9UI33	204,8	170 - stack
Splicing factor U2AF 65 kDa subunit (U2 auxiliary factor 65 kDa subunit) (U2 snRNP auxiliary factor large subunit) (hU2AF(65))	7	64,98	P26368	53,5	50 - 70

RNF4 (1-105) interacting proteins after Canavanine treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
Src substrate cortactin (Amplaxin) (Oncogene EMS1) (SRC8)	6	42,64	Q14247	61,6	70 - 90
Striatin-4 (Zinedin) (STRN4)	2	26,66	Q9NRL3	80,5	170 - stack
Syntenin-2 (Syndecan-binding protein 2) (SDCB2)	2	24,71	Q9H190	31,6	stack
Talin-1 (TLN1)	2	27,24	Q9Y490	269,6	170 - stack
Tektin-1 (TEKT1)	2	26,12	Q969V4	48,3	170 - stack
Thyroid receptor-interacting protein 13 (TRIP-13) (Thyroid hormone receptor interactor 13) (Human papillomavirus type 16 E1 protein-binding protein) (HPV16 E1 protein-binding protein) (16E1-BP)	15	26,70	Q15645	48,5	50 - 70 and stack
Transcriptional-regulating factor 1 (Transcriptional-regulating protein 132) (Zinc finger transcription factor TReP-132)	3	26,56	Q96PN7	132,2	stack
Ubiquitin	19	39,95	P62988	8,6	> 70 incl. stack
Uncharacterized protein C15orf41 (Protein HH114) (CO041)	3	22,81	Q9Y2V0	32,2	170 - stack
Valyl-tRNA synthetase (EC 6.1.1.9) (Valine--tRNA ligase) (ValRS) (Protein G7a) (SYV)	2	52,44	P26640	140,4	120 - 170
Vigilin (High density lipoprotein-binding protein) (HDL-binding protein) (VIGLN)	10	83,12	Q00341	141,4	120 - 170
WD repeat protein 13 - Homo sapiens (Human) (WDR13)	4	23,89	Q9H1Z4	53,7	stack
Zinc finger X-linked protein ZXDA	3	31,53	P98168	84,7	170 - stack

Table A.8: Summary of mass spectrometry analysis of isolated proteins interacting with GST-RNF4 (1-105) from canvanine and MG132 treated cells

