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„A novel approach to identify substrates for the sumoylated Ubc9“

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für meine Eltern

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ZUSAMMENFASSUNG

Unser Labor konnte zeigen, dass Sumoylierung des E2 konjugierenden Enzyms Ubc9 zur Diskriminierung von SUMO Substraten in Säugetieren beiträgt. Sumoyliertes Ubc9 (S*Ubc9) zeigt keinen Effekt bei der Modifikation der meisten SUMO Substrate, aber interessanterweise inhibiert es die Sumoylierung von RanGAP1 und verstärkt signifikant die Sumoylierung des Transkriptionsregulators Sp100 (Knipscheer et al., 2008). Sp100 war allerdings das einzige Substrat zu dieser Zeit, das mit dem sumoylierten Ubc9 verstärkt modifiziert wurde. Um Einblicke in die Substratreichweite des S*Ubc9 zu bekommen, wurden *in vitro* Sumoylierungsreaktionen mit Ubc9 und S*Ubc9 auf einem Protein Microarray ("ProtoArray® Human Protein Microarray v5.0") von Invitrogen durchgeführt. Über 9000 menschliche Proteine sind darauf gespottet. Protein Microarrays wurden entwickelt, um schnell und sensitiv neue Substrate für Phosphorylierung und Ubiquitinierung zu identifizieren (Gupta et al., 2007; Ptacek et al., 2005; Zhu et al., 2001). In dieser Arbeit wurden Protein Microarrays als neuer Ansatz zur Identifizierung von Substraten für das S*Ubc9 verwendet. Bevor jedoch *in vitro* Sumoylierungsreaktionen mit dem S*Ubc9 auf dem ProtoArray® durchgeführt werden konnten, wurden alle notwendigen Komponenten in E.coli exprimiert und aufgereinigt. Dies beinhaltete SUMO1, HA getaggtes SUMO1, das heterodimere E1 Enzym Aos1/Uba2, Ubc9 und S*Ubc9. Ubc9 wurde in großem Maßstab sumoyliert und die modifizierte wurde von der unmodifizierten Form mittels chromatographischer Methoden getrennt. Die Bedingungen für den ProtoArray® wurden in *in vitro* Sumoylierungsreaktionen mit bereits bekannten Substraten wie Sp100 und E2-25K ausgetestet. Der ProtoArray® selbst wurde vom Invitrogen ProtoArray® Service durchgeführt. Sie verwendeten unser Konzept und die etablierten Bedingungen für die Reaktion. Neu identifizierte Substrate wurden in unserem *in vitro* System verifiziert und werden Einblicke in die biologische Funktion des S*Ubc9 liefern.

ABSTRACT

Our laboratory has discovered a novel mechanism of how sumoylation of the E2 conjugating enzyme Ubc9 contributes to target discrimination in mammals. Whereas sumoylated Ubc9 (S*Ubc9) does not interfere with the sumoylation of most SUMO substrates, it impairs modification of RanGAP1 and significantly enhances modification of the transcriptional regulator Sp100 (Knipscheer et al., 2008). However, Sp100 was the only substrate so far preferentially targeted by S*Ubc9. In order to get insights into the substrate range of S*Ubc9, *in vitro* sumoylation reactions with Ubc9 and S*Ubc9 were performed on a protein microarray chip ("ProtoArray® Human Protein Microarray v5.0") purchasable from Invitrogen. Over 9000 human proteins were spotted on such a microarray. They were developed as rapid and sensitive means to identify novel substrates for phosphorylation and ubiquitination (Gupta et al., 2007; Ptacek et al., 2005; Zhu et al., 2001). In this work, the ProtoArray® was applied for sumoylation reactions as a novel approach to identify substrates for S*Ubc9. Before performing the *in vitro* sumoylation reaction with S*Ubc9 on the ProtoArray®, all components were expressed and purified in E. coli. This included SUMO1, HA tagged SUMO1, the heterodimeric E1 enzyme, Ubc9 and S*Ubc9. Ubc9 was *in vitro* sumoylated in large scale and separated from the unmodified form by using chromatographic methods. Conditions for the ProtoAarray were tested in *in vitro* sumoylation reactions on known SUMO substrates like Sp100 and E2-25K. The ProtoArray® itself was performed by the Invitrogen ProtoArray® service using our concept and the established conditions for the reaction. Newly identified substrates were verified in our *in vitro* system and will provide novel insights in the biological function of S*Ubc9.

TABLE OF CONTENT

1. INTRODUCTION

Post-translational modifications of proteins are an easy and rapid way to reversibly modulate the target's activity, stability or localisation. The most common post-translational modifications are phosphorylation, acetylation, methylation, ADP-ribosylation, ubiquitination and sumoylation. All of them play an essential role in cellular processes including cell signalling, maintenance of chromatin structure, regulation of gene expression, stress response, ribosome biogenesis and DNA repair. Reversible modifications with ubiquitin and its relatives, the ubiquitin-like proteins, are needed for the dynamic control of cellular events and for adaption to changing environmental conditions without de novo protein synthesis. Some of the well-known ubiquitin-like proteins are NEDD8, the interferon-inducible ISG15, FAT10 and the subject of this thesis, SUMO (small ubiquitin-related modifier) [1, 2].

1.1. The SUMO family

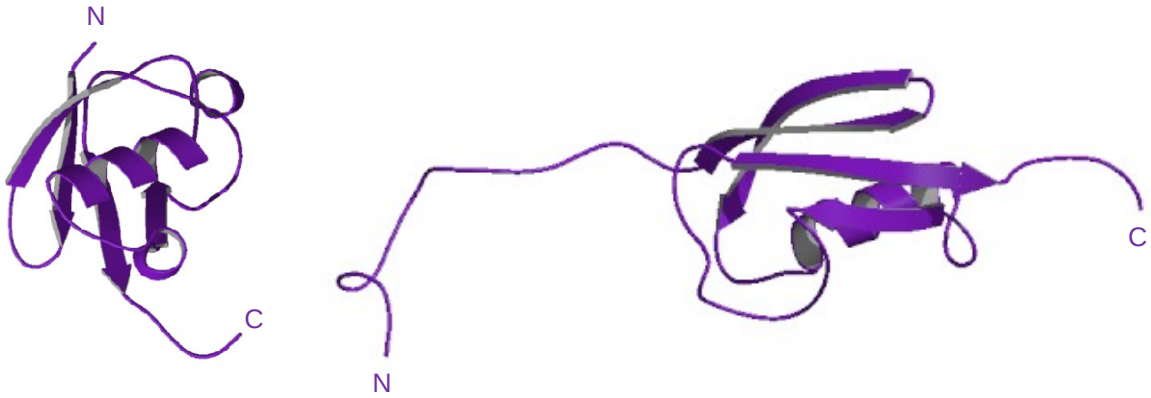


Figure 1: Tertiary structure of Ubiquitin (right panel) and SUMO1 (left panel) from ModBase [3]

SUMO is a small protein, 10.5kD in size, which gets covalently attached to its substrates. It is ubiquitously expressed in all eukaryotes, but missing in eubacteria and archea. One single SUMO gene has been identified in yeast and invertebrates, whereas in vertebrates four isoforms are known: SUMO1, SUMO2, SUMO3 and SUMO4 [1, 2, 4, 5]. SUMO1-3 are expressed ubiquitously, although there are tissue specific variations [6]. SUMO4 expression is restricted to kidney, spleen and lymph nodes [7, 8]. Interestingly, the loss of SUMO1 in mice is not lethal and can be compensated by SUMO2/3 [9]. Paralog specific substrates exist, but the set of substrates for SUMO1 and SUMO2/3 is mostly overlapping. Between mammalian SUMO2 and SUMO3 sequence similarity is about 95% and both share a sequence similarity with mammalian SUMO1 of ~50%. Notably, all 4 SUMO isoforms have only about 18% sequence identity with Ubiquitin, but they share a highly similar tertiary structure, as shown in Figure 1. Due to their non-related amino acid sequence, Ubiquitin and SUMO differ in the overall charge. At physiological conditions, SUMO is charged negatively in contrast to Ubiquitin, which is charged positively. In addition, the SUMO family members possess an extended,

flexible N-terminus, which is 14 residues longer than the one of Ubiquitin (also seen in Figure 1). SUMO is mostly conjugated to its substrate as a single moiety. SUMO2/3 chains have been observed, but the relevance is still unclear. Furthermore, most of the SUMO1 pool in a cell is conjugated to its substrates, whereas SUMO2/3 is mainly free and may build up a reservoir to react on specific stress signals [10].

1.2. SUMO modification cycle

Sumoylation is dependent on the equilibrium of conjugation and deconjugation. All SUMOs are conjugated to their substrates via an ATP-dependent enzymatic cascade closely related to ubiquitination. Deconjugation is achieved by a set of isopeptidases. In Figure 2 the conjugation and deconjugation cycle is shown schematically.

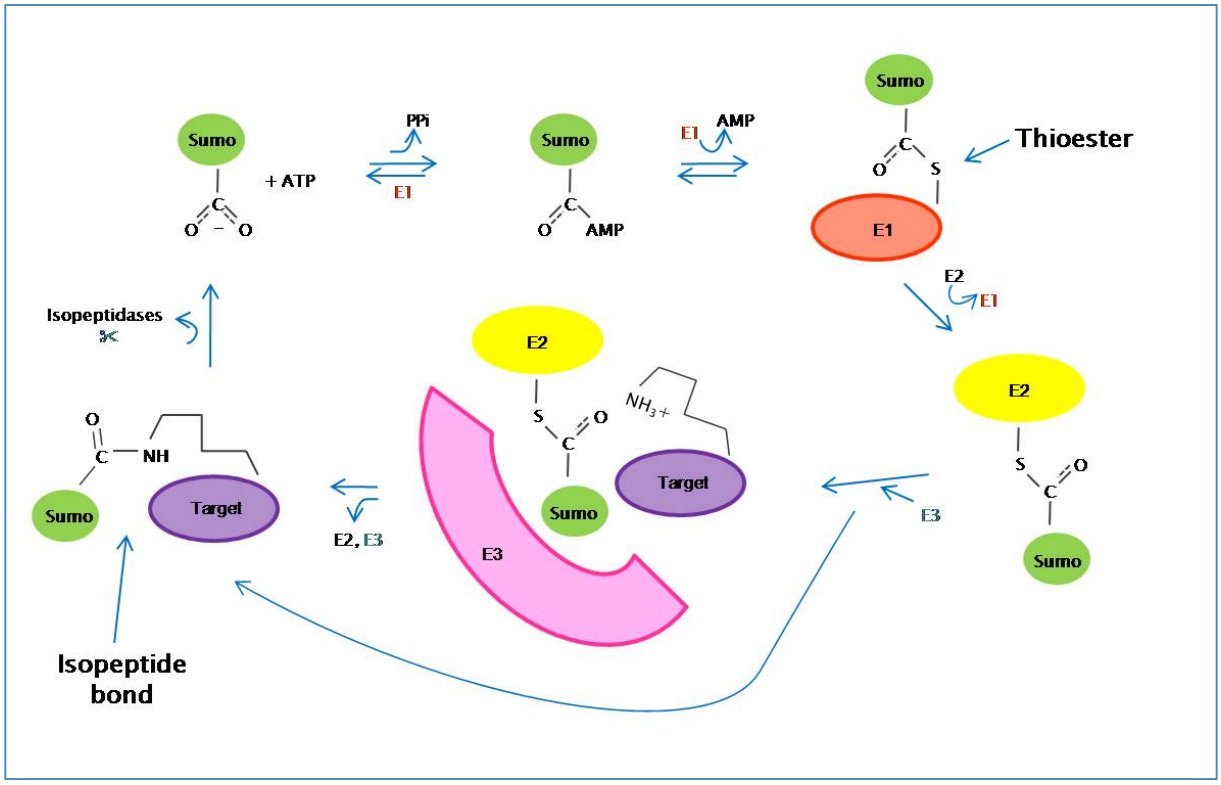


Figure 2: Sumoylation cycle
All SUMO members first need to be processed to free their Gly-Gly motif. SUMO is then activated in an ATP-dependent manner by forming a thioester with the E1 enzyme. Subsequently, SUMO is transferred to the E2 again resulting in a thioester bond. In the final step, SUMO is transferred with or without the help of an E3 ligating enzyme to the target lysine in the substrate resulting in an isopeptide bond. SUMO specific isopeptidases are able to reverse SUMO conjugation.

The enzymatic cascade responsible for SUMO conjugation involves an activating E1 enzyme, a conjugating E2 enzyme and usually, but not mandatory, a ligating E3 enzyme. SUMO's E1, E2 and E3 are specific for sumoylation and not able to substitute ubiquitin specific enzymes. SUMO is expressed as a precursor, which needs to be cleaved by SUMO specific proteases prior to target attachment. Upon processing, the C-terminal Gly-Gly motif is freed, which is a prerequisite for conjugation. The E1 enzyme then promotes the activation of SUMO in an ATP-dependent manner. All eukaryotic organisms contain only one single SUMO-activating

enzyme, a heterodimer between Aos1 and Uba2. For activation a thioester bond is transiently formed between the C terminal glycine of SUMO and the catalytic cysteine C173 of the E1 subunit Uba2 [11, 12]. In the next step of conjugation SUMO is transferred to the sole E2 conjugating enzyme Ubc9. The catalytic cysteine C93 of Ubc9 forms again a thioester bond with the C-terminal glycine of SUMO, which is then ready to get conjugated to its substrate. Upon SUMO conjugation, the substrate linkage is formed as an isopeptide bond between the C-terminal glycine of SUMO and the target lysine. In some cases Ubc9 itself is sufficient for conjugation [13], but there are several SUMO substrates depending on E3 ligase activity for efficient modification. E3 ligases accelerate the SUMO transfer from the E2 enzyme to the substrate and usually ensure substrate specificity. Most E3 ligases interact with both the SUMO loaded Ubc9 and the substrate, bring them in close vicinity and thereby push the SUMO transfer from the E2 enzyme to the substrate [14]. This mechanism is best understood for the Siz/Pias E3 ligase family, which possess a SP-RING finger - a domain related to the RING finger of ubiquitin E3 ligases. Other SUMO E3 ligases, like RanBP2, accelerate sumoylation by optimal positioning of the SUMO loaded E2 to the substrate for efficient transfer [15, 16]. In order to guarantee the reversibility of sumoylation, SUMO specific proteases, also known as isopeptidases, are able to cleave the isopeptide bond between SUMO and its substrate. In humans six SUMO specific proteases (Senp1-3 and Senp5-7) have been described for deconjugation, all of which belong to the family of ubiquitin-like proteases (Ulp). They share a C-terminal 200 amino acid core domain, which contains the catalytic triad (Cys-His-Asn), whereas their N-terminal regions are different. Besides SUMO isopeptide bond cleavage, they are responsible for SUMO processing and SUMO chain editing [17, 18].

1.3. SUMO consensus motif

The target lysine, where SUMO is covalently linked to, is often located in a SUMO consensus motif (ψKxE/D, ψ is a bulky aliphatic residue and x can be any residue). This motif is directly contacted by Ubc9. The target lysine reaches into the catalytic pocket of Ubc9 and the aliphatic and acidic amino acids interact with the residues on the surface of Ubc9 [19]. However, the SUMO consensus motif can only be recognized by Ubc9 when present in an extended loop structure or an unstructured area [20]. Two variants of the SUMO consensus motif have been identified: a phosphorylation dependent SUMO motif (PDSM) and a negatively charged one (NDSM). The PDSM contains a phosphorylated serine and proline in direct neighbourhood to the classical SUMO consensus motif (ψKxE/DxxSP) [21]. The NDSM is defined due to its acidic amino acids in addition to the SUMO consensus motif [22].

1.4. Non-covalent SUMO interaction

Besides covalent attachment, SUMO can also non-covalently interact with its substrates via a SUMO interaction motif (SIM) or a SUMO binding motif (SBM) in the interaction partners. This motif consists of a β-strand that can bind to SUMOs β_2-strand due to the environment of the hydrophobic core. The SIM core unit is defined as V/IxV/IV/I/L [23, 24, 25, 26]. SUMO-SIM

binding can occur in both, parallel and anti-parallel, orientation [27]. The general motif is further determined for SUMO1 binding by a stretch of acidic amino acids and/or phosphorylated serine residues in the neighbourhood imposing a negative charge. Phosphorylation as well as negatively charged acidic amino acids modulate the specificity and orientation of SIM binding to SUMO1 [28]. In contrast to SUMO1, SUMO2-SIM binding is not dependent on acidic amino acids and phosphorylation [26]. Mainly, the core SUMO2-SIM interaction is enough for a stable non-covalent interaction. Overall, the SUMO-SIM recognition is much more specific than the recognition of ubiquitin binding domains by ubiquitin, NEDD8 or FAT10 [26].

1.5. Consequences of sumoylation

Although often only a small fraction of a certain protein is sumoylated at a given time, the consequences for the respective protein can be determining. They can range from altered intracellular localization to changes in activity or stability. In general, it is assumed that SUMO changes interactions and can either interfere with an existing binding interface between substrate and a binding partner or provide a new one via SIM interaction [20, 29]. Moreover, sumoylation has also been revealed to cause conformational changes by intramolecular SIM binding [30]. Among the best studied functions SUMO plays an essential role in DNA repair and genomic integrity [30, 31, 32] protein stability [33, 34, 35] nuclear-cytosolic transport [36, 37, 38] and transcriptional regulation [39, 40, 41].

1.6. The sole SUMO conjugating enzyme Ubc9

The 18kD Ubc9 is the sole E2 conjugating enzyme for sumoylation. As already mentioned, it possesses two essential functions in the SUMO conjugation pathway: accepting the activated SUMO from the E1 enzyme Aos1/Uba2, thereby forming a thioester linkage, and subsequently transferring SUMO to the lysine of its target. Because of its central role in this enzymatic cascade, it requires a binding interface for the E1 and E3 enzymes. Notably, a patch surrounding the active cysteine C93 of Ubc9 binds directly to the consensus sequence in the substrate. This interaction is not efficient for the transfer of SUMO to the target lysine, but sufficient for some substrates. Like for Aos1 and Uba2, Ubc9 is essential in all eukaryotic organisms tested except S. pombe [42]. The expression levels of Ubc9 differ between organs and tissues [43]. Interestingly, Ubc9 expression is frequently upregulated in human cancer [44]. To date less is known about the molecular mechanisms regulating the expression of such a central enzyme in sumoylation. But there is evidence for the regulation of Ubc9 on the post-translational level. For example during oxidative stress, low H_2O_2 concentrations induce reversible disulfide bond formation between the catalytic cysteines of the Uba2 E1 subunit and Ubc9. The result is a loss of SUMO conjugation and consequently, desumoylation of most cellular SUMO targets [45]. Moreover, Ubc9 was shown to be modified by S-nitrosylation upon treatment with the nitric oxide donor GSNO [46].
Intriguingly, another post-translational modification of Ubc9 is its own sumoylation outlined in 1.7. in more detail.

1.7. Sumoylation of Ubc9

Sumoylated Ubc9 (S*Ubc9) has been identified in every screen for novel SUMO substrates [25, 47, 48, 49]. Sumoylation of the mammalian Ubc9 has then been mapped to lysine K14 in a non-consensus SUMO acceptor site *in vivo* and *in vitro* [29]. The structurally similar ubiquitin E2 conjugating enzyme E2-25K is also sumoylated at lysine K14 in a non-consensus site in its N-terminus. But despite these structural similarities, sumoylation of Ubc9 does not impair its enzymatic activity as shown for E2-25K [20]. However, our laboratory has recently shown that sumoylation of Ubc9 regulates target discrimination in mammals. Sumoylated Ubc9 strikingly enhances the modification of the transcriptional regulator Sp100 comparable to E3 ligases. Sp100 contains a SIM, which is able to interact with SUMO linked to lysine K14 of Ubc9. This additional binding interface increases the affinity between Sp100 and the sumoylated Ubc9, thus resulting in enhanced modification. On the other hand, other substrates are not affected by sumoylated Ubc9 or even impaired. For example, sumoylation of Ubc9 has no effect on E2-25K modification but impairs activity on RanGAP1. These findings suggest a role for Ubc9 contributing to target recognition and possibly controlling sumoylation in an E3 ligase independent way [29].

1.8. Controlling SUMO conjugation

In contrast to the large number of SUMO substrates, only a few SUMO E3 ligases are known and it is currently unclear, how substrate specificity is performed. One mechanism to ensure substrate specificity is by controlling substrates and enzymes in a spatial and temporal manner. Another explanation is that several E3 ligases await identification. Intriguingly, recent studies point to a central role of Ubc9 in target selection [19, 29].

In our working model, we propose that the affinity between SUMO loaded Ubc9 and the substrate determines the modification efficiency. In the classical way, an increase in affinity is achieved via E3 ligases, which bind both the E2 and the substrate and bring them in close proximity for an efficient SUMO transfer (Figure 3, E3 dependent). Recent studies have revealed different mechanism of how substrate affinity can be increased in an E3 independent manner. The best understood example is RanGAP1, which stably interacts with Ubc9 via an additional binding interface next to the interaction with the SUMO consensus site in the substrate. This additional interface is required for efficient modification (Figure 3, E3 independent, left pannel) [19]. Alternatively, thioester (Figure 3, E3 independent, middle panel) or isopeptide (Figure 3, E3 independent, middle panel) bonded SUMO to Ubc9 can increase the affinity to the substrate. This depends on a functional SIM motif in the substrate. To date substrates like Daxx [30], TDG [50], USP25 [51], BLM and HIPK2 [52] were described to depend on a functional SIM for efficient sumoylation, which enhances the affinity for SUMO loaded Ubc9. For the sumoylated Ubc9 currently only Sp100 was identified as substrate [29].

