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Lisa Kirschner

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ZUSAMMENFASSUNG

Die Modifikation von Proteinen mit dem Protein SUMO (Small-Ubiquitin-like-Modifier) ist wichtig für verschiedenste zelluläre Prozesse wie Transkription, Struktur von Chromatin, DNA Reparatur, Abwicklung des Zellzyklus, Signaltransduktion und Nuklearem Transport. Störungen dieses Systems wurden in diversen Krankheiten beobachtet wie zum Beispiel unterschiedlichen Arten von Krebs und verschiedenen neurodegenerativen Krankheiten.

Die Konjugation mit SUMO ist ein höchst dynamischer Prozess welcher auf einem ständigen Gleichgewicht zwischen Konjugation und Dekonjugation beruht. SUMO Modifikation ist eine ATP-abhängige Reaktion, die eine enzymatische Kaskade aus einem E1 aktivierenden Enzym, einem E2 konjugierenden Enzym, und einem von wenigen E3 ligierenden Enzymen, benötigt. Die meisten - aber nicht alle - Substrate, brauchen die Anwesenheit einer E3 Ligase. Jedoch können ein paar Substrate wie zum Beispiel RanGAP1 oderTDG auch ohne Hilfe einer E3 Ligase effizient mit SUMO modifiziert werden.

Das Ziel meiner Arbeit war es weitere E3-unabhängige SUMO Substrate zu finden. Dies wurde durch die Durchführung eines in-vitro SUMO Assay auf einem sogenannten ProtoArray™ bewerkstelligt. Dieser ProtoArray enthält mehr als 9000 rekombinante humane Proteine. Nach Durchführung der in-vitro SUMO Reaktion wurden sumoylierte Proteine mit fluoreszierendem Antikörper detektiert. Nach der Analyse wurden einige interessante Substrate ausgewählt, in bakterielle Expressionsvektoren kloniert und gereinigt. Mit diesen gereinigten Proteinen wurden weitere in-vitro SUMO Reaktionen ausgeführt.

ABSTRACT

Modification with the Small-Ubiquitin-like-Modifier (SUMO) is implemented in diverse cellular processes as transcription, chromatin structure, DNA repair, cell cycle progression, signal transduction and nuclear transport. Dysfunction in SUMO modification has been observed in various diseases including different types of cancer and many neurodegenerative diseases.
SUMO conjugation (sumoylation) is a highly dynamic event which depends on the equilibrium between conjugation and deconjugation. Modification with SUMO is an ATP-dependent reaction which is governed by an enzymatic cascade including one E1 activating enzyme, one E2 conjugating enzyme and most of the time one out of a few E3 ligating enzymes.

Most but not all SUMO substrates depend on the third class of enzymes, the E3 enzymes. Some substrates for example RanGAP1 or TDG are efficiently modified without an E3 Ligase in vitro.
The aim of this work was to identify novel E3 independent SUMO substrates by performing an in-vitro SUMO assay on a ProtoArray™. The ProtoArray™ is purchasable as a chip spotted with more than 9000 recombinant human proteins. After accomplishing the in-vitro reaction on the chip, sumoylated proteins were detected with fluorescently labelled antibodies. After analysis some promising substrates were selected, cloned into bacterial expression vectors, purified in bacteria and analysed in E3 independent in-vitro SUMO assay.

TABLE OF CONTENTS

INTRODUCTION

Ubiquitin and its relatives, the ubiquitin-like proteins (Ubls) are covalently linked to a variety of proteins and thereby altering their properties. SUMO, which stands for small ubiquitin-related modifier, is one of several ubiquitin-like proteins and the modification system of interest in my diploma thesis.

1.1. DISCOVERY

The SUMO gene (SMT3 in yeast) was initially identified in *Saccharomyces cerevisiae* more than 15 years ago [1]. It became characterized in mammalian cells upon its discovery as binding partner for example for the tumour suppressor promyelocytic leukaemia (PML) in a yeast two hybrid assay [2]. In addition SUMO was found to be covalently attached to the Ran GTPase-activating protein (RanGAP1) in mammals and thereby facilitates the interaction with the nucleoporin RanBP2 [3,4].