RNF4 (1-105) interacting proteins after canavanine and MG132 treatment	*Peptides*	*Score*	*Accession*	M_w *[kDa]*	M_w *range [kDa]*
5'-AMP-activated protein kinase subunit gamma-2 (AMPK gamma-2 chain) (AMPK gamma2) (H91620p) (AAKG2)	16	20,29	Q9UGJ0	63,0	> 120 incl. stack
Centrosomal protein of 131 kDa (Cep131 protein)	5	22,36	Q9UPN4	122,0	120 - 170
Alpha-1B-glycoprotein precursor (Alpha-1-B glycoprotein) (A1BG)	3	36,05	P04217	54,2	stack
AN1-type zinc finger protein 2A (ZFN2A)	3	28,21	Q8N6M9	16,4	70 - 90
Ankyrin repeat domain-containing protein 12 (Ankyrin repeat-containing cofactor 2) (GAC-1 protein) (ANR12)	5	22,96	Q6UB98	235,5	170 - stack
Ankyrin repeat domain-containing protein 26 (ANR26)	14	21,41	Q9UPS8	196,2	> 120 incl. stack
Annexin A4 (Annexin IV) (Lipocortin IV) (Endonexin I) (Chromobindin-4) (Protein II) (P32.5) (Placental anticoagulant protein II) (PAP-II) (PP4-X) (35-beta calcimedin) (Carbohydrate-binding protein P33/P41) (P33/41) (ANXA4)	8	26,19	P09525	35,9	50 - stack
ATP-binding cassette sub-family B member 5 (P-glycoprotein ABCB5) (ABCB5 P-gp) (ABCB5)	2	21,28	Q2M3G0	89,8	170 - stack
ATP-binding cassette sub-family G member 8 (Sterolin-2) (ABCG8)	6	24,90	Q9H221	75,6	70 -120
Bleomycin hydrolase (EC 3.4.22.40) (BLM hydrolase) (BMH) (BH)	2	36,84	Q13867	52,5	stack
Cartilage acidic protein 1 precursor (CEP-68) (ASPIC) (CRAC1)	3	26,24	Q9NQ79	71,4	stack
Coiled-coil domain-containing protein 102B (C102B)	14	85,72	Q68D86	60,4	stack
Cytochrome c oxidase subunit 5A, mitochondrial precursor (EC 1.9.3.1) (Cytochrome c oxidase polypeptide Va) (COX5A)	3	21,65	P20674	16,8	stack
DNA topoisomerase 2-beta (EC 5.99.1.3) (DNA topoisomerase II, beta isozyme)	3	21,65	Q02880	183,2	stack
Dual specificity protein phosphatase 16 (EC 3.1.3.48) (EC 3.1.3.16) (Mitogen-activated protein kinase phosphatase 7) (MAP kinase phosphatase 7) (MKP-7) (DUS16)	2	21,04	Q9BY84	73,1	170 - stack
Galectin-12 (Galectin-related inhibitor of proliferation) (LEG12)	4	21,61	Q96DT0	37,5	> 170 incl. stack
Gamma-aminobutyric acid receptor-associated protein (GABA(A) receptor-associated protein) (MM46) (GBRAP)	7	24,58	O95166	13,9	70 - 90
Homeobox prospero-like protein PROX1 (PROX 1)	12	22,05	Q92786	83,2	> 70 incl. stack

RNF4 (1-105) interacting proteins after Canavanine and MG132 treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
Hydroxyacid oxidase 2 (HAOX2) ((S)-2-hydroxy-acid oxidase, peroxisomal) (Long chain alpha-hydroxy acid oxidase) (Long-chain L-2-hydroxy acid oxidase)	2	31,35	Q9NYQ3	38,8	170 - stack
Inhibitor of nuclear factor kappa-B kinase subunit alpha (EC 2.7.11.10) (I kappa-B kinase alpha) (IkBKA) (IKK-alpha) (IKK-A) (IkappaB kinase) (I-kappa-B kinase 1) (IKK1)	50	22,48	O15111	84,6	> 50 incl. stack
Interleukin-17 receptor A precursor (IL-17 receptor) (I17RA)	3	23,46	Q96F46	96,1	stack
Kelch repeat and BTB domain-containing protein 10 (Kelch-related protein 1) (Kel-like protein 23) (Sarcosin) (KBTBA)	22	27,48	O60662	68,0	120 - stack
Mitogen-activated protein kinase 14 (EC 2.7.11.24) (Mitogen-activated protein kinase p38 alpha) (MAP kinase p38 alpha) (Cytokine suppressive anti-inflammatory drug-binding protein) (CSAID-binding protein) (CSBP) (MAX-interacting protein 2) (MAP kinase MXI2) (SAPK2A) (MK14)	3	20,35	Q16539	41,3	170 - stack
Msx2-interacting protein (SPEN homolog) (SMART/HDAC1-associated repressor protein) (MINT)	3	26,53	Q96T58	402,0	stack
N(2),N(2)-dimethylguanosine tRNA methyltransferase (EC 2.1.1.32) (tRNA(guanine-26,N(2)-N(2)) methyltransferase) (tRNA 2,2-dimethylguanosine-26 methyltransferase) (tRNA(m(2,2)G26)dimethyltransferase) (TRM1)	13	23,48	Q9NXH9	72,2	70 - 170
N-acetylglucosamine-1-phosphotransferase subunits alpha/beta precursor (EC 2.7.8.17) (GlcNAc-1-phosphotransferase alpha/beta subunits) (UDP-N-acetylglucosamine-1-phosphotransferase alpha/beta subunits) (Stealth protein GNPTAB)	2	25,50	Q3T906	143,5	120 -170
NADP-dependent malic enzyme (EC 1.1.1.40) (NADP-ME) (Malic enzyme 1) (MAOX)	5	20,68	P48163	64,1	170 - stack
N-myc-interactor (Nmi) (N-myc and STAT interactor) (NMI)	5	23,45	Q13287	35,0	170 - stack
Nucleoprotein TPR (TPR)	9	37,66	P12270	265,4	170 - stack
Oligodendrocyte transcription factor 3 (Oligo3) (Class B basic helix-loop-helix protein 7) (bHLHB7) (OLIG3)	2	20,50	Q7RTU3	29,3	70 - 90
Pappalysin-1 precursor (EC 3.4.24.79) (Pregnancy-associated plasma protein-A) (PAPP-A) (Insulin-like growth factor-dependent IGF-binding protein 4 protease) (IGF-dependent IGFBP-4 protease) (IGFBP-4ase) (PAPPA)	3	23,63	Q13219	181,0	stack
Periodic tryptophan protein 1 homolog (Keratinocyte protein IEF SSP 9502) (PWP1)	2	52,01	Q13610	55,8	70 - 90
Probable transcription factor PML (Tripartite motif-containing protein 19) (RING finger protein 71)	4	20,55	P29590	97,5	170 - stack