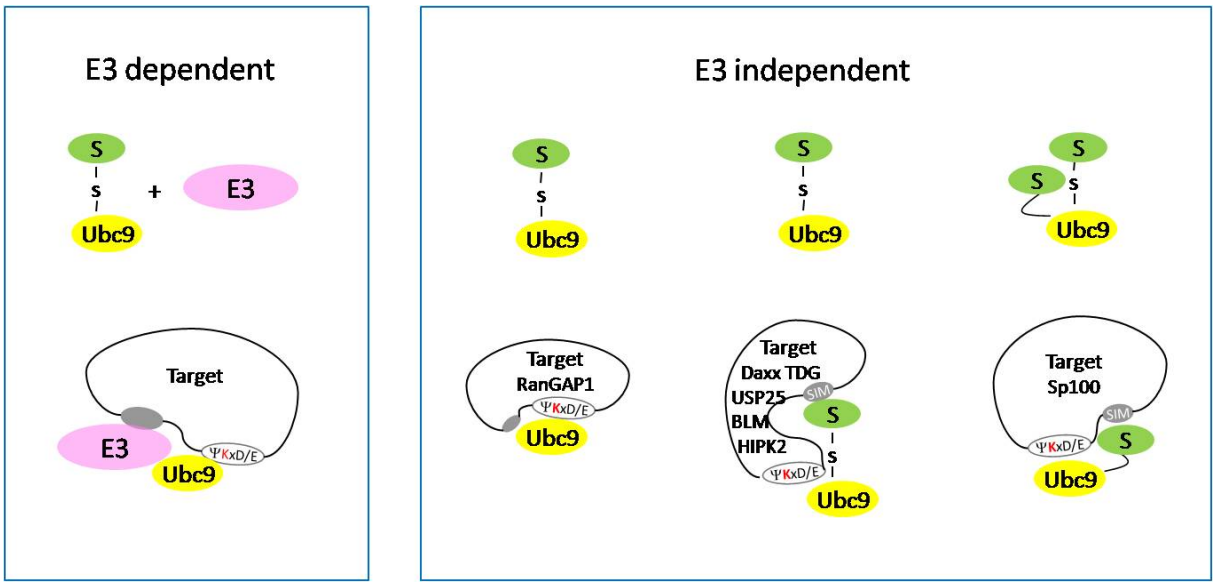


Figure 3: Model of E3 dependent and independent target recognition in SUMO conjugation (adapted from Knipscheer et al., 2008)[29]
Ubc9 is shown in yellow, SUMO in green, RING type E3 ligase in pink. Ubc9´s catalytic cleft directly binds to SUMO consensus motifs (ψKxE/D). This interaction is not sufficient for an efficient SUMO transfer from Ubc9 to the target. RING E3 ligases bind to both, the substrate and Ubc9, and stabilise the interaction for efficient SUMO transfer (E3 dependent). An increase in substrate/Ubc9 affinity can also be achieved by E3 independent mechanism via additional binding interfaces (grey) with either Ubc9 (E3 independent, left panel), the SUMO~Ubc9 thioester (E3 independent, middle panel) or the SUMO modified Ubc9 (E3 independent, right panel). Interaction via SUMO bound to Ubc9 depends on a SIM in the target.

2. AIM OF THIS WORK

Our laboratory has discovered a novel mechanism of how sumoylation of Ubc9 contributes to target discrimination. Whereas sumoylated Ubc9 does not interfere with the sumoylation of most SUMO substrates, it impairs modification of RanGAP1 and significantly enhances modification of the transcriptional regulator Sp100 [29]. To date Sp100 is the sole substrate for this mechanism and the aim of this thesis was the identification of additional substrates targeted by S*Ubc9. In order to address this aim, *in vitro* sumoylation reactions with Ubc9 and S*Ubc9 were performed on a protein microarray chip ("ProtoArray® Human Protein Microarray v5.0") purchasable from Invitrogen. Such microarrays contain over 9000 human proteins and were developed as rapid and sensitive means to identify novel substrates for phosphorylation and ubiquitination [53, 54, 55]. In this study the ProtoArray® was applied for sumoylation reaction. The advantage of this particular array is that proteins were expressed in insect cells as N-terminal GST fusions using a baculovirus expression system. Purification was under native conditions to preserve protein structure. The proteins were printed in duplicates at a reported level on the ProtoArray®, which guarantees reliable and reproducible results [56]. To perform an *in vitro* sumoylation reaction with S*Ubc9 on this ProtoArray®, all components needed to be expressed and purified in an E. coli expression system. This included the SUMO1, HA tagged SUMO1, the E1 enzyme and Ubc9. To obtain S*Ubc9, Ubc9 was *in vitro* sumoylated and separated from the unmodified form. Conditions for the ProtoAarray were established in *in vitro* sumoylation reactions on known SUMO substrates. The ProtoArray® itself was performed by the Invitrogen ProtoArray® service using our established conditions for the reaction. Newly identified substrates were verified in our *in vitro* system and will provide novel insights in the biological function of S*Ubc9.

3. RESULTS

3.1. Purification of recombinant proteins

For producing high amounts of modified Ubc9, all components for the sumoylation reaction were expressed in E. coli and purified to near homogeneity. This included the purification of SUMO1, the heterodimeric E1 enzyme Aos1/Uba2 and the E2 enzyme Ubc9.

3.1.1. Large scale purification of recombinant SUMO1

SUMO1 cloned under the control of the T7 promoter was transformed into the bacterial E.coli strain BL21 gold and expression was induced by addition of IPTG in a total of 4 litres culture. In the first purification step, the negatively charged SUMO1 was bound to Q Sepharose (GE Healthcare), a strong anion exchange matrix. The retained SUMO1 was subsequently eluted with Tris-HCl buffer, pH 7.5, containing high salt. In the final step the SUMO1 containing fractions were applied to a HiLoad S200 size exclusion chromatography column on a FPLC (Fast protein liquid chromatography) Äkta system (GE Healthcare).
Figure 4A demonstrates the monitored fractionation of the SUMO1 size exclusion chromatography. On the x-axis the elution volume is plotted in sample fraction collected. On the y-axis the protein concentration in mAU (milli absorbance unit) is plotted measured by light absorbance of the aromatic amino acids tryptophan, tyrosine and phenylalanine at 280nm. Salt concentration is detected by monitoring the conductivity of the buffer in mS/cm (millisiemens / cm). The curve obtained from the SUMO1 gelfiltration indicated three peaks, with significant absorbance above 100mAU. The fractions of these peaks were analysed by resolving 10µl of each fraction on a 12.5% SDS-PAGE followed by Coomassie staining as shown in Figure 4B. Whereas in the first peak (fraction 19 to 30) mainly bacterial proteins were eluted, the second peak (fractions 31 to 28) contained near homogenous recombinant SUMO1. SUMO1 migrates much slower as its molecular weight of 10.5kD because of the flexible N-terminus. The third peak (fraction 44 to 47) overlaps with the conductivity peak and represents the “salt peak”. The SUMO1 containing fractions 33 to 37 were pooled, aliquoted, shock frozen in liquid nitrogen and stored at -80°C for further analysis.
In a subsequent SDS-PAGE shown in Figure 4C, different amounts of the pooled SUMO1 containing fractions were analysed for purity and for its concentration by comparison to known concentrations of a reference protein. Estimated concentration of SUMO1 was 2mg/ml.

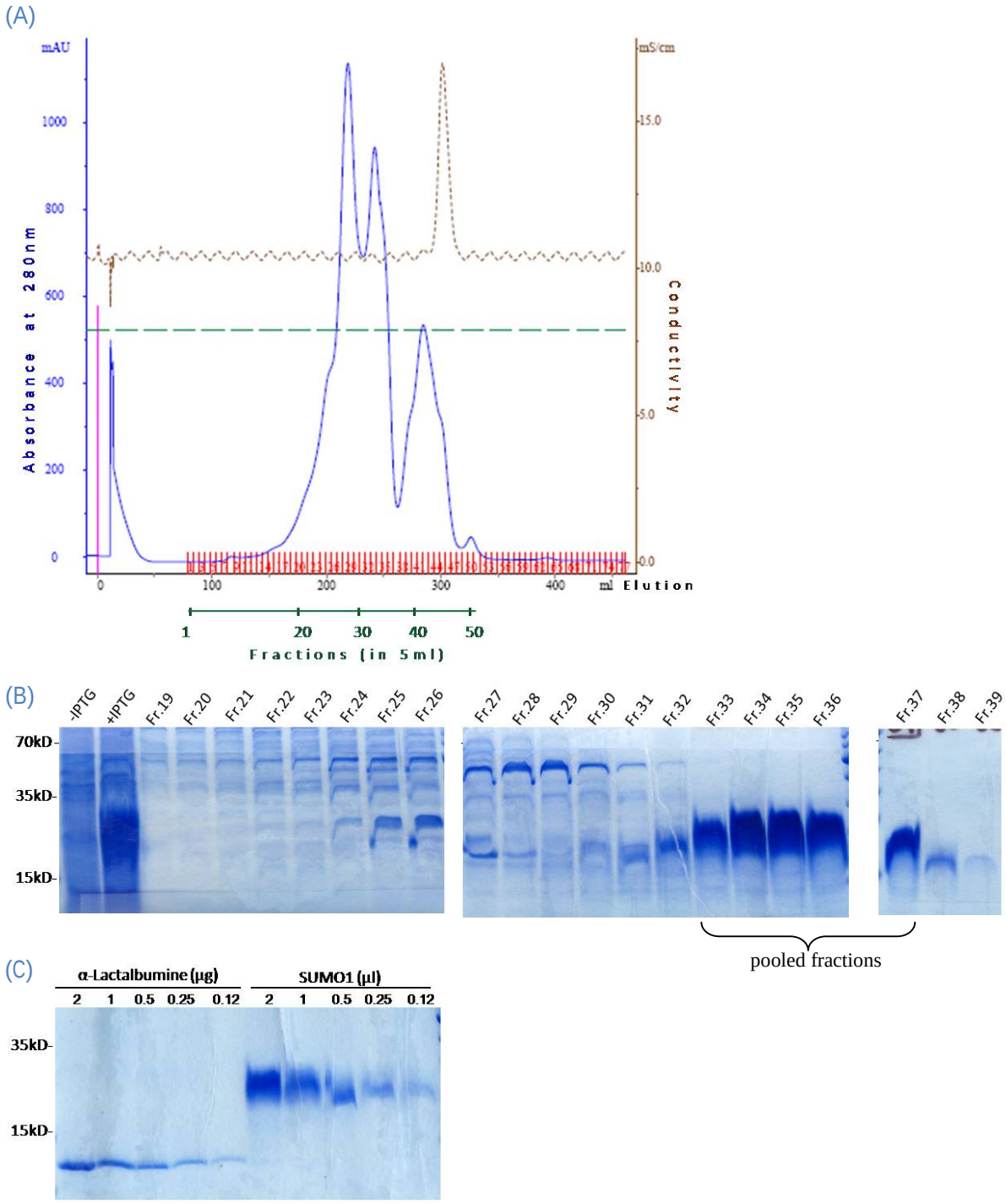


Figure 4: Purification of SUMO1
SUMO1 was IPTG induced and expressed in E. coli BL21 gold. The purification steps included enrichment on Q Sepharose and subsequent size exclusion chromatography on a FPLC Äkta purifier.
(A) Chromatogram of SUMO1 size exclusion chromatography on a HiLoad S200 column.
(B) 10µl aliquots of uninduced and induced (-/+IPTG) SUMO1 expression and fractions collected from the HiLoad S200 column run were separated on a 12.5% SDS-PAGE and Coomassie stained, indicated fractions were pooled, aliquoted and frozen to -80°C for further analysis.
(C) Concentration of SUMO1 in comparison to the reference protein α-Lactalbumine, Coomassie staining of α-Lactalbumine and SUMO1 resolved on a 12.5% SDS-PAGE is shown.

3.1.2. Purification of the recombinant E1 enzyme Aos1/Uba2

Both Aos1 and Uba2, which were cloned under the control of the T7 promoter as His tag fusions, were transformed into the bacterial E.coli strain BL21 gold and expression was induced with IPTG for 6 hours in a total of 2 litres culture each. The proteins were purified separately but following the same protocol including the binding of Aos1 and Uba2 to Ni^{2+} beads (ProBond^{TM} Resin, GE Healthcare), which binds His tagged proteins. Elution was done with a buffer containing a high concentration of imidazole, which displaces His-tagged proteins from the beads. In the final step, the eluted protein containing fractions were applied to a S200 size exclusion chromatography column on a FPLC Äkta system.

Figure 5A and Figure 5C demonstrate the chromatogram of His-Aos1 and Uba2-His separated on a size exclusion column, respectively. His-Aos1 eluted in one broad peak with a shoulder. 10µl of each peak fraction were resolved on a 10% SDS-PAGE followed by Coomassie staining as shown in Figure 5B. All fractions contained highly concentrated, nearly homogenous His-Aos1. Fractions 7 to 17 were pooled, aliquoted, shock frozen in liquid nitrogen and stored at -80°C for further purification. Uba2-His eluted in a peak with several shoulders and again 10µl of each peak fraction was resolved on a 10% SDS-PAGE followed by Coomassie staining (shown in Figure 5D). Fractions 9 to 16 contained high amounts of Uba2 and were therefore pooled, aliquoted, shock frozen and stored at -80°C.

Figure 5E demonstrates a dilution series of pooled His-Aos1 and Uba2-His, respectively. Equal amounts (4mg of each) Aos1 and Uba2 were incubated for 1 hour on ice for forming the active heterodimeric E1 complex, which was subsequently purified by size exclusion chromatography. Figure 6A demonstrates the monitored fractionation of the E1 complex on a gelfiltration column. The peak fractions were analysed by separation on a 5-20% SDS-PAGE with subsequent Coomassie staining as shown in Figure 6B. Fractions containing equal molarities of the His-Aos1/Uba2-His complex (fractions 35 to 37), were pooled, aliquoted, shock frozen and stored at -80°C for further analysis.

To determine the protein concentration of the purified E1 complex, comparison to a reference protein was performed on a 5-20% SDS-PAGE followed by Coomassie staining (Figure 6C). Estimated concentration of the E1 enzyme was 0.5 mg/ml.

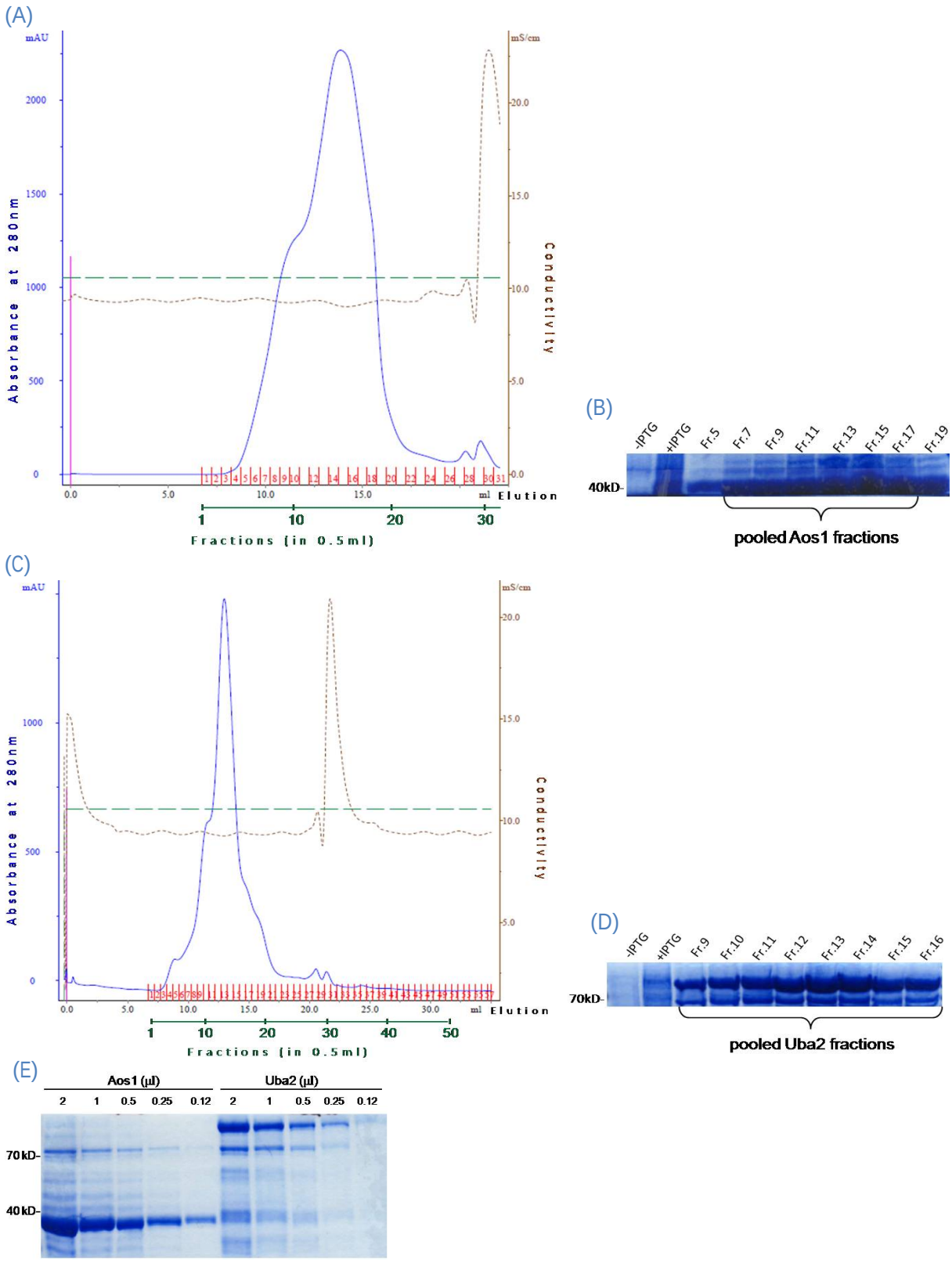


Figure 5: Purification of His-Aos1 and Uba2-His

His-Aos1 and Uba2-His were IPTG induced and expressed in E. coli BL21 gold. The purification steps included enrichment on ProBond™ Resin and subsequent size exclusion chromatography on a FPLC Äkta purifier.

(A) Chromatogram of His-Aos1 on a S200 column.

(B) 10µl aliquots of uninduced and induced (-/+IPTG) Aos1 expression and peak fractions collected from (A). Samples were separated on a 10% SDS-PAGE and Coomassie stained. Indicated fractions were pooled, aliquoted and used for further E1 purification.

(C) Chromatogram of Uba2-His on a S200 column.

(D) as in (B) for Uba2-His.

(E) Dilution series of His-Aos1 and Uba2-His resolved on a 5-20% SDS-PAGE followed by Coomassie staining.

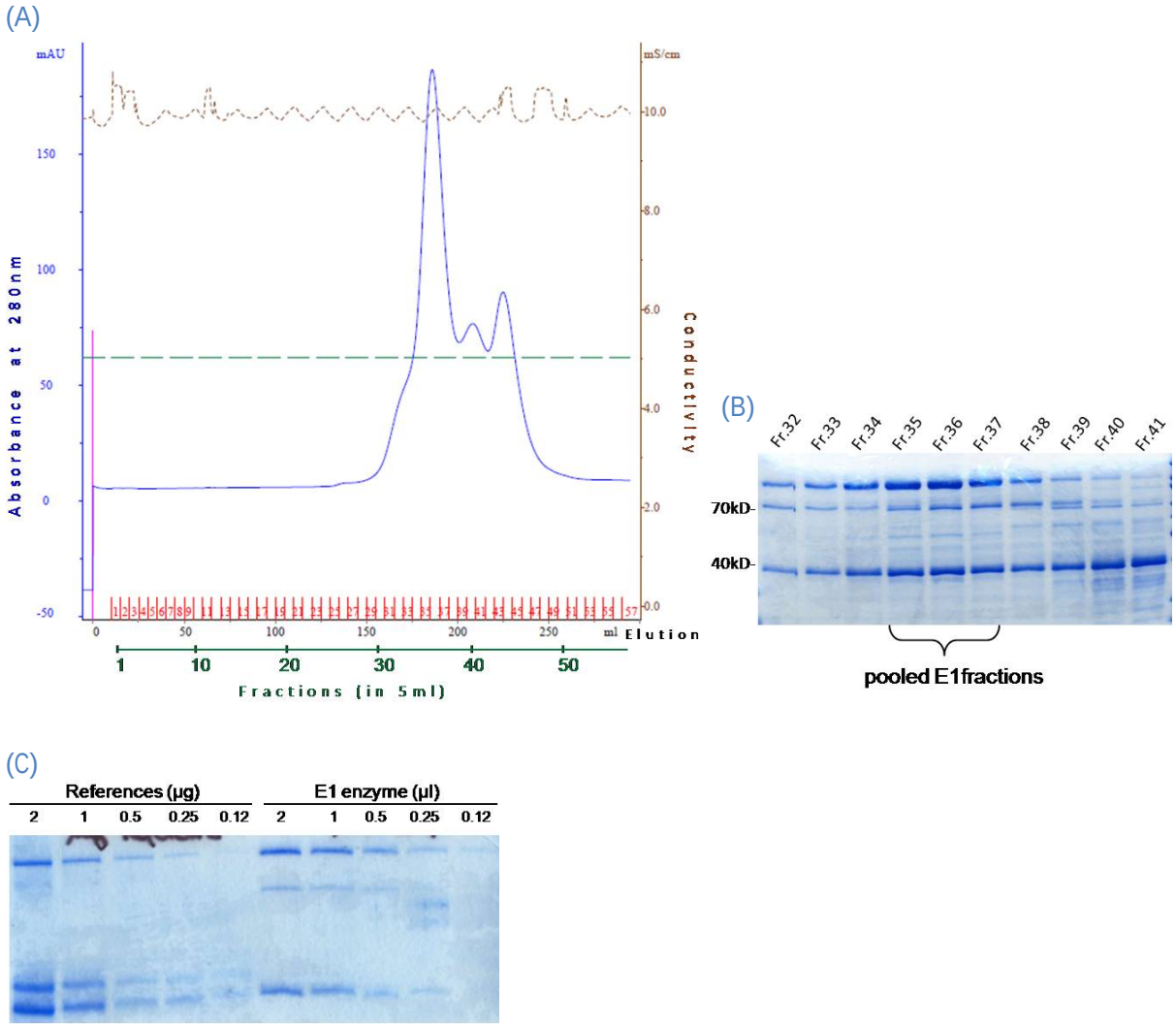


Figure 6: E1 complex formation
4 mg of each His-Aos1 and Uba2-His were pooled and incubated 1 hour on ice followed by size exclusion chromatography on a FPLC Äkta purifier.
(A) Chromatogram of the His-Aos1/Uba2-His complex separated on a HiLoad S200 column.
(B) 10µl aliquots of peak fractions were separated on a 5-20% SDS-PAGE and Coomassie stained. Indicated fractions were pooled, aliquoted and frozen to -80°C.
(C) Concentration determination of the heterodimeric E1 enzyme in comparison to the reference proteins ovalbumine and phosphorylase by Coomassie staining of ovalbumine, phosphorylase and the heterodimeric E1 resolved on a 5-20% SDS-PAGE shown.