Sumoylation is essential for cell viability in budding yeast, nematodes and higher eukaryotes [5]. It regulates diverse biological processes including transcriptional regulation, nuclear transport, apoptosis, cell cycle, signal transduction and the maintenance of genome integrity [6,7,8].
This large variety of biological consequences can be explained by changes in binding interfaces upon covalent attachment of SUMO which results in e.g. changes of intracellular localization, stability and/or activity of the modified target protein. SUMO can negatively and positively influence protein-protein interactions by either providing a new [9] or interfering with an existing binding interface as it is the case for the ubiquitin conjugating enzyme E2-25K [10]. Sumoylation can also have an important role in assembly of multi-protein complexes as it was shown for PML [11].

Modification with SUMO is a rapid, dynamic and energetically relative (it also costs ATP) inexpensive way to reversibly alter the functions of proteins [5,12]. Through sumoylation the complexity of the eukaryotic proteome is enormously increased.
SUMO modification seems to alter the long-term fate of the substrates, even though the SUMO conjugation may be rapidly deconjugated [8]. SUMO conjugation depends on the equilibrium between conjugation and deconjugation [12]. Although only a small fraction of the target proteins

are modified with SUMO at a given time, this small proportion could be enough to affect a change in the physiological state [9].

1.2. THE SUMO FAMILY

SUMO belongs to a highly conserved protein family. SUMO proteins are ~11kD in size; due to the long flexible N-terminus they add approximately 15 to 20kDa to the molecular weight of its substrates in SDS-PAGE. SUMO resembles the three-dimensional structure of ubiquitin (Figure 1), but share only less than 20% of the amino-acid sequence with ubiquitin [1,13].

SUMO proteins are ubiquitously expressed throughout all eukaryotes. Some organisms such as yeast, Caenorhabditis elegans and Drosophila melanogaster possess a single SUMO gene whereas other organisms such as vertebrates and plants have several SUMO genes [6]. Arabidopsis thaliana even contains eight SUMO genes [14,15]. In humans four distinct SUMO proteins are encoded in the genome: SUMO1 to SUMO4 [5,6]. SUMO1 (also known as GMP-1, PIC1, Sentrin, Ubl1, Smt3c and hSmt3) to SUMO3 are ubiquitously expressed, however, SUMO4 is thought to be expressed mainly in the kidney, lymph node and spleen[16,17].

SUMO has distinct positive and negative surface charge distributions. SUMO proteins have an unstructured stretch of 10-25 amino acids at their N termini. The N-terminal extension is flexible in solution and can be completely deleted in yeast without severe effects in vegetatively growing cells [18]. The three dimensional signal of the C-terminal core structure of SUMO presents the typical β-grasp fold shared by the different Ubiquitin-like modifiers [9] (Figure 1).

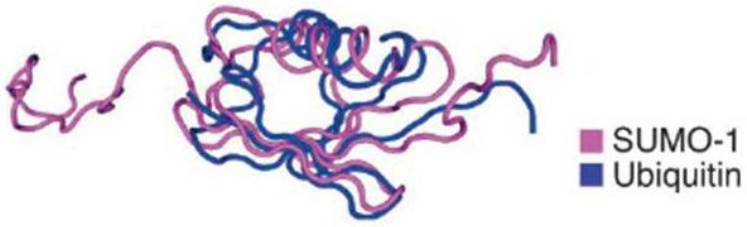

Figure 1 Structual alignment of the backbones of SUMO1 (in pink) and Ubiquitin (in blue) from Protein Modification by SUMO by Erica Johnson 2004. The N-Termini are on the left and SUMO1 possess here an unstructured stretch. C-Termini are on the right.

In cells SUMO is expressed as an inactive precursor carrying a C-terminal stretch with variable length (2-11 amino acids) downstream of the invariant Gly-Gly motif. To activate the protein this C-terminal extension has to be removed by SUMO-specific proteases to free the Gly-Gly motif which is essential for target modification [13].

The mature forms of SUMO2 and SUMO3 are almost identical whereas SUMO1 shares only 50% sequence identity with the SUMO2/3. SUMO1 and SUMO2/3 were shown to have distinct but overlapping functions [1]. This is supported by the surprising finding that disruption of SUMO1 in mice is not lethal most likely because SUMO2/3 can compensate for its function [19].