RNF4 (1-105) interacting proteins after canavanine and MG132 treatment	*Peptides*	*Score*	*Accession*	*M_w [kDa]*	*M_w range [kDa]*
Protein polybromo-1 (hPB1) (Polybromo-1D) (BRG1-associated factor 180) (BAF180)	2	28,08	Q86U86	192,8	stack
Protein S100-A8 (S100 calcium-binding protein A8) (Calgranulin-A) (Migration inhibitory factor-related protein 8) (MRP-8) (S10A8)	3	28,94	P05109	10,8	stack
Sarcoplasmic/endoplasmic reticulum calcium ATPase 3 (EC 3.6.3.8) (Calcium pump 3) (SERCA3) (SR Ca(2+)-ATPase 3) (AT2A3)	2	20,30	Q93084	113,9	stack
SEC14-like protein 1 (S14L1)	9	20,87	Q92503	81,2	> 90 incl. stack
Selenium-binding protein 1 (SBP1)	4	27,77	Q13228	52,3	stack
Serine/threonine-protein kinase 36 (EC 2.7.11.1) (Fused homolog) – (STK36)	5	20,92	Q9NRP7	143,9	170 - stack
Serine/threonine-protein kinase PLK2 (EC 2.7.11.21) (Polo-like kinase 1) (PLK-2)	7	22,32	Q9NYY3	78,2	> 120 incl. stack
Serine/threonine-protein kinase TAO3 (EC 2.7.11.1) (Thousand and one amino acid protein 3) (Jun kinase-inhibitory kinase) (JNK/SAPK-inhibitory kinase) (Dendritic cell-derived protein kinase) (Cutaneous T-cell lymphoma tumor antigen HD-CL-09) (CTCL tumor antigen HD-CL-09) (TAOK3)	3	24,07	Q9H2K8	105,3	120 - 170
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PP-1A)	18	21,80	P62136	37,5	> 50 incl. stack
Serpin B8 (Cytoplasmic antiproteinase 2) (CAP-2) (Protease inhibitor 8) (SPB8)	3	22,66	P50452	42,8	stack
Small inducible cytokine B16 precursor (Transmembrane chemokine CXCL16) (SR-PSOX) (Scavenger receptor for phosphatidylserine and oxidized low density lipoprotein) (SCYBG)	3	21,11	Q9H2A7	27,6	170 - stack
Small ubiquitin-related modifier 2 precursor (SUMO-2) (Ubiquitin-like protein SMT3B) (SMT3 homolog 2) (Sentrin-2) (SUMO2)	2	20,82	P61957	10,9	170 - stack
Soluble calcium-activated nucleotidase 1 (EC 3.6.1.6) (SCAN-1) (Apyrase homolog) (Putative NF-kappa-B-activating protein 107) (Putative MAPK-activating protein PM09) (CANT1)	4	22,39	Q8WVQ1	44,8	50 - 70
Thyroid receptor-interacting protein 13 (TRIP-13) (Thyroid hormone receptor interactor 13) (Human papillomavirus type 16 E1 protein-binding protein) (HPV16 E1 protein-binding protein) (16E1-BP)	10	23,12	Q15645	48,5	> 50 incl. stack
Tumor protein p73-like (p73L) (p63) (Tumor protein 63) (TP63) (p51) (p40) (Keratinocyte transcription factor KET) (Chronic ulcerative stomatitis protein) (CUSP) (P73L)	2	32,46	Q9H3D4	76,7	90 - 120
Ubiquitin	34	75,34	P62988	8,6	70 - stack