3.1.3. Large scale purification of the recombinant E2 enzyme Ubc9

Ubc9, which was cloned under the control of the T7 promoter, was transformed into the bacterial E.coli strain BL21 gold and expression was induced with IPTG in a total of 12 litres bacterial culture. The positively charged Ubc9 was bound to the strong cation exchange matrix SP Sepharose (GE Healthcare). The retained Ubc9 was eluted with a phosphate buffer, pH 6.5, containing high salt. The Ubc9 containing eluate was applied to a HiLoad S200 size exclusion chromatography column on a FPLC Äkta system.

Figure 7A demonstrates the monitored fractionation of the Ubc9 size exclusion chromatography. As described above, the x-axis represents the elution volume in sample fractions and the y-axis the protein concentration in mAU (milli absorbance unit) at 280nm. Salt concentration is indicated as conductivity in mS/cm. Ubc9 eluted in a single peak with high absorbance. 10µl aliquots of each fraction were resolved on a 12.5% SDS PAGE and

Coomassie stained as shown in Figure 7B. The induction and expression of Ubc9 (-/+IPTG) worked very well and the fractions of the peak contained high amounts of recombinant Ubc9. Fractions 33 to 35 with the highest amount of Ubc9 were pooled, aliquoted, shock frozen in liquid nitrogen and stored at -80°C for further purification.

Different amounts of the pooled Ubc9 were finally analysed for its concentration by comparison to a reference protein with known concentration (Figure 7C). Estimated concentration of Ubc9 was 2mg/ml.

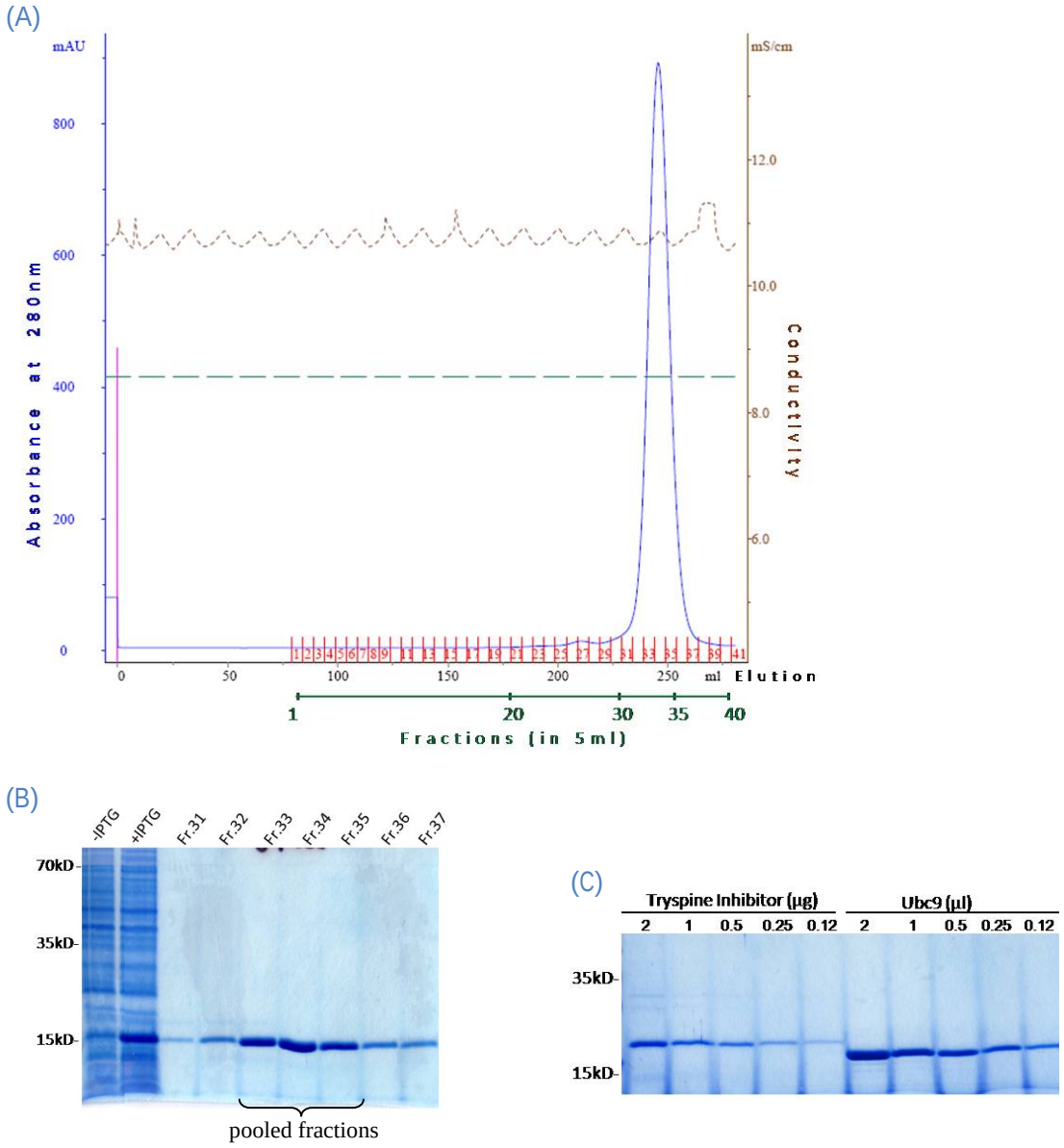


Figure 7: Purification of Ubc9

Ubc9 was IPTG induced and expressed in E. coli BL21 gold. The purification steps included enrichment on SP Sepharose and subsequent size exclusion chromatography on a FPLC Äkta purifier.

(A) Chromatogram of Ubc9 size exclusion chromatography on a HiLoad S200 column.

(B) 10µl aliquots of uninduced and induced (-/+IPTG) Ubc9 expression and fractions collected from the HiLoad S200 column run were separated on a 12.5% SDS-PAGE and Coomassie stained, indicated fractions were pooled, aliquoted and stored at -80°C for further analysis.

(C) Concentration of Ubc9 in comparison to the reference protein Trypsine Inhibitor, Coomassie staining of Trypsine Inhibitor and Ubc9 resolved on a 12.5% SDS-PAGE is shown.

3.1.3.1. Enzymatic analysis of Ubc9

Ubc9 possesses catalytic activity and therefore it is essential to test the enzymatic activity of the newly purified protein. This was performed by comparing its enzymatic activity with a previously purified Ubc9 in an *in vitro* sumoylation reaction. E2-25K was used as substrate, since it is efficiently modified without an E3 ligase.
A dilution series from 7 to 0.06 µM of the "old" and the "new" purified Ubc9 was mixed each with 6.6µM E2-25K, 19µM SUMO1, 70nM E1 and ATP and incubated for 2 hours at 30°C. Reactions were stopped with 2x Laemmli buffer and proteins were separated on SDS-PAGE followed by Coomassie staining.

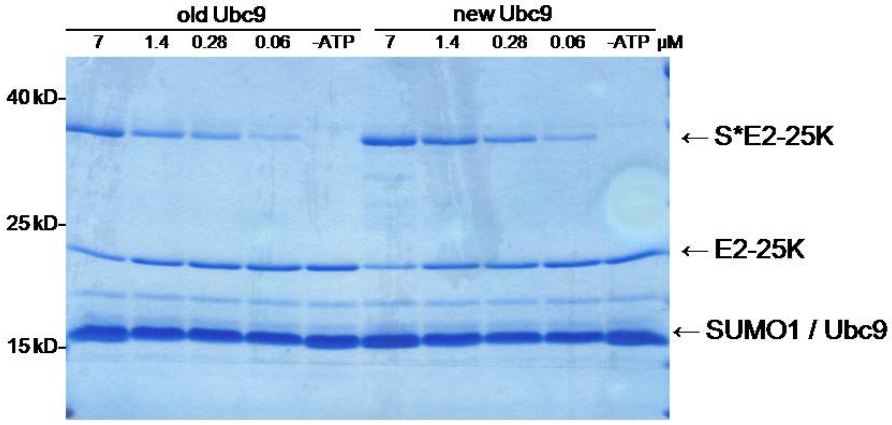


Figure 8: Enzymatic analysis of Ubc9 activity by *in vitro* sumoylation assays with E2-25K as substrate
In vitro sumoylation: 19µM SUMO1, 70nM E1, 6.6µM E2-25K and different amounts of old Ubc9 and new Ubc9 were incubated at 30°C for 2h in 20µl reaction volume without and with ATP. Reaction mix was separated on 5-20% SDS-PAGE and Coomassie stained.

As shown in Figure 8, the newly prepared Ubc9 was as efficient in E2-25K sumoylation as an older Ubc9 preparation. At the highest Ubc9 concentration approximately 80% of E2-25K was modified after 2 hours (lane 1 and 6). Even at the lowest Ubc9 levels E2-25K sumoylation was detected (lane 4 and 8). This experiment confirmed that the newly purified Ubc9 is as active as Ubc9 from an earlier preparation and can be used for further analysis.

3.1.4. Purification of the S*Ubc9

As described above, all proteins required for the large scale purification of sumoylated Ubc9 were newly purified including SUMO1, the SUMO E1 activating enzyme Aos1/Uba2 and the SUMO conjugating enzyme Ubc9. In the next steps, the large scale *in vitro* sumoylation reaction of Ubc9 was performed and S*Ubc9 was separated from its unmodified form. It is important to mention that both Ubc9 and its sumoylated form had to be handled in the same manner for maintaining comparable enzymatic activity.

3.1.4.1. *In vitro* sumoylation of Ubc9

Prior to the large scale *in vitro* Ubc9 sumoylation, small scale reactions were performed to identify the optimal conditions. 55.5µM Ubc9, 62µM SUMO1, 70nM E1 and 5mM ATP were incubated at 37°C and 20µl aliquots were taken every hour. Consequently, these aliquots were resolved on 5-20% SDS-PAGE and stained with Coomassie.

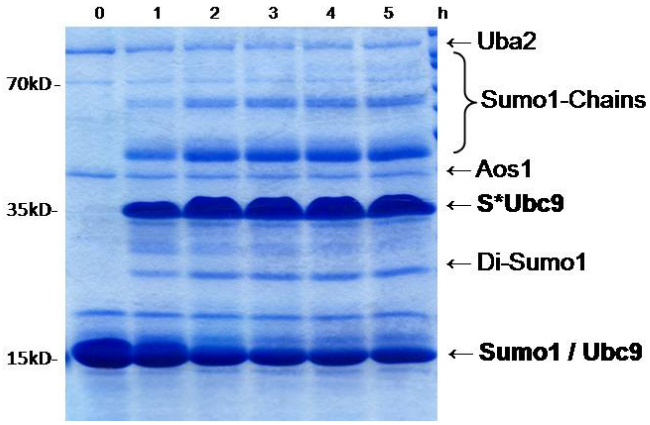


Figure 9: Small scale *in vitro* sumoylation of Ubc9
In vitro sumoylation: 55.5µM Ubc9, 62µM SUMO1 and 70nM E1 were incubated at 37°C for up to 5 hours in 200µl reaction volume with 5mM ATP and $MgCl_2$, every hour one aliquot of 20µl was taken, separated on 5-20% SDS-PAGE and Coomassie stained.

In Figure 9 the modification efficiency of Ubc9 sumoylation can be followed over time. At time point 0 (lane 1) the starting situation is shown. Both, Ubc9 and SUMO1 migrate at a similar size in a 5-20% SDS PAGE and were therefore not distinguishable in a Coomassie stained gel. The E1 enzyme subunits His-Aos1 and Uba2-His migrate at 40kD and 90kD, respectively. The sumoylated Ubc9 migrates at 35kDa and was detectable already after one hour at 37°C in the presence of ATP. At this time point approximately 30% of Ubc9 was modified. In a parallel reaction, di-SUMO1 and SUMO1 chains appeared. At 2 hours approximately 60% of Ubc9 were modified, but also the amount of SUMO1 chains increased. At later time points the amount of modification neither for Ubc9 nor the SUMO1 chains increased, most likely because of ATP breakdown. For our use, approximately 50% of modified Ubc9 were required since the aim was to obtain comparable amounts of unmodified Ubc9, too. Therefore, the ideal condition for our approach was the incubation of the Ubc9 sumoylation reaction for two hours.
For the large scale preparation of sumoylated Ubc9, the reaction mix was hundredfold increased (55µM Ubc9, 62µM SUMO1 and 70nM E1 in 20 ml SAB-buffer) and incubated for 2 hours at 37°C with ATP. The pH of the reaction mix was 8.0. The reaction is monitored in Figure 10 showing aliquots before and after two hours incubation time.

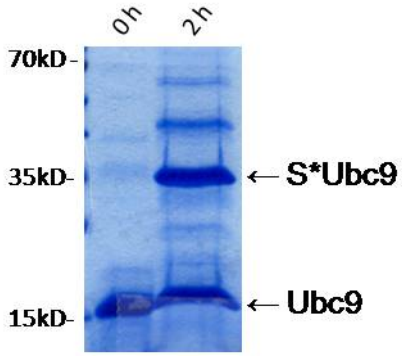


Figure 10: Large scale *in vitro* sumoylation of Ubc9
In vitro sumoylation: 55µM Ubc9, 62µM SUMO1 and 70nM E1 were incubated at 37°C for 2 hours in 20ml reaction volume with ATP and $MgCl_2$. 10µl aliquots of the reaction were separated on 12.5% SDS-PAGE and stained with Coomassie. Time point 0 and 2 h are shown.

3.1.4.2. Separation of the modified from the unmodified Ubc9

To separate the modified from the unmodified Ubc9, the large scale reaction was stopped by incubation on ice. The reaction mix was diluted 1 : 2.5 with 20mM Tris.HCl (pH 8.0) to decrease the salt concentration from 100mM to 40mM NaCl. These conditions allow the sumoylated Ubc9 but not its unmodified form to bind to the positively charged ion exchange HiPrep Q Sepharose column. At pH 8.0 the attachment of the negatively charged SUMO changes the isoelectric point of the rather positively charged Ubc9. Therefore Ubc9 was expected to be obtained in the "flow through", whereas free SUMO1, SUMO1 chains and the sumoylated Ubc9 would bind to the column. After washing with one column volume, elution was performed with a salt gradient from 40 mM to 1M NaCl for 5 column volumes. Figure 11A demonstrates the chromatogram of the HiPrep Q Sepharose run. Sample collection started immediately after sample injection because of the unmodified Ubc9 in the flow through. The chromatogram shown in Figure 11A demonstrate a flattened curve as 'flow through' followed by a high peak with three shoulders and a third separated small peak due to the salt gradient elution. For verification, 10µl aliquots of every fourth fraction in the 'flow through' or every second fraction in the elution was resolved on a 12.5% SDS-PAGE and Coomassie stained as shown in Figure 11B. As expected, the flow through contained the unmodified Ubc9, whereas the large peak with the three shoulders contained S*Ubc9, SUMO1 and SUMO1 chains. It was not possible to separate these three fused peaks. Their charge is quite similar at pH 8.0. The third separated smaller peak showed no protein content in Coomassie staining (Data not shown). Fractions 24 to 80 were pooled to obtain all Ubc9, whereas fractions 114 to 122 were pooled to obtain the highest amount of S*Ubc9 with the least SUMO1 chain contamination. The pooled fractions were used for further purification.

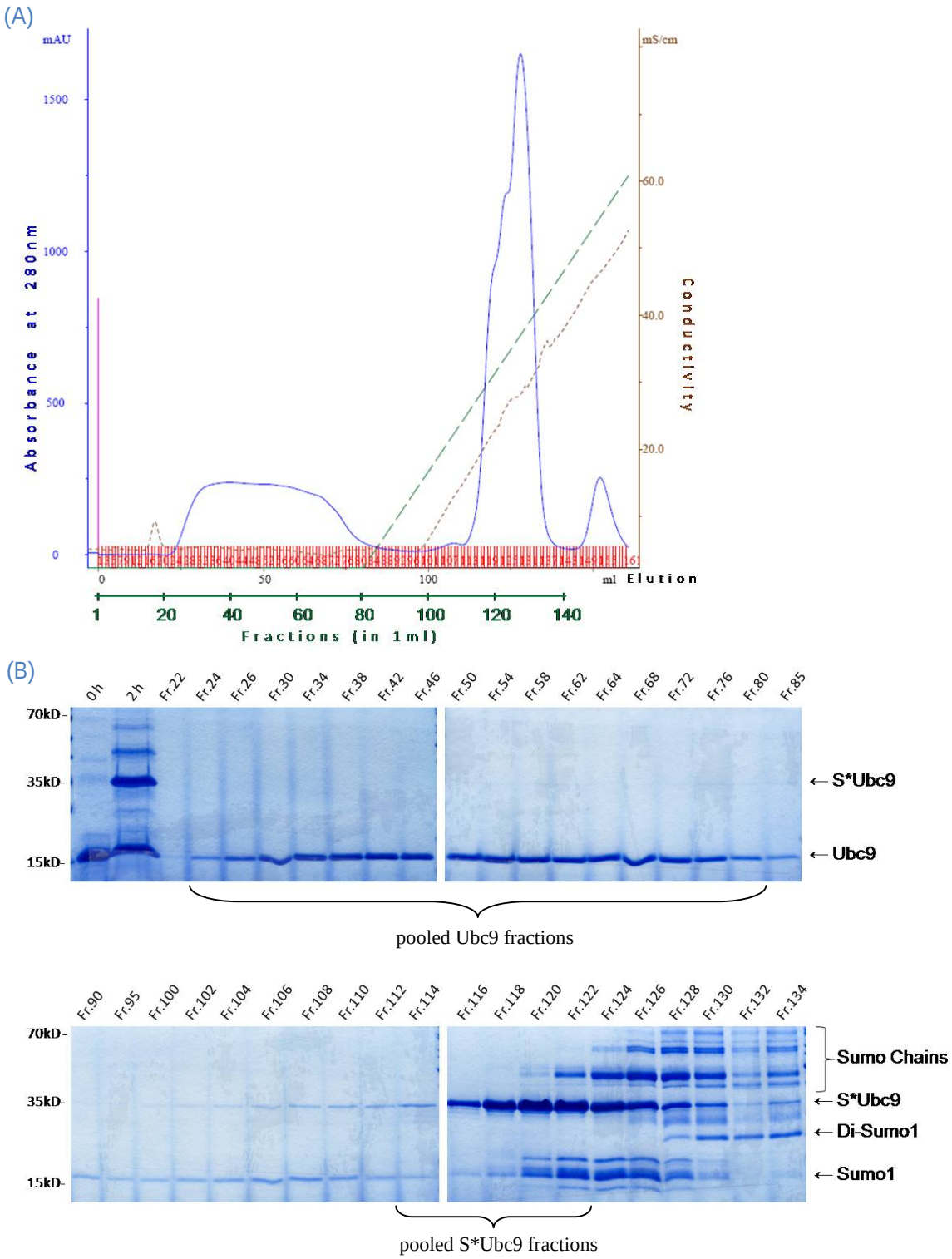


Figure 11: Large scale *in vitro* sumoylation of Ubc9 and initial purification via anion exchange chromatography using an FPLC Äkta purifier system.
(A) Chromatogram of a large scale Ubc9 *in vitro* sumoylation reaction separated on a HiPrep Q Sepharose column. *In vitro* sumoylation reaction was performed with 55µM Ubc9, 62µM SUMO1 and 70nM E1 and incubated at 37°C for 2 hours in 20ml reaction volume with ATP and $MgCl_2$. Prior to applying to the Q Sepharose column, the reaction mix was diluted 1: 2.5 with 20mM Tris.HCl, pH 8.0.
B) 10µl aliquots of the large scale *in vitro* reaction (lane 1 and 2) and 10µl aliquots of fractions collected in (A) were separated on a 12.5% SDS-PAGE and stained with Coomassie. Indicated fractions containing Ubc9 and S*Ubc9 were pooled and used for further purification.