Cells contain large pools of free unconjugated SUMO2/3; however there is virtually no free pool of SUMO-1, at any given time. This suggests that the majority of SUMO1 is constitutively conjugated to its substrates [20,21], whereas SUMO2/3 gets conjugated upon different stress stimuli [22].

Many SUMO substrates are sumoylated at one or multiple sites with a single SUMO (mono-SUMO) although poly SUMO chain formation is described mainly for SUMO2/3 targets upon different stress stimuli [23].

1.3. MECHANISM OF SUMO CONJUGATION AND DECONJUGATION

SUMO entities are conjugated to their target proteins via a three step enzyme pathway. These enzymes are specific for SUMO modification. SUMO modification knows one E1, one E2 and several E3 enzymes [24,25,26,27,28,29] (Figure 2).

Sumoylation results in the formation of an isopeptide bond between the C-terminal Gly residue of the modifier protein (SUMO) and the ε-amino group of a Lysine residue in the substrate.

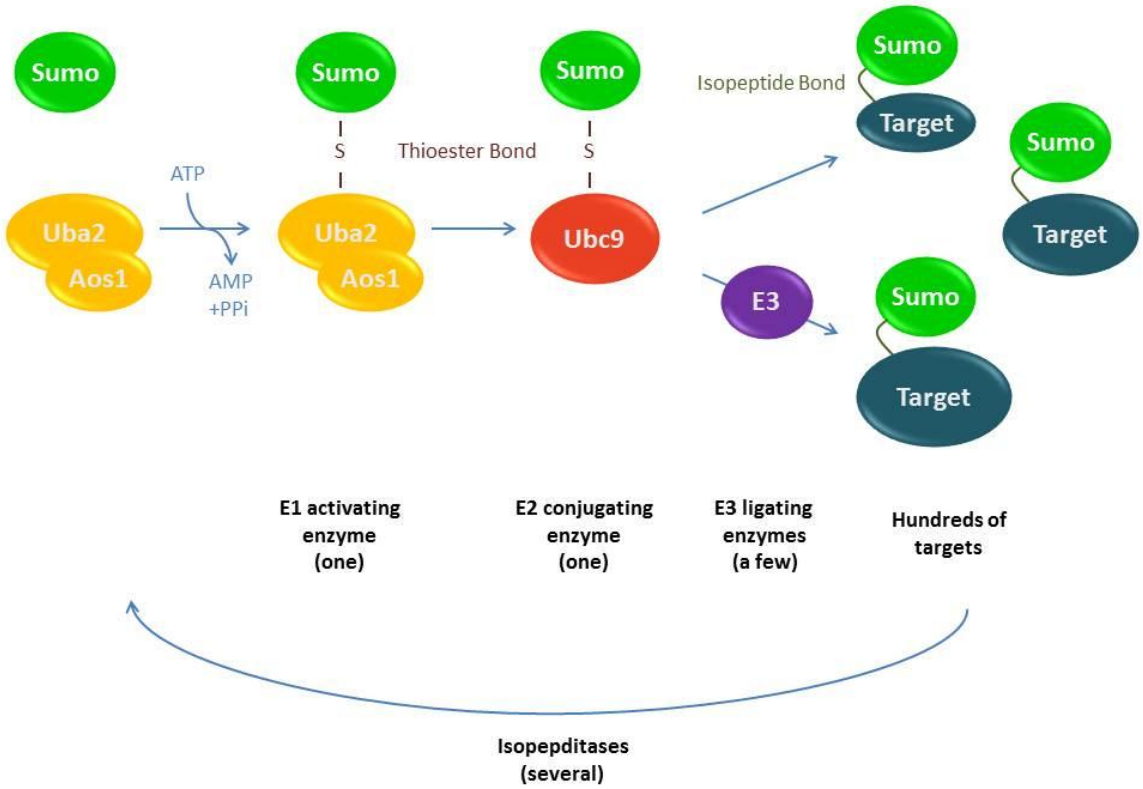

Figure 2 SUMO Modification Cycle: SUMO is activated in an ATP dependent reaction resulting in SUMO~AMP and releasing pyrophosphate. The E1 enzyme attacks the activated SUMO protein with its active cysteine, which results in the formation of a thioester bond between the E1 enzyme and SUMO. In the next step SUMO is transferred to the E2 enzyme and they are also connected via a thioester bond. In the final step, SUMO is transferred to the substrate and an isopeptide bond is formed. This step is usually facilitated by E3 ligases. The process of sumoylation is reversible and is performed by several isopeptidases.