RNF4 (1-105) interacting proteins after Canavanine and MG132 treatment	*Peptides*	*Score*	*Accession*	M_w *[kDa]*	M_w *range [kDa]*
Ubiquitously transcribed Y chromosome tetratricopeptide repeat protein (Ubiquitously transcribed TPR protein on the Y chromosome) (UTY)	4	30,35	O14607	149,5	stack
Uncharacterized protein C10orf111 (CJ111)	5	22,61	Q8N326	17,8	170 - stack
WD repeat protein 13 (WDR13)	8	23,16	Q9H1Z4	53,7	> 70 incl. stack
Zinc finger protein 639 (Zinc finger protein ZASC1) (Zinc finger protein ANC_2H01) (ZN639)	3	20,71	Q9UID6	56,0	90 - 120
Zinc finger protein basonuclin-1 (BNC1)	2	22,29	Q01954	110,9	170 - stack

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ABSTRACT

Ubiquitin and the small ubiquitin related modifier (SUMO) belong to a group of small proteins that can be covalently attached to lysine side chains of other proteins, thereby changing their function, localization, interaction partners or stability. The conjugation reactions are mediated by an enzymatic cascade of specific activating, conjugating and ligating enzymes. A ubiquitin chain of at least four K48-linked ubiquitin molecules target substrate proteins for degradation by the proteasome.

Several interconnections exist between the ubiquitin and SUMO system, with the latest discoveries made in yeast by identifying E3 ubiquitin ligases that target SUMO conjugates for ubiquitylation and subsequent degradation by the proteasome. These ubiquitin ligases for SUMO conjugates (ULS) recognize especially high molecular weight SUMO conjugates, probably modified with SUMO chains.

In mammals, out of the three conjugatable SUMO paralogs, only SUMO-2/3 are able to form chains. Upon stress induction, the free pool of SUMO-2/3 is rapidly conjugated to cellular target proteins. These conjugates are under proteasomal control, implicating that the ULS pathway is conserved in humans.

This work identified the RING finger protein RNF4 as a human ULS protein, confirming previous observations in which RNF4 complemented yeast ULS deletion phenotypes. RNF4 comprises a RING domain which is present in many E3 ligases and a stretch of up to four SUMO interaction motifs (SIMs) that confer binding to SUMO.

In order to demonstrate ULS activity for RNF4, an *in vitro* ubiquitylation assay for SUMO conjugates has been developed. For that purpose, SUMOylated proteins were generated and purified as *in vitro* substrates from *E. coli*.

RNF4 efficiently *in vitro* ubiquitylated SUMO modified PML while unmodified PML was not recognized as a substrate. This result is in line with recent studies in cells demonstrating that RNF4 targets PML in a SUMO-dependent manner after arsenic trioxide treatment, a drug which is applied in acute promyelocytic leukemia (APL).

By investigating the SUMO binding properties of the RNF4 SIM domain, it became apparent that the interaction was especially enhanced by the presence of SUMO chains of more than two SUMOs. In addition, a SIM type specific recognition was noticed for different SUMO paralogs, which emphasizes the idea that there is also a SUMO paralogs specific regulation. Finally, in an attempt to find other ULS regulated cellular proteins, an RNF4 SIM domain construct was used to isolate poly- or multi-SUMOylated proteins from cells subjected to diverse cell stresses.