Additional purification steps for Ubc9 and S*Ubc9 included cation exchange and size exclusion chromatography. The cation exchange chromatography was not only applied to further purify the proteins of interest, but also to concentrate them before buffer exchange on a size exclusion column. To increase binding of the positively charged Ubc9 to the negatively charged matrix of SP sepharose, the pH 8.0 of the Tris buffer was diluted 1:1 with 75mM BisTris (pH 6.5) resulting in full recovery of Ubc9 on the column. Elution was performed with a salt gradient (25mM to 1M NaCl) for four column volumes. The chromatogram of Ubc9 on the SP sepharose XL column is shown in Figure 12A. The corresponding fraction resolved by separation on a 12.5% SDS PAGE and Coomassie staining are demonstrated in Figure 12B. Ubc9 was concentrated in three fractions, which were applied to a preparative S200 gel filtration column, equilibrated in the physiological transport buffer. Figure 12C demonstrates the chromatogram of this separation. Fractions 43 to 49 were pooled, aliquoted, shock frozen in liquid nitrogen and stored at -80°C for further analysis. Figure 12D shows the concentration determination of Ubc9 in comparison to the reference protein Trypsine Inhibitor at indicated concentration. In conclusion, the purified Ubc9 has approximately a concentration of 2mg/ml.

In parallel, a similar procedure was performed for the S*Ubc9. Conjugation of the negatively charged SUMO reduces the positive charge of Ubc9. Also the relatively high salt concentration required for elution of S*Ubc9 in the previous purification step would avoid binding to the cation exchange column. Therefore S*Ubc9 depends on prior dialysis to 20mM BisTris (pH 6.5) to achive efficient binding to the SP sepharose XL column. Elution was performed with a salt gradient (25mM to 1M NaCl) for four column volumes. In this step, S*Ubc9 was not only concentrated but also separated from the majority of contaminations, like the negatively charged free SUMO1 and SUMO1 chains. The chromatogram of this column run is shown in Figure 13A and the analysed fractions resolved on a 12.5% SDS-PAGE and Coomassie stained are shown in Figure 13B. S*Ubc9 fractions still contained trace amounts of SUMO1 and SUMO1 chains, most likely due to non-covalent interactions of Ubc9 with SUMO1/SUMO1 chains. However, fraction 29 to 31 were pooled and applied to the preparative S200 gel filtration column equilibrated in transport buffer. The chromatogram of this size exclusion chromatography is shown in Figure 13C. Fractions 38 to 45 were pooled, aliquoted, shock frozen in liquid nitrogen and stored at -80°C for further experiments. Figure 13D shows the concentration determination by comparison to Trypsine Inhibitor and also demonstrates the purity of the preparation. The final concentration of S*Ubc9 was approximately 0.8mg/ml.

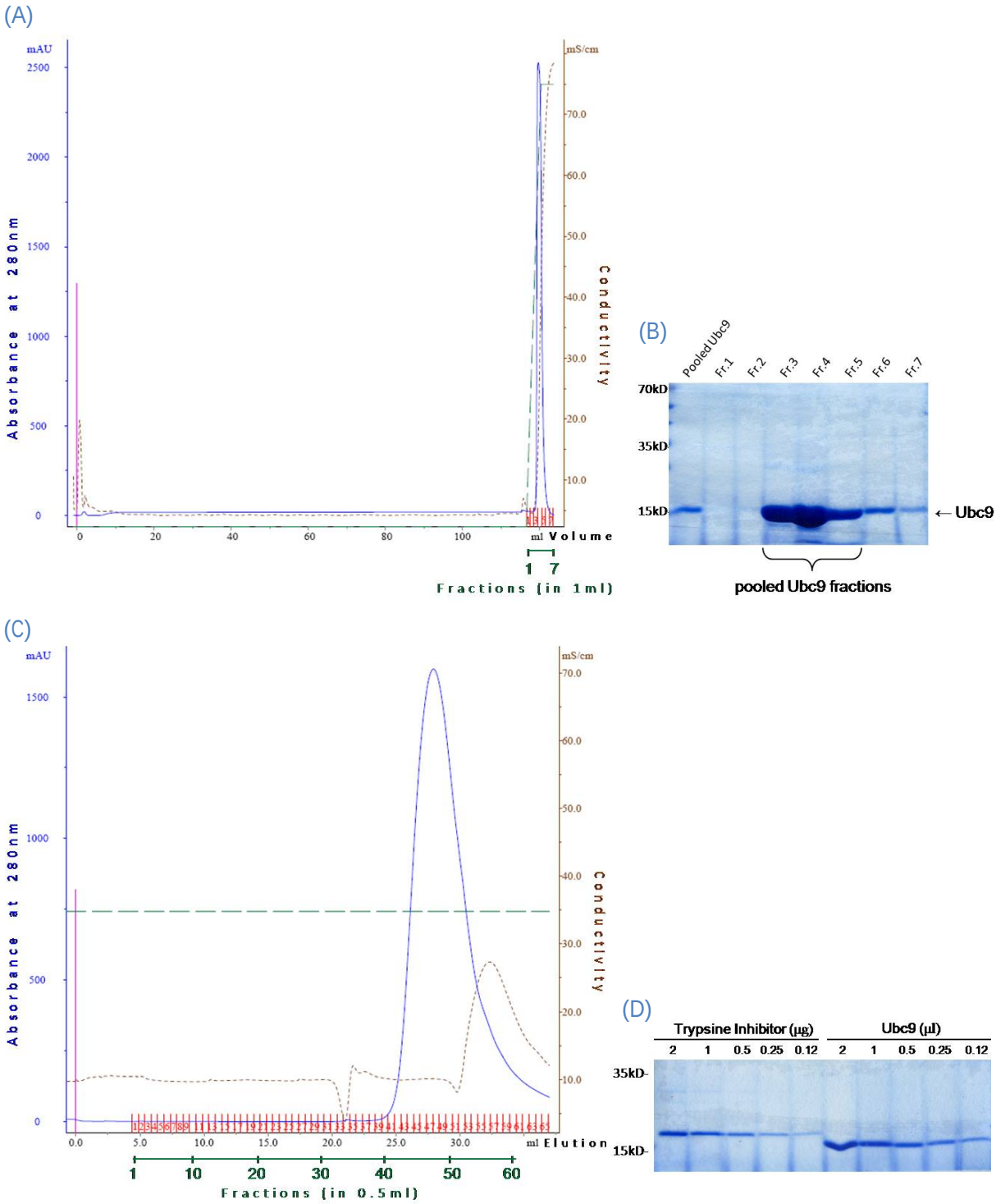


Figure 12: Ubc9 concentration and buffer exchange via cation exchange chromatography followed by gelfiltration
(A) Chromatogram of Ubc9 separated on a cation exchange SP sepharose XL column.
(B) 10µl aliquots of the peak fractions were separated on a 12.5% SDS-PAGE and stained with Coomassie. Indicated fractions were pooled for further purification.
(C) Chromatogram of Ubc9 on a S200 size exclusion chromatography column. Peak fractions 43 to 49 were pooled, aliquoted and frozen at -80°C for further experiments.
(D) Coomassie stain of Ubc9 and Trypsine Inhibitor, as reference protein, separated on a 12.5% SDS-PAGE for concentration determination.

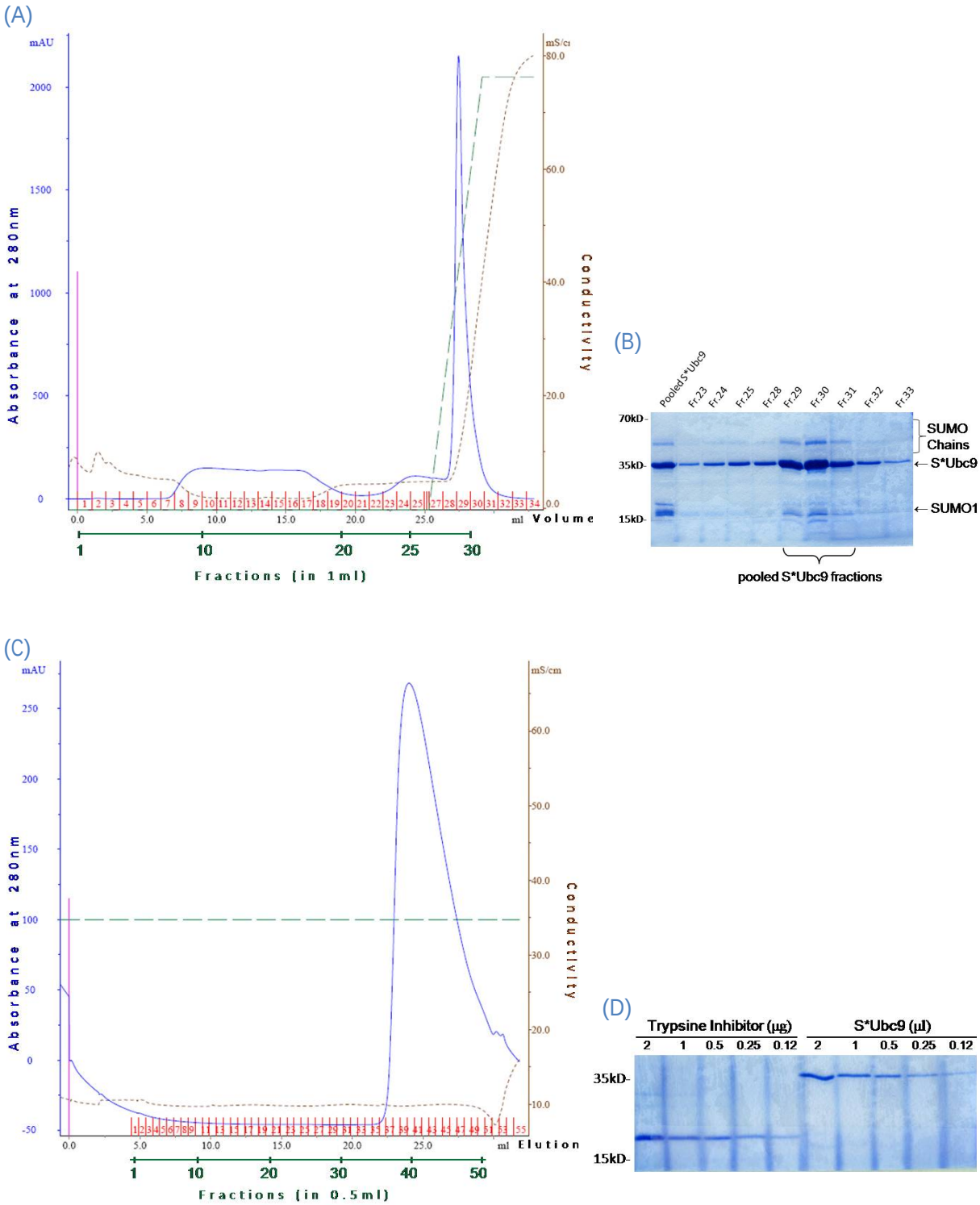


Figure 13: S*Ubc9 concentration and buffer exchange via cation exchange chromatography followed by gelfiltration
(A) Chromatogram of S*Ubc9 separated on a cation exchange SP sepharose XL column.
(B) 10µl aliquots of the most important fractions after the SP sepharose XL run to follow the S*Ubc9 purification. Coomassie staining of S*Ubc9 separated on a 12.5% SDS-PAGE is shown. Indicated fractions were pooled and used for further purification.
(C) Chromatogram of S*Ubc9 size exclusion chromatography on a S200 column. Fractions 38 to 45 of the peak were pooled, aliquoted and frozen at -80°C.
(D) Concentration of S*Ubc9 in comparison to the reference protein Trypsine Inhibitor. Coomassie staining of Trypsine Inhibitor and S*Ubc9 separated on a 12.5% SDS-PAGE is shown.

Figure 14 shows a side-by-side comparison of the purified Ubc9 and the S*Ubc9 in a dilution series to confirm comparable molarities for further analysis.

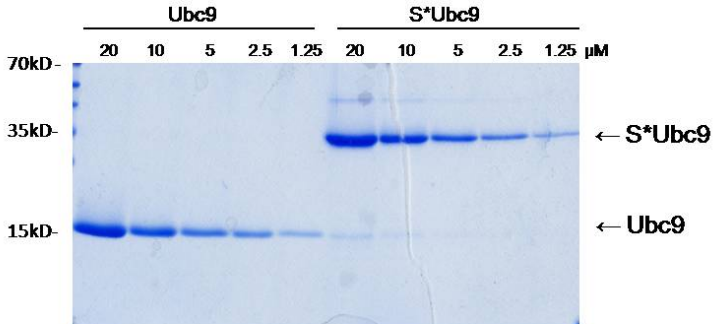


Figure 14: Side-by-side comparison of Ubc9 and S*Ubc9
Dilution series in a range from 20µM (3.6µg Ubc9 and 5.9µg S*Ubc9) to 1.25 µM of Ubc9 and S*Ubc9 respectively in 10µl sample volume separated on a 12.5% SDS PAGE followed by Coomassie staining.

3.2. Analysis of the modified and unmodified Ubc9 to identify conditions for the ProtoArray®

Prior to applying the newly purified enzymes in *in vitro* sumoylation assays on the ProtoArray®, the enzymes had to be tested for their enzymatic activity and subsequently for their activity on known SUMO substrates to find the ideal conditions for the ProtoArray®.

3.2.1. Analysis of SUMO thioester formation ability

Different enzyme purifications can vary in enzymatic activity. Therefore it is important to compare Ubc9 and its sumoylated form from preparations done in parallel. To finally confirm comparable enzymatic activities, SUMO thioester formation assays were performed. In these assays, the efficiency of the SUMO transfer from the E1 enzyme to the E2 enzyme is measured in a time or concentration dependent manner.
Figure 15 shows such a thioester assay with HA tagged SUMO1, the E1 enzyme and similar concentrations of Ubc9 or S*Ubc9, respectively, in a time dependent reaction from 0 to 540 sec. Since the thioester linkage is a relatively labile bond, the reaction was performed under non-reducing conditions. Sample separation was done in urea buffer on a 12.5% SDS-PAGE for stabilizing the thioester bond. Detection was done in an immunoblot with anti-Ubc9 antibodies.

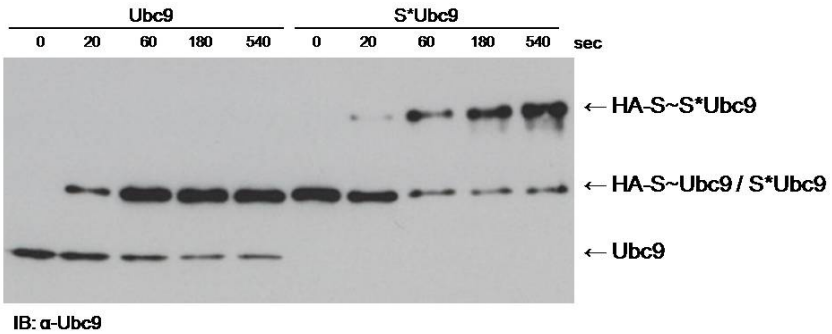


Figure 15: SUMO thioester formation activity of Ubc9 and S*Ubc9
4µM HA-SUMO1, 140nM E1, ATP and 500nM Ubc9 or S*Ubc9 in 20µl were incubated for indicated time points at 30°C. Reactions were stopped with urea containing sample buffer and separated on a non-reducing 12.5% SDS Page. Detection was with anti-Ubc9 antibodies.

At the concentrations used, the reaction is relatively fast and already after 20 seconds thioester formation can be detected. After 60 seconds it appears that the majority of E2 input is forming a thioester bond for both the Ubc9 (lane 3) and the S*Ubc9 (lane 8). For the S*Ubc9 it appears that thioester formation is further increased over time (lane 9 and 10). Since we only obtained a decrease in the unmodified form but did not detect a further increase for the thioester formation, we concluded that most of the S*Ubc9 is already loaded at 60 seconds and the increase is lost due to irregular blotting or detection. In summary, Ubc9 and S*Ubc9 showed comparable enzymatic activities in thioester formation.

3.2.2. Analysis of known SUMO substrates

To further analyse the activity of Ubc9 and S*Ubc9, both enzymes were tested on substrate modification ability. Our laboratory has found that the transcriptional regulator Sp100 is significantly enhanced modified in the presence of S*Ubc9 compared to Ubc9. Therefore the newly purified enzymes were tested in a concentration dependent manner on Sp100 modification. As shown in Figure 16, Sp100 sumoylation was already detected with 50nM S*Ubc9 (lane 8) whereas five-fold higher amounts of unmodified Ubc9 were required to reach comparable modification levels (lane 4).

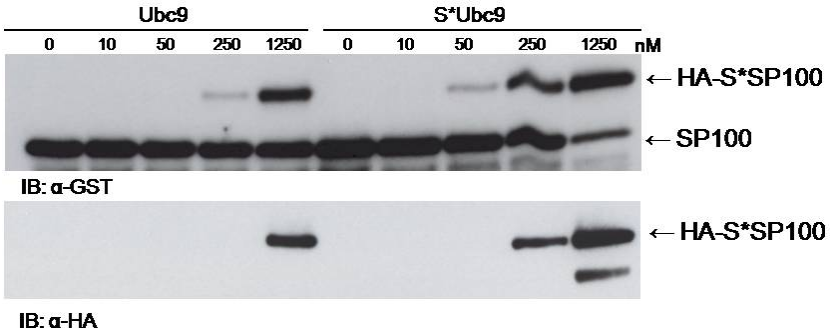


Figure 16: *In vitro* sumoylation assay with GST-Sp100
4.4µM HA-SUMO1, 70nM E1, 200nM GST-SP100, ATP and indicated concentrations of Ubc9 or S*Ubc9 in 20µl reactions were incubated at 30°C for 2 hours. Samples were separated on a 7% SDS Page and detection was with either anti-GST or anti-HA antibodies.

Daxx sumoylation was shown to depend on a functional SIM and it is assumed that the SIM is rather required for recruitment of the SUMO~Ubc9 thioester than for recruting the S*Ubc9 conjugate [29]. Since Daxx sumoylation was shown to be slightly enhanced in the presence of high concentrations of S*Ubc9, it was also included in our analysis. Figure 17 demonstrates the Daxx *in vitro* sumoylation assay with different concentrations of Ubc9 and S*Ubc9. In our analysis we could confirm the slight enhancment of Daxx sumoylation in the presence of S*Ubc9.

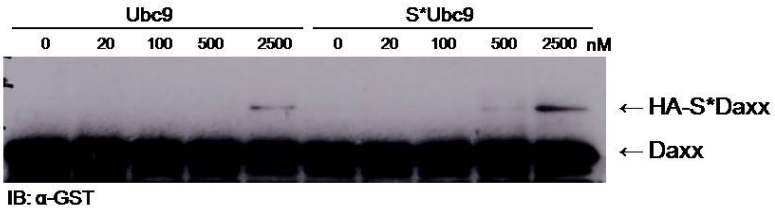


Figure 17: *In vitro* sumoylation assay with GST-Daxx
4µM HA-SUMO1, 200nM E1, 500nM GST-Daxx, ATP and indicated concentrations of Ubc9 or S*Ubc9 in 20µl reactions were incubated at 30°C for 2 hours. Samples were separated on a 5-20% SDS Page and detection was with anti-GST antibody.

E2-25K sumoylation was shown to be unaffected by S*Ubc9 [29] and was therefore included in our investigation. Figure 18 demonstrates the *in vitro* sumoylation assay with E2-25K as substrate. Again we were able to confirm the results obtained before.

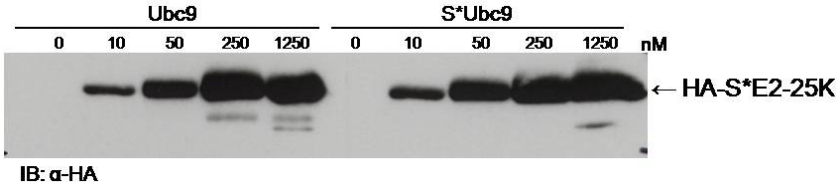


Figure 18: *In vitro* sumoylation assay with E2-25K
4.4µM HA-SUMO1, 70nM E1, 200nM E2-25K, ATP and indicated concentrations of Ubc9 or S*Ubc9 in 20µl reactions were incubated at 30°C for 2 hours and subsequently separated on a 5-20% SDS Page. Staining was with anti-HA antibody.

In summary, the newly purified enzymes were catalytically active in SUMO thioester formation and had similar activities on substrate sumoylation as it was described before. Therefore the enzymes were ready to use for the ProtoArray® service.

3.3. The ProtoArray®

The aim of this thesis was the identification of additional substrates targeted by S*Ubc9. Thus, a protein microarray chip, the "ProtoArray® Human Protein Microarray v5.0" from Invitrogen was used. In this thesis, the ProtoArray® was applied for sumoylation reactions as a novel approach to identify substrates for the S*Ubc9. The ProtoArray® itself was performed by the Invitrogen ProtoArray® service using our concept, enzymes and conditions for the sumoylation reaction.

3.3.1. Novel concept to identify S*Ubc9 substrates

In collaboration with Invitrogen, we combined their knowledge and experience in applying the array for the identification of ubiquitin E3 ligase dependent substrates with our experience in *in vitro* sumoylation assays. In addition, we decided on detection via a HA-tagged SUMO1 instead of the biotin tagged modifier usually used by Invitrogen. We expected to reduce unspecific background and increase sensitivity with the HA detection system. Instead of performing the reaction in duplicates, we tested two different E2 concentrations (100nM and 500nM) to include an additional evaluation level. The concept of the *in vitro* sumoylation on the ProtoArray® is outlined in Figure 19.