1.3.1. SUMO-ACTIVATING ENZYME E1

First, the mature SUMO needs to be activated at its C-terminal Gly-Gly by the SUMO- specific activating enzyme E1. The E1 is a heterodimer consisting of Aos1 (also called SAE1) and Uba2 (or SAE2). Aos1 resembles the N-terminus of the ubiquitin activating enzyme E1 and Uba2 corresponds to the C-terminus that contains the active cysteine [30].

Formation of a SUMO- adenylate requires ATP. SUMO attacks ATP with its C-terminal carboxyl group, this results in a SUMO C-terminal adenylate and pyrophosphate is released. The thiol group of the active cysteine in the E1 attacks the SUMO adenylate. It thereby releases AMP and forms a high-energy thioester bond between the E1 and the C-terminus of SUMO [5,31].

1.3.2. SUMO-CONJUGATING ENZYME E2

The E2 conjugating enzyme Ubc9 accepts SUMO from the E1 enzyme and subsequently catalyses the transfer to the substrate by selecting the target Lysine for modification. Ubc9 is the only known SUMO-conjugating enzyme in yeast and invertebrates and most likely in vertebrates as well [25,26]. This is supported by the findings that the Ubc9 gene is essential in all tested organisms

[32,33,34] except in fission yeast where it is not crucial [35,36]. Interestingly, in the ubiquitin pathway multiple E2 enzymes are involved in ubiquitin conjugation and play roles in different cellular pathways [37].

Upon accepting SUMO from the E1 enzyme a thioester bond between the catalytic Cysteine residue of Ubc9 and the C-terminal carboxy group of SUMO is formed. Since this bond is highly reactive it serves as a donor for the final reaction where SUMO is transferred to the substrate. This results in the formation of a stable isopeptide bond between the C-terminal Glycine residue of SUMO and a Lysine side chain of the substrate [5,8].

Ubc9 has a strong overall positive charge and its three dimensional structure strongly resembles ubiquitin E2 enzymes [38]. The patch around Ubc9´s active Cysteine directly recognizes SUMO consensus sequences often found in SUMO substrates [39] (discussed in section 1.3.5). However, additional residues in Ubc9 might have a role in correctly orienting the thioester linked SUMO and/or stabilizing the substrate interaction to facilitate isopeptide bond formation [39,40]. The residues of Ubc9 which are in direct contact with the substrate Lysine seem to have a role in catalysis rather than in substrate binding [41].

Nevertheless, the interaction of the SUMO consensus site with the catalytic cleft in Ubc9 is usually not sufficient for efficient SUMO transfer. Therefore most targets depends on E3 ligases for efficient modification [42].

1.3.3. E3 SUMO LIGASES

SUMO E3 ligases catalyse the transfer of SUMO from Ubc9 to a substrate promoting the sumoylation efficiency. E3 ligases are involved in most sumoylation events in vivo. *The E2 contributes to target recognition, but the E3 ligases usually confer substrate specificity and accelerate catalysis* [42].

The classical definition of an E3 enzyme is that it binds the E2 and the substrate thereby promoting the transfer of SUMO from the E2 to the target. None of the known SUMO E3s form covalent intermediates with SUMO; however they bring together Ubc9 and the substrate. Selected SUMO E3 ligases seem not to interact directly with the substrate and rather stabilize and orient the SUMO charged E2 for optimal transfer [40]. However, no direct role of E3s in lysine activation during SUMO conjugation has been demonstrated. This suggests that the catalytic potential which is required for conjugation comes from the E2. [41]