ZUSAMMENFASSUNG

Die zwei verwandten Proteine Ubiquitin und SUMO gehören zu einer Gruppe kleiner Proteine, die kovalent an die Seitenketten von Lysinen anderer Proteine (Substrate) konjugiert werden können. Dies kann zu Veränderungen der Proteinfunktion, -lokalisation, -interaktion oder -stabilität führen. Die Konjugation erfolgt über spezifische Enzymkaskaden von aktivierenden, konjugierenden und ligierenden Enzymen. Ubiquitin-Ketten, die über K48 geknüpft werden, dienen als Signal für den proteasomalen Abbau von Substratproteinen. Die Ubiquitin- und SUMO-Systeme sind auf vielerlei Art miteinander verbunden, wobei die neueste Entdeckung in der Hefe zur Identifizierung spezialisierter Ubiquitin E3-Ligasen führte. Diese ubiquitylieren SUMO-modifizierte Proteine und bewirken dadurch deren proteasomalen Abbau. Diese sogenannten Ubiquitin-Ligasen für SUMO-Konjugate (ULS) erkennen vor allem hochmolekulare SUMO-Konjugate, die wahrscheinlich mit SUMO-Ketten modifiziert sind. In Säugern sind von den drei konjugierbaren SUMOs nur SUMO-2 und -3 in der Lage, Ketten auszubilden. Freie SUMO-2/3-Moleküle werden durch mehrere Arten von Zellstressen relativ schnell an Substrate konjugiert. Diese Konjugate stehen unter proteasomaler Kontrolle, was darauf schließen lässt, dass der ULS-Abbauweg bis zum Menschen konserviert ist.

In dieser Arbeit konnte das RING-Finger Protein RNF4 als humanes ULS-Protein identifiziert werden. Dies bestätigte vorangegangene Beobachtungen, in denen RNF4 die Deletionsphänotypen von Hefe-ULS-Proteinen komplementiert.

RNF4 enthält eine für viele E3-Ligasen typische RING-Domäne sowie einen Bereich von bis zu vier SUMO-Interaktionsmotiven (SIMs), welche die Bindung an SUMO ermöglichen.

Um die ULS-Aktivität von RNF4 eindeutig zeigen zu können, wurde ein *in vitro*-Versuch entwickelt, in dem SUMO-Konjugate ubiquityliert werden. Dazu wurden gereinigte SUMO-modifizierte Proteine benötigt, die in E. coli hergestellt wurden.

RNF4 ubiquityliert effizient und spezifisch SUMO-modifiziertes PML, während nicht-modifiziertes PML nicht ubiquityliert wird. Dieses Ergebnis wird durch aktuelle Studien bestätigt, in denen gezeigt wurde, dass RNF4 nach Arsentrioxid (ATO)-Zugabe den SUMO-abhängigen Abbau von PML induziert. ATO wird zur Behandlung der akuten promyelozytischen Leukämie eingesetzt.

Untersuchungen der SUMO-bindenden Eigenschaften des SIM-haltigen RNF4-Bereichs zeigten, dass RNF4 bevorzugt an SUMO-Ketten mit mindestens zwei SUMO-Molekülen bindet. Zusätzlich wurde eine unterschiedliche SIM-Typ-Präferenz verschiedener SUMOs festgestellt, was die Frage nach einer SUMO-spezifischen Regulation aufwirft. Schließlich wurde der SIM-haltige Bereich von RNF4 dazu eingesetzt, poly- oder multi-SUMOylierte Proteine als potentielle ULS-Substrate aus Zellen zu isolieren, die zuvor verschiedenen Stressarten ausgesetzt wurden.

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Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Dr. Gerrit Praefcke, Institut für Genetik, Zülpicher Str. 47, 50674 Köln, betreut worden.

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