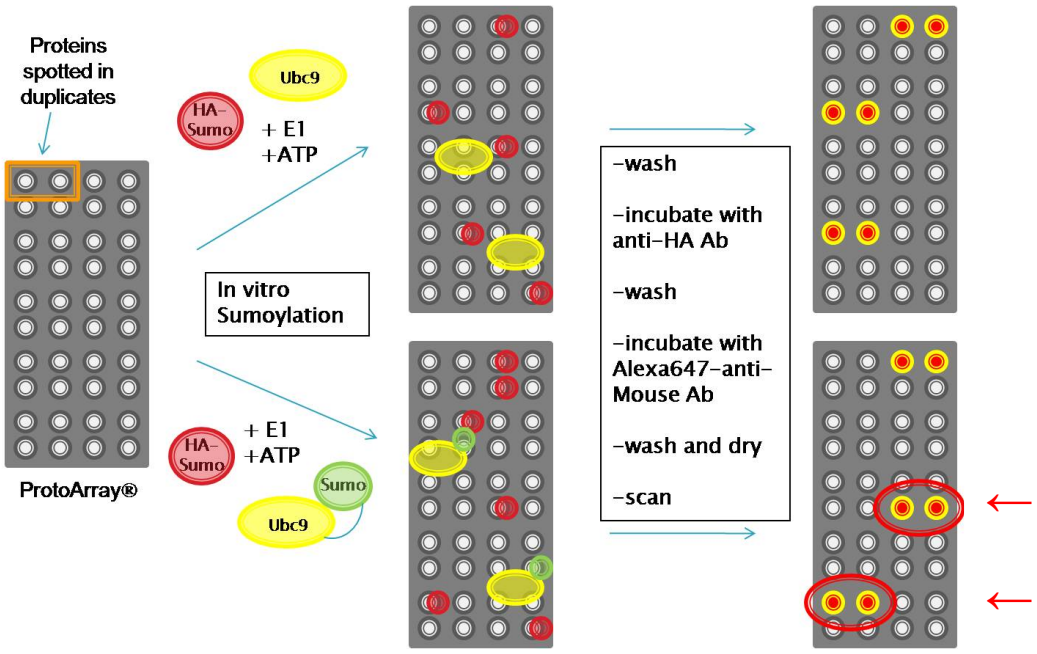


Figure 19: Concept of *in vitro* sumoylation on the ProtoArray®

A ProtoArray® is incubated with an *in vitro* sumoylation reaction mix containing 4µM HA tagged SUMO1, 100nM E1 and either Ubc9 or S*Ubc9, each at two different concentrations, in the presence of an ATP regenerating system. Two additional arrays were included as control, one containing the sumoylation reaction mix without E2 enzymes the other was performed with only the detection reagents. Arrays were incubated for 1hour at 30°C. After washing steps to remove all unconjugated components, the arrays were incubated with anti-HA antibody for 1h to select on HA-SUMO1 modified proteins. After additional washing steps, the arrays were incubated with Alexa647 labelled goat anti-mouse antibody for another hour. After final washing steps, the arrays were dried and scanned. Substrates for the sumoylated Ubc9 were expected to show higher values with S*Ubc9 than with Ubc9 (indicated with a red arrow).

The final conditions used for the ProtoArray® included 4µM HA-SUMO1, 100nM E1, 100nM or 500nM Ubc9/S*Ubc9 incubated with an ATP regenerating system in our SAB buffer (SUMO-Assay-Buffer) buffer with 20mM HEPES, 110mM KOAc, 2mM $Mg(OAc)_2$, 1mM EGTA, pH 7.3, supplemented with 1mM DTT, 1mg/ml Leupeptin/Pepstatin, 1mg/ml Aprotinin, 0.05% Tween, 0.2mg/ml Ovalbumine. The reaction was performed for 1 hour at 30°C.

As controls, one array was incubated with the detection reagent alone to define the background and one with a reaction mix lacking the E2 enzyme to detect E2 independent reactions.

Assay	HA-SUMO	E1	Ubc9	S*Ubc9
Negative control				
E1 control	4 µM	100 nM		
Low Ubc9	4 µM	100 nM	100 nM	
High Ubc9	4 µM	100 nM	500 nM	
Low S*Ubc9	4 µM	100 nM		100 nM
High S*Ubc9	4 µM	100 nM		500 nM

Table 1: Summary of conditions for different ProtoArrays

Detection of HA-SUMO1 conjugated substrates was performed with mouse anti-HA antibodies (Covance) and Alexa 647 labelled rabbit-anti mouse secondary antibodies (Invitrogen). Excitation of the fluorophore was at 650nm and detection was done at 668nm with a GenePix® 4000B fluorescence scanner from Molecular Devices Corporation.

3.3.2. Results of the ProtoArray® and subsequent validation

Data analysis was performed by Invitrogen ProtoArray® Service using the software ProtoArray® Prospector. Candidates were considered as putative SUMO substrates, when they produced a signal that was at least two fold above background. This criterion must have been met in all E2 dependent reactions tested. To define putative substrates for the sumoylated Ubc9, it was expected that they demonstrate a concentration dependent increase with S*Ubc9 and show greater signal intensity with S*Ubc9 compared to the respective Ubc9 concentrations.

In order to validate the ProtoArray® screen, two putative targets preferentially modified with S*Ubc9 and two targets not impaired by S*Ubc9 were chosen and tested in our classical *in vitro* sumoylation assay. These targets were first cloned in bacterial expression vectors, expressed in E. coli, purified and finally analysed in our *in vitro* system. The coding DNAs (cDNA) of these substrates were either provided from other laboratories or were obtained from HeLa mRNA after reverse transcription. Cloning was performed by PCR (polymerase chain reaction) amplification with specific primers into a pGEX vector for bacterial expression of glutathione-S-transferase (GST) -fused proteins. Plasmids for Rcc1, HP1α and RNF4 were obtained from the Melchior, Jenuwein and Palvimo laboratories, respectively. cDNA for eIF5 was kindly provided by Helene Klug and Lisa Kirschner. All four GST-tagged proteins were expressed in the bacterial strains BL21 gold or Rossetta C41 and purified by affinity to Glutathion-Sepharose 4B. Elution was done with 20mM glutathione. The final step included purification via gelfiltration on a S200 column (data not shown).

3.3.2.1. ProtoArray® substrates enhanced modified with S*Ubc9

One of the most promising substrates identified in the ProtoArray® screen was RNF4. As shown in Figure 20A, RNF4 demonstrated much brighter signals in the presence of S*Ubc9 compared to Ubc9 dependent sumoylation.

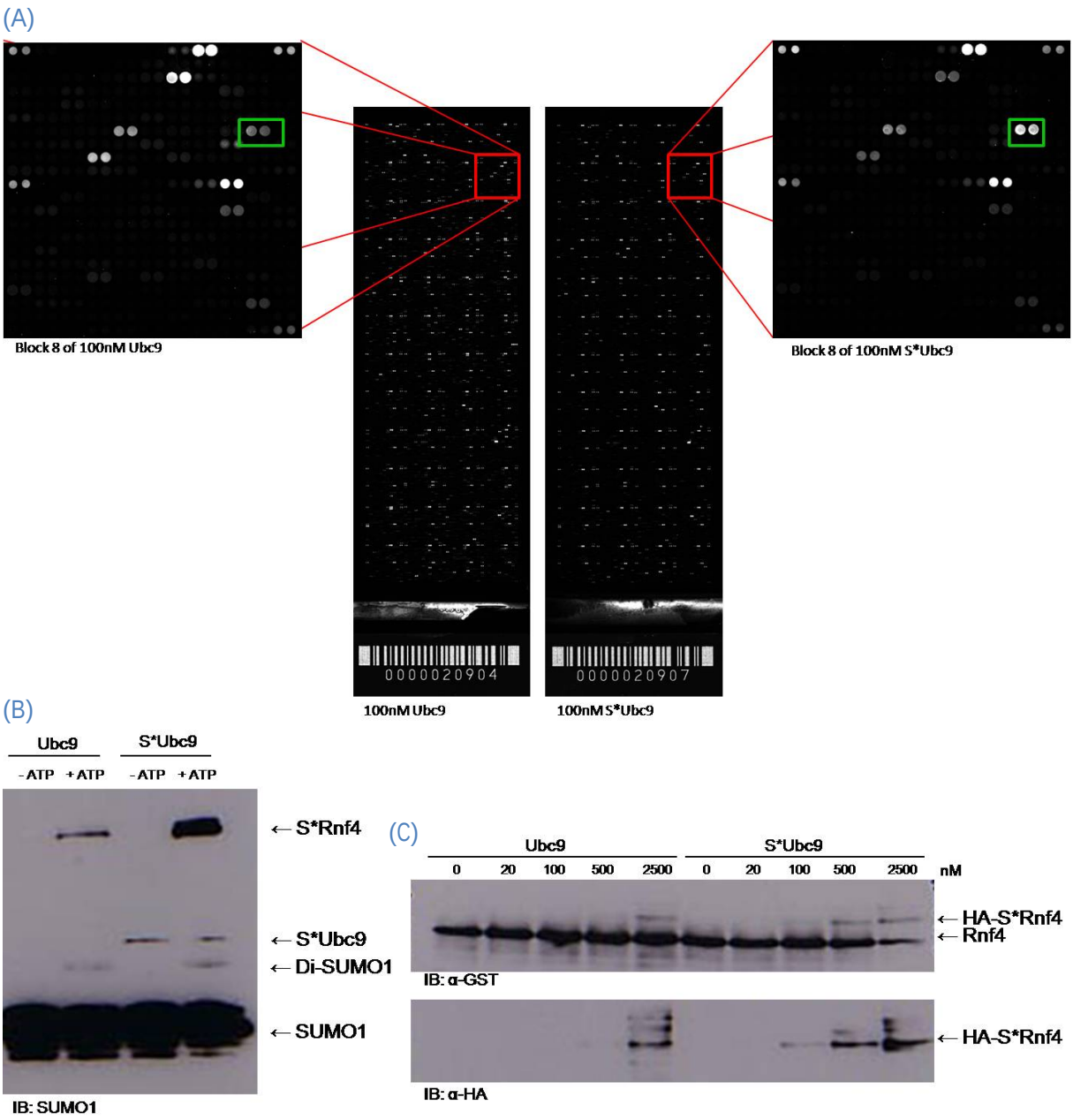
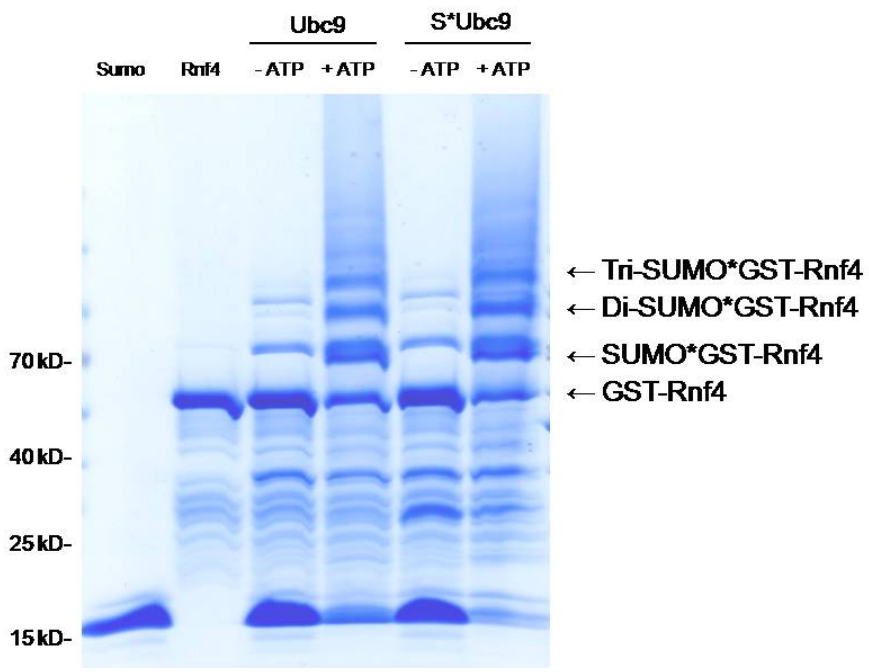


Figure 20: Sumoylation of GST-RNF4
(A) Block 8 of two Protein Arrays (red box) incubated with either 100nM Ubc9 (left panel) or S*Ubc9 (right panel) dependent *in vitro* sumoylation reactions. Block 8 is further enlarged and dots indicating sumoylated GST-RNF4 are marked with a green box.
(B) *In vitro* sumoylation of recombinant GST-RNF4: 21.63µM SUMO1, 100nM E1, 5.75 µM GST-RNF4 and 500nM Ubc9 or S*Ubc9 were incubated at 30°C for 2 hours in 20µl reaction volume with or without ATP. Samples were resolved on a 5-20% SDS-PAGE and detection was with anti-SUMO1 antibodies.
(C) 4µM HA-SUMO1, 100nM E1, 200nM GST-RNF4, ATP and different concentrations of Ubc9 or S*Ubc9 were incubated at 30°C for 2 hours in 20µl reaction volume. Samples were resolved on a 10% SDS-PAGE and detection was with anti-GST and anti-HA antibodies.

Figure 20B and 20C shows the validation of the bacterially purified GST-RNF4 in *in vitro* sumoylation reactions with different Ubc9/S*Ubc9 concentrations. Indeed, also the bacterially purified GST-RNF4 was enhanced sumoylated in the presence of S*Ubc9 and therefore confirms the result obtained by 'on chip' sumoylation. GST-RNF4 is a novel substrate for S*Ubc9.

GST-RNF4 is very efficiently sumoylated as demonstrated in a Coomassie stain of the *in vitro* modified protein in Figure 21A. RNF4 sumoylation results in at least three different slower migrating species, which were applied to mass spectrometry analysis performed by Dr. Gerhard Mittler at the MPI in Freiburg. He identified lysines K108 and K121 attached to the C-terminus of SUMO1 (Figure 21B). In addition, peptides, in which SUMO1 is linked to SUMO1, were found suggesting the involvement of SUMO chain formation rather than a third lysine in RNF4 to be modified.

(A)



(B)

1 MSTRNPQRKR RGGAVNSRQT QKRTRETTST PEISLEAEPI ELVETVGDEI
51 VDLTCESLEP VVVDLTHNDS VVIVEERRRP RRNGRRLRQD HADSCVVSSD
101 DEELSKDKDV YVTTHTPRST KDEGTTGLRP SGTVSCPICM DGYSEIVQNG
151 RLIVSTECGH VFCSQCLRDS LKNANTCPTC RKKINHKRYH PIYI

Figure 21: RNF4 sumoylation site mapping by mass spectrometry analysis
(A) 21.63µM SUMO1, 500nM E1, 5.75 µM GST-RNF4 and 2.5µM Ubc9 or S*Ubc9 were incubated at 30°C for 2 hours in 20µl reaction volume with or without ATP. Samples were resolved on a 5-20% SDS-PAGE and Coomassie stained. Bands representing SUMO1*RNF4, Di-SUMO1*RNF4 and Tri-SUMO1*RNF4 were applied to mass spectrometry analysis.
(B) Protein sequence of rat RNF4. Lysines indicated in yellow were mapped as sumoylation sites in RNF4. Instead of a third modification site SUMO*SUMO chain linkage was identified (Mass spectrometry analysis was performed by Dr. Gerhard Mittler at the MPI, Freiburg)

A second substrate demonstrating enhanced signals in 'on chip' sumoylation reactions with S*Ubc9 was eIF5. Figure 22A shows the signals of eIF5 obtained with 100nM Ubc9 and 100nM S*Ubc9, respectively, on the ProtoArray®.

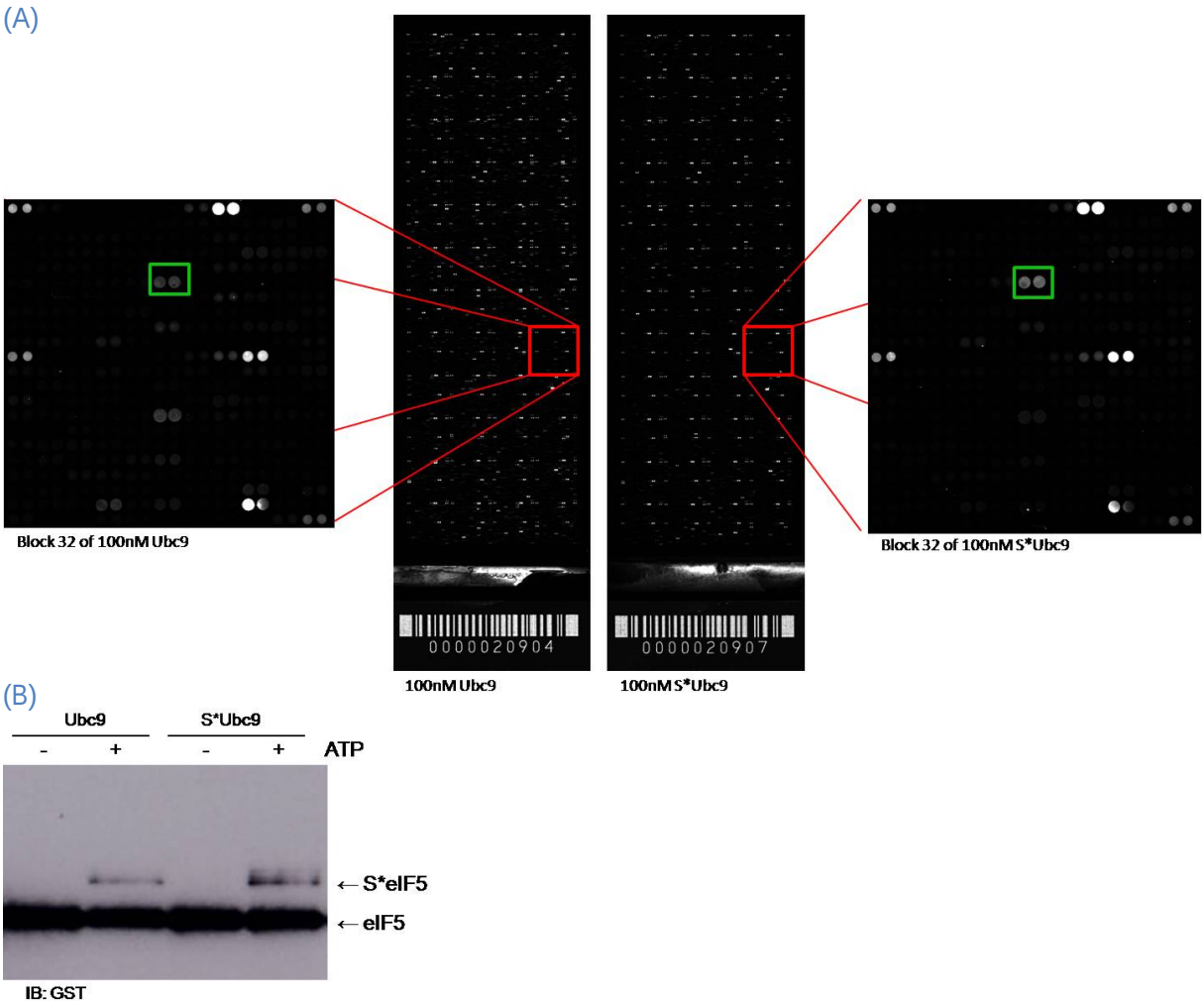


Figure 22: Sumoylation of GST-eIF5
(A) Block 32 of two Protein Arrays (red box) incubated with either 100nM Ubc9 (left panel) or S*Ubc9 (right panel) dependent *in vitro* sumoylation reactions. Block 32 is further enlarged and dots indicating sumoylated GST-eIF5 are marked with a green box.
(B) *In vitro* sumoylation of recombinant eIF5: 17.3µM SUMO1, 500nM E1, 200nM GST-eIF5 and 2.5µM of either Ubc9 or S*Ubc9 were incubated at 30°C for 2 hours in 20µl reaction volume with or without ATP. Samples were resolved on a 5-20% SDS-PAGE and detection was with anti-GST antibody.

GST-eIF5 expressed and purified from bacteria was analysed in our *in vitro* sumoylation reactions with Ubc9 and S*Ubc9. As shown in Figure 22B, GST-eIF5 is a very inefficient SUMO substrate and depends on high E2 concentrations to obtain modification. Here, only slight enhancement in sumoylation was obtained in the presence of S*Ubc9. However, the enhancement identified 'on chip' was significantly stronger.

3.3.2.2. ProtoArray® substrates not enhanced modified by S*Ubc9

Rcc1 and HP1α were two examples of 'on chip' substrates not enhanced in the presence of S*Ubc9 (see Figure 23A and Figure 24A). Interestingly, in case of Rcc1 sumoylation was even weaker with S*Ubc9. To validate the ProtoArray® screen, also these substrates were analysed in our *in vitro* assay.

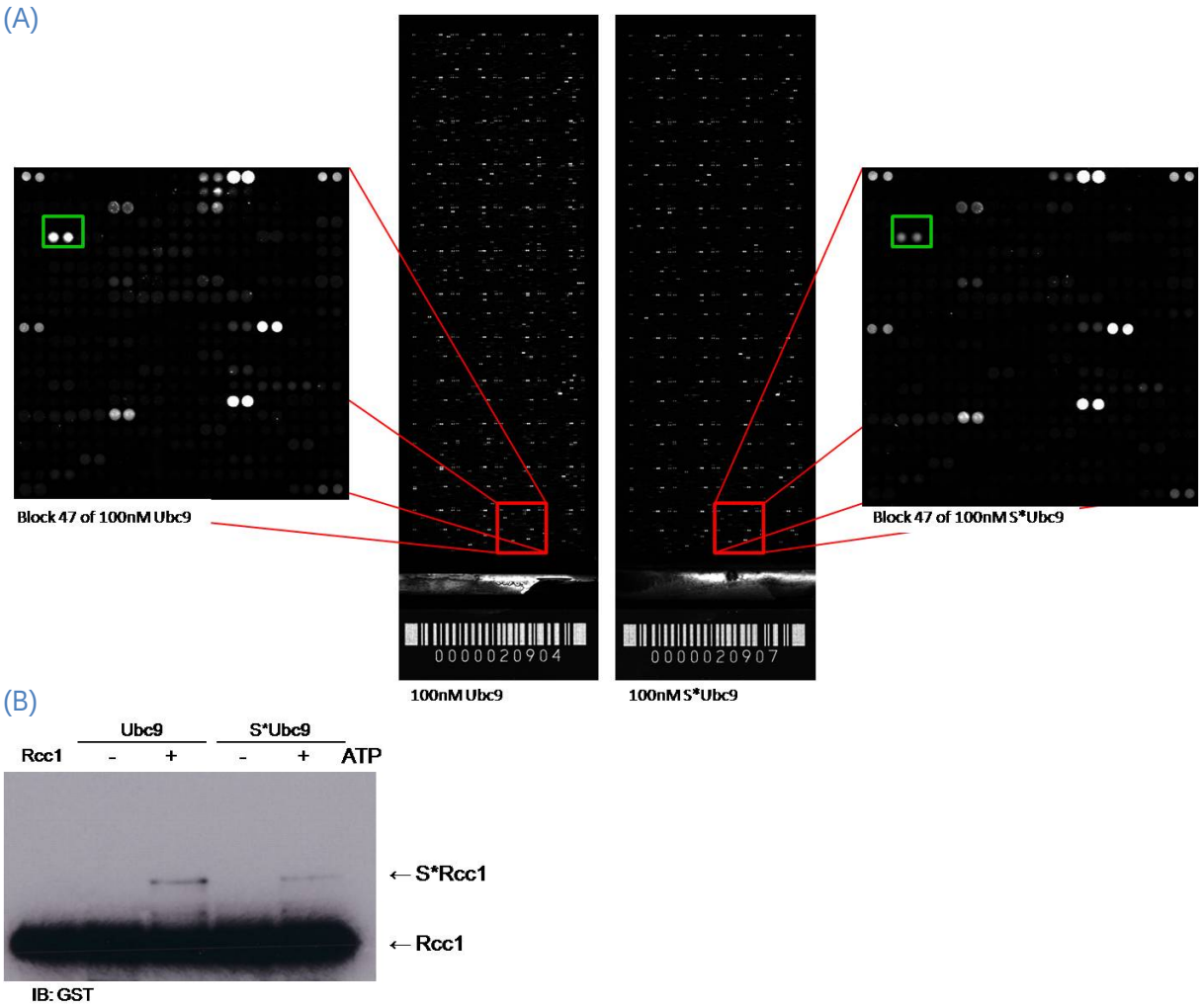


Figure 23: Sumoylation of GST-Rcc1
(A) Block 47 of two Protein Arrays (red box) incubated with either 100nM Ubc9 (left panel) or S*Ubc9 (right panel) dependent *in vitro* sumoylation reactions. Block 47 is further enlarged and dots indicating sumoylated Gst-Rcc1 are marked with a green box.
(B) 17.3µM SUMO1, 500nM E1, 700nM GST-Rcc1 and 2.5µM of either Ubc9 or S*Ubc9 were incubated at 30°C for 2 hours in 20µl reaction volume with or without ATP. Samples were resolved on a 5-20% SDS-PAGE and detection was with anti-GST antibody.

Figure 23B shows the *in vitro* sumoylation reaction with bacterially expressed GST-Rcc1 as substrate. Modification was only detected at high enzyme concentrations, but the signal obtained for S*Ubc9 was slightly reduced and therefore consistent with the 'on chip' analysis.

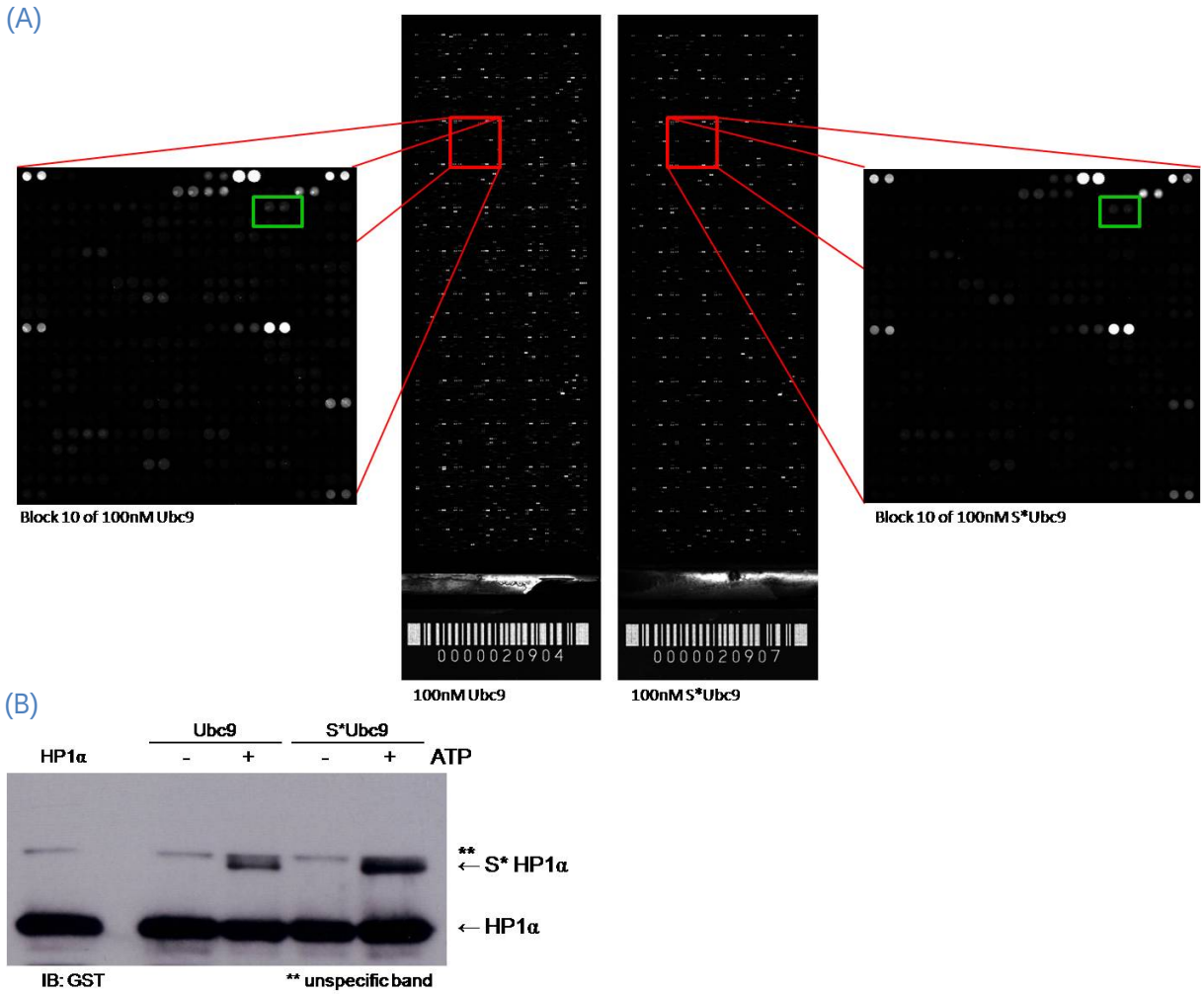


Figure 24: Sumoylation of GST-HP1α
(A) Block 10 of two Protein Arrays (red box) incubated with either 100nM Ubc9 (left panel) or S*Ubc9 (right panel) dependent *in vitro* sumoylation reactions. Block 10 is further enlarged and dots indicating sumoylated GST-HP1α are marked with a green box.
(B) 17.3µM SUMO1, 500nM E1, 950nM GST-HP1α and 2.5µM of either Ubc9 or S*Ubc9 were incubated at 30°C for 2 hours in 20µl reaction volume with or without ATP. Samples were resolved on a 5-20% SDS-PAGE and detection was with anti-GST antibody.

In vitro sumoylation of the bacterially expressed GST-HP1α is shown in Figure 24B. Again high concentrations of modifying enzymes were needed to detect sumoylation. The slight increase in sumoylation in the presence of S*Ubc9 is most likley due to differences in loading than enhancement of S*Ubc9.

In summary, four substrates identified in the ProtoArray® were analysed by bacterial expression and purification and subsequent tesing for *in vitro* sumoylation with Ubc9 and S*Ubc9. We identified one novel substrate to be enhanced modified in the presence of S*Ubc9 and could verify sumoylation of three novel SUMO substrates. The modification efficiency of these substrates was not as efficient as assumed from the obtained `on chip´ signal.

4. DISCUSSION

Our laboratory has recently demonstrated a novel mechanism of how sumoylation of the SUMO E2 conjugating enzyme Ubc9 contributes to target discrimination. Whereas sumoylated Ubc9 does not interfere with the sumoylation of most substrates, it impairs modification of RanGAP1 and significantly enhances modification of the transcriptional regulator Sp100 via a functional SIM motif [29]. The goal of this thesis was the identification of additional substrates targeted by S*Ubc9 to gain insights in its target range and to learn thereby about its biological function. In this thesis, a novel approach for SUMO substrate identification was established. *In vitro* sumoylation reactions with Ubc9 and S*Ubc9 were performed on protein microarray chips purchasable from Invitrogen. Such microarrays contain over 9000 human proteins expressed and purified as GST-tagged proteins in insect cells. To perform such a screen, all proteins required for *in vitro* sumoylation reactions were expressed and purified from E.coli. This included untagged and HA-tagged SUMO1, the heterodimeric E1 activating enzyme Aos1/Uba2 and Ubc9. To obtain S*Ubc9, Ubc9 was *in vitro* sumoylated and separated from the unmodified form by applying different biochemical purification steps. Ubc9 and S*Ubc9 from the same preparation were used for further analysis. Before realisation on chips, all enzymes had to be tested in thioester formation assays for their enzymatic activities and in *in vitro* sumoylation assays for their ability to modify known substrates. The protein array screen was performed by the Invitrogen ProtoArray® Service and data was analysed with the ProtoArray® Prospector software. Candidates showing signals at least two fold above background were considered as putative SUMO substrates. This criterion must have been met in all E2 dependent reactions. To define putative substrates for the S*Ubc9, it was required that they demonstrate signals with higher intensity for S*Ubc9 compared to Ubc9.
To validate the results from the ProtoArray® screen, four substrates have been chosen - two preferentially modified with S*Ubc9 and two preferentially modified with Ubc9. These substrates needed to be cloned in bacterial expression vectors, expressed in E. coli and purified by affinity and size exclusion chromatography. Subsequently, the substrates were tested in our classical *in vitro* modification assays, separated on SDS-PAGE and analysed by immunoblotting.
The substrates selected for S*Ubc9 were the RING finger ubiquitin E3 ligase RNF4 and the eukaryotic translation initiation factor eIF5.

RNF4 (also known as SNURF) is ubiquitously expressed in humans and its deregulation has been implicated in several human diseases including cancer [57, 58]. It acts as co-activator for steroid receptors and other transcription factors like Sp1 [59, 60]. RNF4 possesses E3 ubiquitin ligase activity, which is dependent on its RING finger [61, 62]. Intriguingly, one of its substrates is the PML (promyelocytic leukemia protein) modified with SUMO2 chains upon arsenic treatment, which results in ubiquitin-mediated degradation. Thus, RNF4 is proposed to be an E3 ligase specific for polysumoylated proteins [63, 64]. For recognition of poly-SUMO chains,

four functional SIMs in RNF4 were identified [63]. The presence of SIMs in RNF4 strongly supports our finding of RNF4 as a novel substrate for the S*Ubc9. S*Ubc9 was shown to enhance the affinity for selected SIM containing SUMO substrates [29]. Therefore, we have chosen RNF4 to test it in our classical *in vitro* sumoylation reaction. We obtained GST-RNF4 cloned in a vector for bacterial expression from the Palvimo laboratory and subsequently purified it from E.coli. Indeed, GST-RNF4 sumoylation was significantly enhanced in the presence of S*Ubc9 in our *in vitro* reactions. Hence, RNF4 represents a novel substrate for the S*Ubc9. This finding raises several challenging questions, like where and when in the cell RNF4 gets sumoylated in a S*Ubc9 dependent manner, which SIM is responsible for efficient sumoylation and what are the consequences on its function as ubiquitin E3 ligase.

The second substrate selected for the S*Ubc9 was the eukaryotic translation initiation factor 5 (eIF5). Mammalian eIF5 is a monomeric 49 kDa protein, which interacts with the ribosomal 40S initiation complex at the mRNA start codon and promotes the hydrolysis of bound GTP. This leads to the release of initiation factors and allows joining of the 60S ribosomal subunit to form the active 80S initiation complex [65]. eIF5 has been shown to get phosphorylated by the Caseine Kinase 2 (CK2). Mutants lacking this phosphorylation site attenuate cell cycle progression and proliferation [66]. Our ProtoArray® screen has discovered eIF5 as a novel SUMO substrate and it appears to be enhanced sumoylated by S*Ubc9. To verify this result, eIF5 was reverse transcribed from HeLa cell mRNA, amplified by PCR and cloned into a bacterial pGEX expression vector. GST-eIF5 was expressed and purified from E. coli and tested in our *in vitro* sumoylation assay. Sumoylation of eIF5 could be verified. However, the modification efficiency of eIF5 was very low and dependent on high enzyme concentrations. S*Ubc9 only slightly enhanced sumoylation of GST-eIF5. One difference between the assay on the ProtoArray® and our *in vitro* system is that proteins on the array are purified from insect cells, which can positively influence protein folding. Interestingly, eIF5 has a putative SIM in close proximity to two CK2 phosphorylation sites. A recent study has shown that phosphorylation next to selected SIMs enhance SUMO binding [28]. One intriguing possibility is that phosphorylation is obtained by purification from insect cells, but is lacking by purification from bacteria, thus leading to different modification efficiencies. In the future, it will be important to compare the different eIF5 purifications side by side or *in vitro* phosphorylate eIF5 prior to *in vitro* sumoylation. If phosphorylated eIF5 stimulates S*Ubc9 dependent sumoylation, it will be exciting to investigate the resulting biological consequences. Both phosphorylation sites and the putative SIM are located in the glutamic acid-rich C-terminal region of eIF5, which is essential for the biological function of eIF5.

The second class of identified substrates analysed in this study were proteins not enhanced in modification with S*Ubc9. In this case, we selected to analyse HP1α and Rcc1. Rcc1 (regulator of chromosome condensation), located in the nucleoplasm, is the guanine nucleotide exchange factor for the Ras related GTPase Ran and plays an essential role in nucleo-cytoplasmic transport and in cell cycle regulation [67]. HP1α (Heterochromatin protein

1 homolog alpha, also referred as Chromobox protein homolog 5) plays an essential role in heterochromatin formation by recognizing and binding to histone H3 tails methylated at lysine K9 [68]. HP1α also interacts with lamin B receptor (LBR), which contributes to the association of the heterochromatin with the inner nuclear membrane [69].
We obtained bacterial expression vectors for both GST-Rcc1 and GST-HP1α from the Melchior and Jenuwein laboratories, respectively. Both proteins were purified after expression in E. coli and tested in our standard sumoylation assays. Indeed, both proteins were weakly sumoylated at high E2 concentration levels. These results suggest that either the ProtoArray® screen is more sensitive compared to our analysis and/or the difference in signal efficiency is due to expression in insect cells as suggested for eIF5.

In summary, this study established a novel approach for the identification of SUMO substrates. Initial analysis of four substrates validates their modification in classical *in vitro* sumoylation assays. Moreover, one substrate - RNF4 - was found to be significantly enhanced with S*Ubc9.

5. EXPERIMENTAL PROCEDURES

5.1. Plasmids, antibodies and bacterial strains

5.1.1. Plasmids

Protein	Plasmid	Selection/Marker	Source
SUMO1ΔC4	pET11a	Amp	Pichler et al. 2002
HA-SUMO1 ΔC4	pET23a	Amp	Melchior Lab
Ubc9	pET23a	Amp	Pichler et al. 2002
His-Aos1	pET28a	Kan	Pichler et al. 2002
Uba2-His	pET28a	Kan	Melchior Lab
GST-Sp100	pGEX	Amp	Pichler et al. 2002
E2-25K	pGEX4T	Amp	Pichler et al. 2005
GST-Daxx (residue 572-740)	pGEX4T	Amp	Pichler et al. 2008
GST-RNF4	pGEX5X	Amp	Palvimo Lab
GST-RCC1	pGEX	Amp	Melchior Lab
GST-HP1α	pGEX6P1	Amp	Jenuwein Lab
GST-eIF5	pGEX6P1	Amp	outlined in 5.4.7.

5.1.2. Antibodies

Antibody	Source
mouse α-SUMO1	Melchior Lab
rabbit α-GST	Melchior Lab
goat α-Ubc9	Pichler et al. 2002
monoclonal mouse α-HA.11	Covance
HRP-coupled α-mouse, α-goat and α-rabbit antibodies	Jackson Laboratories
Alexa647 labelled α-mouse antibody	Invitrogen

5.1.3. Competent bacterial strains

DH5α: E.coli F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–

BL21 gold: E. coli F^- ompT gal dcm lon $hsdS_B(r_B^-\ m_B^-)$ λ(DE3) pLysS(cm^R)

Rosetta C41: E.coli F^- ompT $hsdS_B(R_B^-\ m_B^-)$ gal dcm λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (Cam^R)

5.2. Molecular cloning methods

5.2.1. Isolation of plasmid DNA from E.coli

Plasmid DNA was isolated from E.coli either using the QIAprep Spin Miniprep Kit or according to the protocol of a MiniPrep outlined below:
MiniPrep: Colonies were inoculated in 5ml LB/Amp and incubated o/n at 37°C. 2ml of the o/n culture were centrifuged for 5min at 5000rpm, 4°C, and the pellet was resuspended in 200µl Buffer P1 (50mM Tris.HCl, pH 8.0, 10mM EDTA, 100µg/ml RNAse A) followed by 5 min incubation on ice. Then 200µl Buffer P2 (200mM NaOH, 1% SDS) were added, carefully mixed by inverting a few times, followed by incubation for 5min at RT. 200µl Buffer P3 (3M KCH_3COO, pH 5.5) were added, mixed by vortexing and then incubated for 5min on ice. The mix was centrifuged for 10min at 13000rpm. After transfer of the supernatant to a new vial, 420 µl isopropanol were added, mixed and incubated 10min at RT followed by centrifugation for 20min at 13000rpm. The pellet was washed with 300µl 70% EtOH and dried on air before resuspending it in 30µl H_2O and storage at -20°C.
DNA concentration was measured by using the NanoDrop ND-1000 spectrophotometer from ThermoScientific or another photometer.

5.2.2. Restriction digestions

Digestion of the PCR product and plasmid DNA with restriction enzymes:

Reaction mix:

- 20µl PCR product or 10 µl plasmid DNA
- 5µl Buffer (1-4) from New England BioLabs
- 5µl 10x BSA from New England BioLabs
- 5µl 10x RNAse A
- 1.5µl restriction enzyme 1 from New England BioLabs
- 1.5µl restriction enzyme 2 from New England BioLabs
- 12µl or 22 µl ddH_2O respectively

50µl total volume

All components were added to the reaction mix as indicated. The reaction mix was incubated for 2h at the recommended temperature. The reaction mix was then analysed by resolving on a 0.8% agarose gel and DNA fragments were excised from the gel. Elution of the fragments was done with QIAquick gel extraction kit.

5.2.3. Ligation of DNA fragments

10µl of the digested PCR product was mixed with 2µl digested plasmid DNA, 2µl T4 DNA Ligase Buffer (from New England BioLabs) and 1µl T4 DNA Ligase (400000U/ml, from New

England BioLabs), filled up to 20µl and incubated for 2h at RT. Ligation mix was added to 100µl competent E.coli DH5α and transformation was done as described below.

5.2.4. Sequencing

DNA sequencing reactions were carried out by VBC Biotech Sequencing, Vienna, or by the Sequencing Facility of the Max-Planck Institute, Freiburg.

Sequencing PCR mix:

- 300ng template DNA
- 2.4µl 5x ABI sequencing buffer
- 2pmol standard primer for pGEX
- 0.5µl Big Dye v1.1 mix
- filled up with ddH_2O

12µl total volume

Sequencing PCR program:

initial denaturation	94°C	5min	
denaturation	94°C	10sec	30x
annealing	50°C	5sec	
elongation	60°C	4min	
storage	4°C	forever	

After the sequencing PCR was finished, 12µl ddH_2O were added and free nucleotides were removed by a gel filtration on Sephadex G-50 Superfine from GE Healthcare. Capillary electrophoresis and subsequent data analysis was done by the Sequencing Facility at the Max-Planck Institute Freiburg.

5.2.5. mRNA isolation from HeLa cells

done by Helene Klug and Lisa Kirschner

Materials:

- 6 Plates ($10cm^2$) of Hela cells, 100% confluent
- 1x PBS: 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.76mM KH_2PO_4, pH adjusted to 7.4
- TRIzol reagent
- Chloroform
- Isopropanol and 70% EtOH
- ddH_2O

6 Plates (10cm^2) of Hela Cells, which were 100% confluent, were washed once with PBS. 1 ml of TRIzol reagent was added on each plate, the cells were resuspended and transferred to a fresh Eppendorf tube. 200µl of chloroform were added, mixed for several times and then incubated for 2 minutes, followed by centrifugation in an Eppendorf table top centrifuge for 10min, at 4°C and 13200rpm. The upper phase was carefully transferred to a new Eppendorf tube. After addition of 500µl of isopropanol, the mix was incubated for 2min and subsequently centrifuged for another 10min at 4°C and 13200rpm. The isopropanol was discarded and the RNA pellet was washed once with 70%EtOH. Then the EtOH was removed completely and the pellet was air dried for about 10min. The RNA was resuspended in 20µl of ddH_2O and denatured at 75°C for 10min. Then the samples were always kept on ice.
RNA concentration was measured by using the NanoDrop ND-1000 spectrophotometer from ThermoScientific or another photometer at OD 260. Measured RNA concentration was ~1250ng/µl

5.2.6. cDNA synthesis

done by Helene Klug and Lisa Kirschner

Materials:

- 5µg total RNA from Hela cells
- RevertAid^{TM} First Strand cDNA Synthesis Kit
- 10pmol/µl reverse primer
- 10pmol/µl forward primer
- DEPC treated water
- Phusion polymerase from Finnzymes
- 5x Phusionbuffer HF from Finnzymes
- 100mM dNTPs

5µg total RNA from Hela cells + 20pmol reverse primer were mixed with DEPC-treated water to a total volume of 12µl and incubated for 5min at 70°C. Then the reaction mix was chilled on ice and 4µl 5x reaction buffer, 1µl RiboLockTM ribonuclease inhibitor (20U/µl) and 2µl 10mM dNTP mix were added. The reaction mix was then incubated at 37°C for 5min. After addition of 1µl RevertAidTM M-MuLV reverse transcriptase (200U/µl), the mixture was incubated at 42°C for 1h. The reaction was stopped by heating at 70°C for 10min and subsequently diluted 1:10 with DEPC treated water.

PCR reaction with Phusion polymerase from Finnzymes:

Reaction mix:

- 2µl 5x Phusionbuffer HF
- 0.2µl 100mM dNTPs

- 0.5µl 10pmol/µl forward primer
- 0.5µl 10pmol/µl reverse primer
- 5µl cDNA synthesised with RevertAid Kit
- 0.1µl Phusion polymerase
- 1.7µl ddH_2O

total volume of 20µl

PCR program:

initial denaturation	98°C	30sec	
denaturation	98°C	10sec	35x
annealing	65°C	30sec	
elongation	72°C	3min	
storage	4°C	forever	

The PCR-products were analysed on a 1.2% agarose gel and PCR products with the expected size were used for cloning.

5.2.7. Cloning of eIF5 into pGEX6P1

eIF5 was amplified with eIF5 forward primer (5'AAA CGC GGA TCC GCG ATG TCT GTC AAT GTC AAC CGC) and eIF5 reverse primer (5' AAA CCG CTC GAG CGG TTA AAT GGC ATC AAT ATC GAT GTC 3') from cDNA as described previously. The eluted PCR product (QIAquick gel extraction kit) was cloned into pGEX6P1 with BamHI and XhoI (New England Biolabs) restriction enzymes and verified by mini prep analysis and sequencing.

5.3. Biochemical methods

5.3.1. SDS polyacrylamid gel electrophoresis (PAGE)

Materials:

- ddH_2O
- 2M Tris.HCl pH 8.8
- 30% Acrylamid : Bisacrylamid (30 : 0.8) Rotiphorese® from Carl Roth
- 20% SDS
- 10% APS
- TEMED
- 0.5M Tris.HCl pH 6.8
- 1x western blot running buffer: 25mM Tris, 0.19M Glycine and 0.05% SDS

- 2x Laemmli sample buffer: 50mM Tris pH 6.8, 2% SDS, 0.1% Bromphenole Blue, 10% glycerin and 100mM DTT
- Protein markers: PageRuler™ prestained protein ladder and PageRuler™ unstained protein ladder from Fermentas Life Science

Gel recipes (for 10 gels):

SDS running gel:

	5%	7%	10%	12.5%	20%
ddH_2O	42.9ml	38.35ml	31.3ml	25.5ml	7.9ml
2M Tris.HCl pH 8.8			14ml		
30% Acrylamid : Bisacrylamid	11.7ml	16.3ml	23.33ml	29.2ml	46.7ml
20% SDS			350µl		
+ 10% APS			700µl		
+ TEMED			70µl		

SDS stacking gel:

	2x
ddH_2O	45.4ml
0.5M Tris.HCl pH 6.8	6ml
30% Acrylamid : Bisacrylamid	8ml
20% SDS	300µl
+ 10% APS	300µl
+ TEMED	60µl

SDS-PAGE: run for 1.2 – 1.5h at 25mA/ gel.

5.3.2. Coomassie staining

- Fixing solution: 40% ethanol, 10% acetic acid
- Coomassie dye solution: 0.08 % Coomassie Brilliant Blue G250, 1.6% ortho-phosphoric acid, 8% ammonium sulfate, 20% methanol
- Destaining solution: 1% acetic acid

Gels were fixed for 1h in the fixing solution and subsequently washed in ddH_2O twice for at least 10 min. Then the gels were transferred into the Coomassie dye solution and stained o/n. Destaining was done with the destaining solution for at least one day.

5.3.3. Semi-dry immunoblotting

Materials:

- 1x transfer buffer: 25mM Tris, 0.193M glycine, 20% methanol, 0.36 % SDS
- Whatman® gel blotting paper
- Whatman® Protran® nitrocellulose membrane
- Ponceau solution: 0.5% Ponceau S in 1% acetic acid
- PBS+0.2%Tween20: 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.76mM KH_2PO_4, pH adjusted to 7.4 + 0.2% Tween20
- 5% skimmed milk in PBS+0.2%Tween20
- Primary antibody diluted in 5% skimmed milk in PBS+0.2%Tween20
- Secondary antibody diluted 1:10000 in 5% skimmed milk in PBS+0.2%Tween20
- SuperSignal® West Pico Chemiluminescent Substrate detection reagent from Thermo Scientific
- Amersham Hyperfilm^{TM} ECL from GE Healthcare

Proteins were transferred onto a nitrocellulose membrane via semi-dry blotting with 0.1 A / gel for 1h. Afterwards, the transfer was checked by Ponceau staining and the membrane was washed with PBS+0.2% Tween20.
The membrane was subsequently incubated in 5% skimmed milk in PBS+0.2%Tween20 for 1h at RT or o/n at 4°C.
Proteins were detected by incubating in primary antibody solution for 2 hours followed by three washes with PBS+0.2% Tween20 for 5 min and subsequent incubation with secondary antibody solution for 1 hour. After three washes with PBS+0-2% Tween20 for 10 minutes each, signals were detected with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific).

5.3.4. Expression and purification of recombinant proteins

5.3.4.1. Medium and antibiotics

LB medium (5l): 50g tryptone, 25g yeast extract, 25g NaCl, pH adjusted to 7.0
Ampicilin (Amp): 100mg/ml in H_2O, stored at -20°C (1000x stock)
Kanamycin (Kan): 60mg/ml in H_2O, stored at -20°C (1000x stock)
Chloramphenicol (Cam): 34mg/ml in ethanol, stored at -20°C (1000x stock)

5.3.4.2. Transformation of plasmid DNA into competent E.coli

200ng plasmid DNA was added to 200µl competent E.coli and the mix was incubated for 20min on ice. A heat shock was done for 1.5 min at 42°C followed by 5 min incubation on ice. 1ml LB was added and E.colis were recovered for 1h at 37°C.

5.3.4.3. Expression and purification of Aos1/Uba2

Materials:

- Competent E.coli BL21 gold
- Plasmid: His-Aos1 in pET 28a, Uba2-His in pET28
- LB/Kan
- 1M IPTG
- Ni-lysis buffer: 50mM NaH_2PO_4, 300mM NaCl, 10mM Imidazol + 0.1mM PMSF, 1mM β-Mercaptoethanol, 1µg/ml Aprotinin, 1µg/ml Leupeptin/ Pepstatin
- 100mM Lysozyme
- ProBond™ resin from Invitrogen (Ni-Beads)
- Ni-wash buffer: 50mM NaH_2PO_4, 300mM NaCl, 20mM Imidazol + 1mM β-Mercaptoethanol, 1µg/ml Aprotinin, 1µg/ml Leupeptin/ Pepstatin
- Ni-elution buffer: 50mM NaH_2PO_4, 300mM NaCl, 250mM Imidazol + 1mM β-Mercaptoethanol, 1µg/ml Aprotinin, 1µg/ml Leupeptin/ Pepstatin
- Transport buffer: 20mM HEPES, 110mM KOAc, 2mM $Mg(Oac)_2$, 1mM EGTA, titrated with KOH to pH 7.0 + 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/Pepstatin
- Puck buffer: 20mM Tris (pH 8.0), 100mM NaCl + 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/Pepstatin
- Äkta S200 10/300 GL preparative gelfiltration column
- Äkta HiLoad 26/60 S200 prep grad preparative gelfiltration column and 5-20% PAA gels

Expression and Purification:

E.coli BL21 gold cells were transformed with either His-Aos1 in pET28a or Uba2-His in pET28. A 5 ml pre-culture was grown at 37°C for several hours and diluted to 40ml LB/Kan. This culture was grown over night at 37°C.
The following day, the overnight culture was diluted 1:50 in 2l LB/Kan and grown to OD600 = 0.5-0.6 at 37°C. Expression was induced with 1 mM IPTG (1ml/L culture of a 1M stock) and the culture was grown for 6h at 25°C.
Bacteria were harvested by centrifugation at 4000rpm for 15min at 4°C. The pellet was resuspended in 40ml (20ml/L) icecold Ni-lysis buffer and frozen in liquid nitrogen followed by storage at -80°C.

To purify the protein, the pellet was thawed and cells were lysed by addition of 1 mM lysozyme and incubation on ice for 1 hour. After 1h ultracentrifugation at 55000rpm (140000 x g) at 4°C, the cleared lysate was applied on 5ml Ni-beads (2.5ml/l culture), equilibrated with Ni-lysis buffer. The mix was incubated for 1h at 4°C while rotating. Beads were harvested by centrifugation at 1000 rpm for 5 min at 4°C and transferred into a column. Beads were washed several times with Ni-wash buffer. Proteins were eluted with Ni-elution buffer and 10x 0.5ml fractions were collected. Fractions were tested for protein by spotting 1µl of each fraction on a nitrocellulose membrane and staining the spots with Ponceau. Protein-containing fractions were pooled and concentrated to 1ml by using a concentrator (Vivaspin 2). The sample was ultracentrifuged for 10min at 55000rpm and subsequently purified using an Äkta S200 10/300 GL column, equilibrated with transport buffer. 0.5ml fractions were collected and analysed via SDS-PAGE according to the chromatogram. Best fractions of either Aos1 or Uba2 were pooled. Aliquots of 1ml were prepared, frozen in liquid nitrogen and stored at -80°C.
The concentration of both Aos1 and Uba2 was determined by resolving serial dilutions of the protein of interest next to a dilution series of a reference protein of known concentration on a 5-20% SDS-PAGE. After staining the gel with Coomassie brilliant blue, protein concentration was estimated.

To reconstitute the dimeric SUMO E1, equal amounts of purified Aos1 and Uba2 (4mg each) were incubated on ice for 1h, followed by purification via the Äkta HiLoad 26/60 S200 preparative gelfiltration column, equilibrated in Puck buffer. Fractions of 5ml were collected and analysed with via SDS-PAGE. Fractions containing Aos1 and Uba2 in equal amounts were pooled, aliquoted, frozen in liquid nitrogen and stored at -80°C.
The concentration of the E1 enzyme was determined by resolving serial dilutions of the protein next to a dilution series of a reference protein of known concentration on a SDS-PAA-gel. After staining the gel with Coomassie brilliant blue, protein concentration was estimated. Estimated concentration of the E1 enzyme was 0.5mg/ml = 4.5 µM.

5.3.4.4. Expression and purification of SUMO1 and HA-SUMO1

Materials:

- Competent E.coli BL21 gold
- Plasmid: SUMO1ΔC4 in pET11a, HA-SUMO1ΔC4 in pET23a
- LB/Amp
- 1M IPTG
- SUMO lysis buffer: 50mM Tris-HCl (pH8.8), 25mM NaCl + 0.1mM PMSF, 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/ Pepstatin
- 100mM Lysozyme

- Q Sepharose™ Fast Flow from GE Healthcare
- SUMO elution buffer: 50mM Tris (pH 7.5), 500mM NaCl + 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/ Pepstatin
- Puck buffer for SUMO1: 20mM Tris (pH 8.0), 100mM NaCl + 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/Pepstatin
- Transport buffer for HA-SUMO1: 20mM HEPES, 110mM KOAc, 2mM $Mg(Oac)_2$, 1mM EGTA, titrated with KOH to pH 7.0 + 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/Pepstatin
- Äkta HiLoad 26/60 S200 prep grad preparative gelfiltration column for SUMO1
- Äkta S200 10/300 GL preparative gelfiltration column for HA-SUMO1
- 12.5% PAA gels

Expression and Purification:

E.coli BL21 gold cells were transformed with either SUMO1ΔC4 in pET11a or HA-SUMO1ΔC4 in pET23a.
A 5 ml pre-culture was grown at 37°C for several hours and diluted to 40ml LB/Amp. This culture was grown at 37°C o/n.
The following day, the overnight culture was diluted 1:100 in 4l LB/Amp and grown to OD600 = 0.5-0.6 at 37°C. Expression was induced with 1 mM IPTG (1ml/L culture of a 1M stock) and the SUMO1 culture was grown at 37°C for 4h, whereas the HA-SUMO1 was grown o/n. Bacteria were harvested by centrifugation at 4000rpm for 15min at 4°C. The pellet was resuspended in 40ml icecold SUMO lysis buffer and frozen in liquid nitrogen followed by storage at -80°C.
The pellet was thawed, sonicated three times for 30 sec at 75% intensity and the lysate was cleared by ultracentrifugation for 1 hour at 55000rpm at 4°C. 10ml Q sepharose (2.5ml/l culture) was transferred to a column and equilibrated with SUMO lysis buffer without PMSF. The supernatant after ultracentrifugation was applied to the Q sepharose. Subsequently, the beads were washed with SUMO lysis buffer without PMSF several times. Bound protein was eluted with SUMO elution buffer and 20x 1ml fractions were collected. Fractions were tested for protein by spotting 1µl of each fraction on a nitrocellulose membrane and staining the spots with Ponceau. Protein-containing fractions were pooled and applied to the Äkta HiLoad 26/60 S200 preparative gelfiltration column, equilibrated in Puck Buffer. Fractions of 5ml were collected.
HA-SUMO1 was applied to the Äkta S200 10/300 GL preparative gelfiltration column, equilibrated in transport buffer. 0.5ml fractions were collected and analysed via SDS-PAGE according to the chromatogram. Best fractions of SUMO1 or HA-SUMO1 were pooled. Aliquots were prepared, frozen in liquid nitrogen and stored at -80°C.

The concentration of both SUMO1 and HA-SUMO1 was determined by resolving serial dilutions of the protein next to a dilution series of a reference protein of known

concentration on a SDS-PAA-gel. After staining the gel with Coomassie brilliant blue, protein concentration was estimated. Estimated concentration of SUMO1 was 2mg/ml = 173 µM. Estimated concentration of HA-SUMO1 was 1.5mg/ml = 100 µM.

5.3.4.5. Expression and purification of Ubc9

Materials:

- Competent E.coli BL21 gold
- Plasmid: Ubc9 in pET 23a
- LB/Amp
- 1M IPTG
- Ubc9 lysis buffer: 25mM Na-phosphate (pH 6.5), 50mM NaCl + 0.1mM PMSF, 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/ Pepstatin
- 100mM Lysozyme
- SP Sepharose™ High Performance from GE Healthcare
- Ubc9 elution buffer: 25mM Na-phosphate (pH 6.5), 300mM NaCl + 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/ Pepstatin
- Puck buffer: 20mM Tris (pH 8.0), 100mM NaCl + 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/Pepstatin
- Äkta HiLoad 26/60 S200 prep grad preparative gelfiltration column and 12.5% PAA gels

Expression and Purification:

E.coli BL21 gold cells were transformed with Ubc9 in pET23a. A 5 ml pre-culture was grown at 37°C for several hours and diluted to 40ml LB/Amp. This culture was grown at 37°C o/n.
The following day, the overnight culture was diluted 1:100 in 4l LB/Amp and grown to OD600 = 0.5-0.6 at 37°C. Expression was induced with 1 mM IPTG (1ml/L culture of a 1M stock) and the Ubc9 culture was grown at 37°C for 4h. Bacteria were harvested by centrifugation at 4000rpm for 15min at 4°C. Pellet was resuspended in 40ml icecold Ubc9 lysis buffer and frozen in liquid nitrogen followed by storage at -80°C. This step was repeated twice to get 3 pellets of Ubc9 out of 12l bacterial culture.
Pellets were thawed and ultracentrifuged for 1h at 55000rpm and 4°C. 24ml SP Sepharose (2ml/l culture) were washed, transferred to a column and equilibrated with Ubc9 lysis buffer without PMSF. Supernatant was applied to the SP Sepharose. Subsequently, beads were washed with Ubc9 lysis buffer (-PMSF) for several times. Elution was done with Ubc9 elution buffer and 30x 1ml fractions were collected. Fractions were tested for protein by spotting 1µl of each fraction on a nitrocellulose membrane and staining the spots with Ponceau. Protein containing eluates were pooled and applied to the Äkta HiLoad 26/60 S200 prep grad

preparative gelfiltration column equilibrated in Puck buffer. 5ml fractions were collected and analysed via SDS-PAGE according to the chromatogram. Best fractions of Ubc9 were pooled. Aliquots of 1ml were prepared, frozen in liquid nitrogen and stored at -80°C.
The concentration of Ubc9 was determined by resolving serial dilutions of the protein next to a dilution series of a reference protein of known concentration on a SDS-PAA-gel. After staining the gel with Coomassie brilliant blue, protein concentration was estimated. Estimated concentration was 2mg/ml.

5.3.4.6. Expression and purification of GST tagged proteins

Materials:

- Competent E.coli BL21 gold: used for the expression of GST-Sp100, GST-E2-25K, GST-Daxx and GST-RNF4 (RING finger protein) → selection on LB/Amp
- Competent E.coli Rosetta C41: for the expression of GST-Rcc1, GST-HP1α and GST-eIF5 → selection on LB/Amp/Cam
- Plasmids: GST-Sp100 in pGEX
  GST-E2-25K in pGEX4T
  GST-Daxx in pGEX4T
  GST-RNF4 in pGEX5X
  GST-Rcc1 in pGEX
  GST-HP1α in pGEX6P1
  GST-eIF5 in pGEX6P1
- LB/Amp or LB/Amp/Cam, 1M IPTG
- for RING finger proteins additionally 10µM $ZnCl_2$
- Lysis buffer: 50mM Tris-HCl (pH 8.0), 100mM NaCl + 0.1mM PMSF, 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/ Pepstatin
- 100mM Lysozyme
- Glutathione Sepharose 4B (GST-beads) from GE Healthcare
- GSH elution buffer: 50mM Tris, 20mM glutathione, reduced (pH 8.0) +1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/Pepstatin
- Transport buffer: 20mM HEPES, 110mM KOAc, 2mM $Mg(Oac)_2$, 1mM EGTA, titrated with KOH to pH 7.0 + 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/Pepstatin
- Äkta S200 10/300 GL preparative gelfiltration column and 6 - 12.5% PAA gels depending on the molecular weight of the protein
- Reference proteins:

α-Lactalbumine [10mg/ml]	14.2 kD
Trypsine Inhibitor [10mg/ml]	20.1 kD
Ovalbumine [10mg/ml]	44.3 kD
BSA [2mg/ml]	66.0 kD
Phosphorylase [1mg/ml]	97.2 kD

E.coli BL21 gold or E.coli Rosetta C41, respectively, were transformed with plasmid DNA as mentioned above. A 5 ml pre-culture was grown at 37°C for several hours and diluted to 40ml LB/Amp or LB/Amp/Cam. This culture was grown at 37°C o/n.
The following day, the overnight culture was diluted 1:50 in 2l LB/Amp or LB/Amp/Cam, respectively. RING finger proteins additionally needed 10µM $ZnCl_2$. The culture was grown to OD600 = 0.5-0.6 at 37°C. Expression was induced with 1 mM IPTG (1ml/L culture of a 1M stock) and the culture was grown at 16°C for 6h. Bacteria were harvested by centrifugation at 4000rpm for 15min at 4°C. Pellet was resuspended in 40ml icecold lysis buffer and frozen in liquid nitrogen followed by storage at -80°C.
To purify the protein, the pellet was thawed and cells were lysed by addition of 1 mM lysozyme and incubation on ice for 1 hour. After 1h ultracentrifugation at 55000rpm at 4°C, the cleared lysate was applied to 4ml GST-beads (1-2ml/l culture), which had been equilibrated with lysis buffer. Supernatant and beads were incubated for 1.5h at 4°C on rotation.
Subsequently, beads were washed several times with lysis buffer (-PMSF). Bound proteins were eluted with GSH elution buffer and 10x 0.5ml fractions were collected. Fractions were tested for protein by spotting 1µl of each fraction on a nitrocellulose membrane and staining the spots with Ponceau. Protein containing eluates were pooled and applied to the Äkta S200 10/300 GL column equilibrated in icecold transport buffer. 0.5ml fractions were collected and analysed via SDS-PAGE according to the chromatogram. Best fractions were pooled, aliquoted, frozen in liquid nitrogen and stored at -80°C.
The concentration was determined by resolving serial dilutions of the protein next to a dilution series of a reference protein of known concentration on a SDS-PAA-gel. After staining the gel with Coomassie brilliant blue, protein concentration was estimated.
Estimated Concentrations: GST-Sp100 2mg/ml = 30µM, GST-Daxx 2mg/ml= 30µM, GST-RNF4 = 23µM, GST-Rcc1 1.5mg/ml = 21.4µM, GST-HP1α 1.2mg/ml = 22.6µM, GST-eIF5 = 0.8mg/ml = 10.6µM.

5.3.4.7. Removing the GST tag by using TEV protease

Materials:

- Glutathione Sepharose 4B (GST-Beads) from GE Healthcare
- Transport buffer: 20mM HEPES, 110mM KOAc, 2mM $Mg(Oac)_2$, 1mM EGTA, titrated with KOH to pH 7.0 + PI
- 30µl TEV protease
- Vivaspin 20 centricon (10000 MWCO PES) from Sartorius Stedim
- Äkta S200 10/300 GL preparative gelfiltration column and 12.5% PAA gels

Expression and harvest of E2-25K was done as described previously. After lysis and ultracentrifugation, the supernatant was incubated with equilibrated GST-beads for 1.5h.
Subsequently, the beads were washed three times with transport buffer. The buffer was then removed and the beads were resuspended in 3ml transport buffer. 30µl TEV protease was added and the reaction mix was incubated o/n at 4°C while rotating.
The following day, the reaction mix including the beads was loaded on a small column and the flow through was collected. The beads were washed two times with 3ml transport buffer and the flow through was again collected. Then, the pooled flow through was concentrated with a Vivaspin 20 centricon to 1ml. After ultracentrifugation to remove precipitates, the sample was applied to the equilibrated Äkta S200 10/300 GL column. 0.5ml fractions were collected and analysed as described previously. Best fractions were pooled, aliquoted, frozen in liquid nitrogen and stored at -80°C.
The concentration was determined by resolving serial dilutions of the protein next to a dilution series of a reference protein of known concentration on a SDS-PAA-gel. After staining the gel with Coomassie brilliant blue, protein concentration was estimated. Estimated concentration of E2-25K was 2.5 µM.

5.3.4.8. *In vitro* sumoylation of Ubc9 and separation of the modified from the unmodified form

Materials:

- Purified SUMO1, Ubc9 and Aos1/Uba2
- Äkta HiPrep 16/60 Q FF anion exchange column
- HiPrep buffer A: 20mM Tris pH 8.0, 40mM NaCl + PI
- HiPrep buffer B: 20mM Tris pH 8.0, 1M NaCl + PI
- 75mM BisTris pH 6.5 + PI
- 20mM BisTris pH 6.5 + PI
- Äkta HiTrap SP XL cation exchange column
- SP XL buffer A: 20mM BisTris pH 6.5, 25mM NaCl + PI
- SP XL buffer B: 20mM BisTris pH 6.5, 1M NaCl + PI
- Äkta S200 10/300 GL preparative gelfiltration column
- Transport buffer: 20mM HEPES, 110mM KOAc, 2mM $Mg(Oac)_2$, 1mM EGTA, titrated with KOH to pH 7.0 + PI
- 12.5% PAA gels

Molarities of the in vitro *sumoylation reaction of Ubc9:*

55.5µM Ubc9
62µM SUMO1
70nM E1
5mM ATP
5mM $MgCl_2$
0.1mM DTT

Reaction mix of the small scale in vitro *sumoylation of Ubc9 (in µg):*

200µg Ubc9
130µg SUMO1
15µg E1
5mM $MgCl_2$
5mM ATP
0.1mM DTT

total volume ~ 200µl

→ reaction mix was incubated for 5h at 37°C, every hour an 20µl aliquot was taken and resolved on a 5-20% SDS-PAGE

Reaction mix of the large scale in vitro *sumoylation of Ubc9 (in mg):*

20mg Ubc9
13mg SUMO1
1.5mg E1
5mM $MgCl_2$
5mM ATP
0.1mM DTT

total volume ~ 20ml

→ reaction mix was incubated for 2h at 37°C, pH of the reaction was pH 8.0

The reaction mix was diluted 1: 2.5 (1+1.5 volumes) in 20mM Tris.HCl (pH8.0) + PI and applied to the Äkta HiPrep 16/60 Q FF column equilibrated in HiPrep buffer A. Fractions of 1ml were collected and analysed via SDS-PAGE. Free Ubc9 did not bind to the column and was washed out with at least 1 column volume of HiPrep buffer A. The bound proteins (S*Ubc9, SUMO1 and E1) were eluted with a NaCl gradient over 5 column volumes with the final concentration of buffer B (1M NaCl). 1ml fractions were collected and analysed via a chromatogram and SDS-PAGE. Fractions containing the highest amounts of Ubc9 and S*Ubc9, respectively, were pooled.

Pooled Ubc9 was diluted 1:1 with 75mM BisTris (pH 6.5) and applied to the Äkta HiTrap SP XL cation exchange column by using the buffer intake tubing. The column was washed with 5 column volumes of SP XL buffer A to wash out all unbound proteins. Ubc9 was eluted with a NaCl gradient over 4 column volumes with the final concentration of SP XL buffer B (1M NaCl). 1ml fractions were collected and analysed with a chromatogram and SDS-PAGE. Fractions, which contain the highest amount of Ubc9, were pooled and, in order to change the buffer, applied to Äkta S200 10/300 GL preparative gelfiltration column equilibrated in transport buffer. Fractions of 0.5 ml were collected and analysed with a chromatogram and subsequent SDS-PAGE. Fractions containing the highest amount of Ubc9 were pooled, aliquoted, frozen in liquid nitrogen and stored at -80°C.
Pooled S*Ubc9 was dialysed with 2l of 20mM BisTris (pH 6.5) for 3h and applied to the Äkta HiTrap SP XL cation exchange column by using the buffer intake tubing. The column was washed with 5 column volumes of SP XL buffer A to wash out all unbound proteins. S*Ubc9 was eluted with a NaCl gradient over 4 column volumes with the final concentration of SP XL buffer B (1M NaCl) as described for the Ubc9. 1ml fractions were collected and analysed with a chromatogram and subsequent SDS-PAGE. Fractions, which contain the highest amount of S*Ubc9, were pooled and again, in order to change the buffer, applied to Äkta S200 10/300 GL preparative gelfiltration column equilibrated in transport buffer. Fractions of 0.5ml were collected and analysed with a chromatogram and subsequent SDS-PAGE. Fractions, which contain the highest amount of S*Ubc9, were pooled, aliquoted, frozen in liquid nitrogen and stored at -80°C.
The concentration was determined by resolving serial dilutions of the protein next to a dilution series of a reference protein of known concentration on a SDS-PAA-gel. After staining the gel with Coomassie brilliant blue, protein concentration was estimated.
Estimated concentration of Ubc9 was 2mg/ml = 111 µM and of S*Ubc9 0.8mg/ml = 27 µM.

5.3.5. *In vitro* thioester assay

Reaction mix:

Solution [Conc.]	Volume		Final Concentration
HA-SUMO1 [100 µM]	0.88 µl		4.4 µM
Aos1-Uba2 [4.5 µM]	0.6 µl		140 nM
Ubc9 [111 µM]	0.09 µl	-	500 nM
S*Ubc9 [27 µM]	-	0.372 µl	500 nM
ATP [100 mM]	1 µl		5 µM
10x thioester buffer (200mM Tris pH 7.6, 500mM NaCl, 100mM $MgCl_2$)	2 µl		20 mM Tris pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$
ddH_20 + 1 mM AP + LP	15 µl		
1 mM DTT	0.81 µl	0.53 µl	0.1 mM DTT
total	20 **µl**		

All components were added to the reaction mix and thioester formation was induced by adding ATP. Thioester formation was stopped by adding 20µl urea buffer (50mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 4M urea) after incubation for 0 / 20 / 60 / 180/ 540 sec at 30°C. Prior to loading, samples were incubated for 15 min at 30°C. Non-reducing SDS-PAGE was done at 15mA/gel and 4°C.

5.3.6. *In vitro* sumoylation assay

Reaction mix to test the enzymatic activity of Ubc9:

Solution [Conc.]	Volume		Final Concentration / Amount
SUMO1 [173 µM]	2.2 µl		19 µM = 4.4 µg
Aos1-Uba2 [4.5 µM]	0.3 µl		70 nM = 150 µg
old Ubc9 [111 µM]	2 µl	-	X
new Ubc9 [111 µM]	-	2 µl	X
E2-25K [60 µM]	2.2 µl		6.6 µM = 3.5 µg
ATP [100mM]	1 µl		5 µM
SAB (1x transport buffer, 2mg/ml ovalbumin, 0.05% Tween, 1mM LP, AP, DTT)	12.3 µl		
total	**20µl**		

X = 7 / 1.4 / 0.28 / 0.06 / 0 µM old and new Ubc9

Reaction mix for Sp100 and E2-25K:

Solution [Conc.]	Volume				Final Concentration
HA-SUMO1 [100 µM]	0.88 µl				4.4 µM
Aos1-Uba2 [4.5 µM]	0.3 µl				70 nM
Ubc9 [111 µM]	2 µl	-	2 µl	-	X
S*Ubc9 [27 µM]	-	2 µl	-	2 µl	X
Sp100 [30 µM] or	0.13 µl		-		200 nM
E2-25K [2.5 µM]	-		1.6 µl		200 nM
ATP [100mM]	1 µl				5 µM
SAB (1x transport buffer, 2mg/ml ovalbumin, 0.05% Tween, 1mM LP, AP, DTT)	x				
total	**20µl**				

X = 1250 / 250 / 50 / 10 / 0 nM

Reaction mix for Daxx:

Solution [Conc.]	Volume		Final Concentration
HA-SUMO1 [100 µM]	0.8 µl		4 µM
Aos1-Uba2 [4.5 µM]	0.88 µl		200 nM
Ubc9 [111 µM]	4 µl	-	X
S*Ubc9 [27 µM]	-	4 µl	X
Daxx [30 µM]	0.325 µl		500 nM
ATP [100mM]	1 µl		5 µM
SAB (1x transport buffer, 2mg/ml ovalbumin, 0.05% Tween, 1mM LP, AP, DTT)	12.995 µl		
total	**20µl**		

X = 2500 / 500 / 100 / 20 / 0 nM

Reaction mix for RNF4 + SUMO1:

Solution [Conc.]	Volume		Final Concentration
SUMO1 [173 µM]	2.5 µl		21.63 µM
Aos1-Uba2 [4.5 µM]	0.44 µl		100 nM
Ubc9 [111 µM]	0.09 µl	-	500 nM
S*Ubc9 [27 µM]	-	0.37 µl	500 nM
RNF4 [23 µM]	5 µl		5.75 µM
ATP [100mM]	1 µl		5 µM
SAB (1x transport buffer, 2mg/ml ovalbumin, 0.05% Tween, 1mM LP, AP, DTT)	10.97 µl	10.69 µl	
total	**20µl**		

Reaction mix for RNF4 + HA-SUMO1:

Solution [Conc.]	Volume		Final Concentration
HA-SUMO1 [100 µM]	0.8 µl		4 µM
Aos1-Uba2 [4.5 µM]	0.44 µl		100 nM
Ubc9 [111 µM]	4 µl	-	X
S*Ubc9 [27 µM]	-	4 µl	X
RNF4 [23 µM]	0.17 µl		200 nM
ATP [100mM]	1 µl		5 µM
SAB (1x transport buffer, 2mg/ml ovalbumin, 0.05% Tween, 1mM LP, AP, DTT)	13.59 µl		
total	**20µl**		

X = 2500 / 500 / 100 / 20 / 0 nM

Reaction mix for Rcc1 + SUMO1:

Solution [Conc.]	Volume		Final Concentration
SUMO1 [173 µM]	2 µl		17.3 µM
Aos1-Uba2 [4.5 µM]	2.2 µl		500 nM
Ubc9 [111 µM]	0.45 µl	-	2.5 µM
S*Ubc9 [27 µM]	-	1.85 µl	2.5 µM
Rcc1 [21.4 µM]	0.66 µl		700 nM
ATP [100mM]	1 µl		5 µM
SAB (1x transport buffer, 2mg/ml ovalbumin, 0.05% Tween, 1mM LP, AP, DTT)	13.69 µl	12.29 µl	
total	**20µl**		

Reaction mix for HP1α + SUMO1:

Solution [Conc.]	Volume		Final Concentration
SUMO1 [173 µM]	2 µl		17.3 µM
Aos1-Uba2 [4.5 µM]	2.2 µl		500 nM
Ubc9 [111 µM]	0.45 µl	-	2.5 µM
S*Ubc9 [27 µM]	-	1.85 µl	2.5 µM
HP1α [22.6 µM]	0.84 µl		950 nM
ATP [100mM]	1 µl		5 µM
SAB (1x transport buffer, 2mg/ml ovalbumin, 0.05% Tween, 1mM LP, AP, DTT)	13.51 µl	12.11 µl	
total	**20µl**		

Reaction mix for eIF5 + SUMO1:

Solution [Conc.]	Volume		Final Concentration
SUMO1 [173 µM]	2 µl		17.3 µM
Aos1-Uba2 [4.5 µM]	2.2 µl		500 nM
Ubc9 [111 µM]	0.45 µl	-	2.5 µM
S*Ubc9 [27 µM]	-	1.85 µl	2.5 µM
eIF5 [10.6 µM]	0.38 µl		200 nM
ATP [100mM]	1 µl		5 µM
SAB (1x transport buffer, 2mg/ml ovalbumin, 0.05% Tween, 1mM LP, AP, DTT)	13.51 µl	12.11 µl	
total	**20µl**		

All components were added to the reaction mix as indicated. ATP was added last in order to start the sumoylation reaction. The reaction mix was incubated for 2h at 30°C and stopped by adding 20µl 2x Laemmli buffer and boiling for 5 min at 95°C. Samples were centrifuged and loaded on PAA gels. Proteins were detected either by Coomassie staining or immunoblotting.

5.4. ProtoArray® assay

5.4.1. Probing the ProtoArray®

Materials:

- 6 ProtoArray® Human Protein Microarrays 5.0 from Invitrogen incubated with:
  - 1x 100nM Ubc9 + reaction mix
  - 1x 100nM S*Ubc9 + reaction mix
  - 1x 500nM Ubc9 + reaction mix
  - 1x 500nM S*Ubc9 + reaction mix
  - 1x Reaction Mix without Ubc9/S*Ubc9
  - 1x Detection reagent alone
- Stock concentrations:
  - HA-SUMO1: 1.5mg/ml = 100 µM
  - E1 enzyme: 0.5mg/ml = 4.5 µM
  - Ubc9: 2 mg/ml = 111 µM
  - S*Ubc9: 0.8mg/ml = 27 µM
- SAB buffer: transport buffer (=20 mM HEPES, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM EGTA, ph 7.3 (KOH)) + 1mM DTT, 1mg/ml Leupeptin/Pepstatin, 1mg/ml Aprotinin, 0.05%Tween, 0.2mg/ml ovalbumine
- ATP regenerating system from Invitrogen
- Blocking buffer from Invitrogen: 50mM HEPES pH 7.5, 200mM NaCl, 0.08% Triton X-100, 25% glycerol, 20mM reduced glutathione, 1mM DTT, 1% BSA
- 0.5% SDS from Invitrogen
- Assay buffer from Invitrogen: 50mM Tris.HCl pH 7.5, 50mM NaCl, 5mM $MgSO_4$, 0.1% Tween20, 1mM DTT, 1% BSA
- α-HA.11 antibody from Covance in Assay Buffer (1:1500 dilution, end conc. = 3.3 – 4.6 µg/ml)
- Alexa647 goat α-mouse antibody from Invitrogen in assay buffer (1:2000 dilution, end conc. = 1µg/ml)

Reaction mixes in detail:

	100nM Ubc9	100nM S*Ubc9	500nM Ubc9	500nM S*Ubc9	- Ubc9/ S*Ubc9	Detection reagent alone
4µM HA-SUMO	4 µl	4 µl	4 µl	4 µl	4 µl	-
100nM E1	2.22 µl	2.22 µl	2.22 µl	2.22 µl	2.22 µl	-
Ubc9	0.09 µl	-	0.45 µl	-	-	-
S*Ubc9	-	0.37 µl	-	1.85 µl	-	-
ATP	5 µl	5 µl	5 µl	5 µl	5 µl	-
SAB	88.69 µl	88.41 µl	88.33 µl	86.93 µl	88.78 µl	100 µl
	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl

All samples were sent to Invitrogen ProtoArray® Service at -20°C, the antibody was sent at 4°C. Probing the ProtoArray® was done by the ProtoArray® Service of Invitrogen as described in the next steps.
The ProtoArray® was equilibrated for 15min at 4°C upon removal from storage at -20°C. Then each chip was blocked with 5ml blocking buffer for 1h at 4°C on a circular shaker at 50rpm. After blocking the chips were washed with 5ml assay buffer for 3min.
For probing the ProtoArray®, 100 µl reaction mix were dropped on the respective array and incubated for 1h at 30°C in a humidified chamber.
The ProtoArray® was then washed 1x with 5ml assay buffer, 3x with 5ml 0.5% SDS for 5min and 2x with 5ml assay buffer.
The ProtoArray® was incubated with α-HA.11 antibody from Covance (stock conc. = 5-7mg/ml, dilution 1:1500, end conc. = 3.3 – 4.6 µg/ml) for 1h at 4°C followed by washing 5x with 5ml assay buffer for 5min.
Subsequently, the ProtoArray® was incubated with Alexa647 goat anti-mouse antibody (stock conc. = 2mg/ml, dilution 1:2000, end conc. = 1µg/ml) for 1h at 4°C followed again by washing 5x with 5ml assay buffer for 5min.
The ProtoArray® was dried by centrifugation at 200 x g for 1min at RT and detection was done by scanning the chip at 668nm with the fluorescence scanner GenePix® 4000B from Molecular Devices Corporation.

5.4.2. Data analysis

Data analysis was done with the software ProtoArray® Prospector from Invitrogen. Additionally, candidates were also identified as SUMO substrates, if 50% of the pixels produced a signal greater than two standard deviations (σ = 755,7) above the background. Candidates must meet this criterion on all of the assayed slides incubated with 100 or

500nM E2 concentration. Once the SUMO substrate list was generated, the spots were calculated for their signal intensity.
Calculation was done as

$$Signal\ intensity = \frac{(mean\ signal\ on\ the\ duplicated\ spot - background)}{mean\ protein\ concentration\ on\ the\ spot}$$

Signal intensity with S*Ubc9 was then correlated to the signal intensity of the corresponding spots on an array incubated with the same concentration of Ubc9. Calculation was done as

$$Correlation\ value = \frac{signal\ intensity\ S * Ubc9}{signal\ intensity\ Ubc9}$$

Thus, two values are generated by correlating 100nM S*Ubc9 with 100nM Ubc9 and 500nM S*Ubc9 with 500nM Ubc9. All candidates with a correlation value ≤ 1 at both concentrations (21 proteins) were chosen as 'high-confidence' substrates, which were preferentially targeted by S*Ubc9 over Ubc9. Conclusions could be drawn from the difference in signal intensity to modification efficiency with the requirement that results were located in the linear range (1510 – 65500).

6. ABBREVIATIONS

ADP	adenosine di-phosphate
Aos	activation of Smt3p
AP	aprotinin
ATP	adenosine tri-phosphate
BLM	Bloom syndrome protein
CK2	caseine kinase 2
Daxx	death domain-associated protein 6
DTT	dithiothreitol
eIF	eukaryotic initiation translation factor
FPLC	fast protein liquid chromatography
GSNO	S-nitrosoglutathione
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HA	hemagglutinin
HIPK2	homeodomain-interacting protein kinase 2
HIS	poly-histidine
HP1α	heterochromatin protein 1 alpha
IB	immunoblot
IPTG	Isopropyl-β-D-thiogalactopyranosid
LP	leupeptin / pepstatin
NDSM	negative charged dependent SUMO motif
o/n	over night
PAA	polyacrylamide
PCR	polymerase chain reaction
PDSM	phosphorylation dependent SUMO motif
PI	protease inhibitors

PIAS	protein inhibitor of activated STAT
PML	promyelocytic leukemia
RanGAP	Ran GTP activating protein
RCC	regulator of chromosome condensation
RING	really interesting new gene
RNF4	RING finger protein 4
S*	sumoylated
SBM	SUMO binding motif
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SIM	SUMO interaction motif
SUMO	small ubiquitin-related modifier
TDG	thymine DNA glycosylase
Uba	Ubiquitin activating
Ubc	Ubiquitin conjugating
Ulp	Ubiquitin-like proteases
USP25	Ubiquitin carboxyl-terminal hydrolase
*	isopeptide bond
~	thioester bond

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