SUMO conjugation can take place in vitro in the absence of an E3, at the Lysine residues that are actually modified in vivo [24]. However, the majority of substrates depend on E3 ligases for efficient sumoylation [43]. RanGAP1 is the prime example which seems not to require an E3 ligase for efficient modification. Interestingly, the RanGAP1-Ubc9 binding is stabilized through an additional binding interface and indeed this interaction is required for its efficient sumoylation.[39]

Four different types of E3s have been identified in the last years which includes the largest group, the SP-RING family (contains the Siz/PIAS family members)[43], the nucleoporin RanBP2 [29], the polycomb group protein 2 [44] and TOPORS [45]. Several of these proteins resemble ubiquitin E3 ligases and are regulated by posttranslational modifications for example phosphorylation and/or S-nitrosylation [46,47,48,49].

1.3.4. SUMO PROTEASES

Sumoylation is a reversible modification and one class of enzymes able to cleave SUMO are described [50]. All known SUMO cleaving enzymes, called isopeptidases, belong to a class of Cysteine proteases with a ~200 amino acid conserved C-terminal domain [51]. In humans they are called sentrin-specific proteases (SENPs). They have three main functions: they remove SUMO from its substrates (isopeptidase cleavage), thereby making the modification reversible, *they are responsible for the pool of matured SUMO in the cell (SUMO processing)* [5], and they edit SUMO chains [52].

1.3.5. THE SUMO CONSENSUS SITE

Many of the substrates share a common motif for modification, a so called SUMO consensus site: ΨKxE where Ψ is a bulky hydrophobic amino acid (Isoleucine, Leucine or Valine), K is the Lysine which is conjugated to SUMO, x is any amino acid and E is Glutamic acid. The SUMO acceptor site was identified after mapping Lysine residues in the SUMO targets/substrates RanGAP1 [53], PML [54], Sp100 [55] and p53 [56]. The catalytic cleft of Ubc9 directly recognizes this tetrapeptide (ΨKxE) on the target [39]. Hence Ubc9 is not only capable of catalysing the transfer of the SUMO protein to the substrate but also selects *the accurate lysine residue for modification* [57].

Sumoylation often occurs on a SUMO consensus motif but far not all SUMO consensus motifs are de facto modified. One example is E2-25K which has three SUMO consensus motifs but is modified at a non-consensus Lysine. Other SUMO substrates like DAXX do not even contain a SUMO consensus site.

Structural studies have shown that a SUMO consensus motif can only be recognized by Ubc9 if it is in an unstructured area or part of an extended loop, as in RanGAP1 [39] but not when located in an α -helix as in E2-25K [10]. Due to this direct interaction of Ubc9 with the SUMO consensus site in its targets it is not surprising that E1 and Ubc9 are often sufficient to sumoylate many substrates at the correct sites in vitro also in the absence of an E3 [24].

Some SUMO substrates show enhanced sumoylation due additional Ubc9 binding sites in close proximity to the classical SUMO consensus motif. Two different extensions to the SUMO-consensus motif are described.

The phosphorylation-dependent sumoylation motif (PDSM). It is found in heat shock factor-1 (HSF1), myocyte enhancer factor 2 (MEF2) and several other proteins [58]. This motif consists of the conventional sumoylation motif followed by a phosphorylated Serine and Proline residue (ΨKxExxpSP). Phosphorylation is required for sumoylation of HSF1 and MEF2A in vivo [59].

The additional negative charge of the phosphate group might enhance substrate binding to the basic surface of Ubc9 and thus promotes SUMO conjugation (MEF2 and HSF1) [60]. Phosphorylation dependent sumoylation provides an additional level of regulation.

A second extended SUMO motif was identified: the negatively charged amino-acid-dependent sumoylation motif (NDSM)[61]. The negatively charged residues at position +3 in the NDSM were found to directly interact with the two positively charged residues (Ubc9 K59 and K61) and this interaction is important for modification. Again an additional binding interface between the substrate and Ubc9 is created[61].

The PDSM motif is more common than the NDSM and the main difference between these motifs is that NDSM is constitutive whereas the PDSM is regulated via its phosphorylation [62].

1.3.6. THE SUMO INTERACTING MOTIF

SUMO does not only bind proteins by covalent interactions also non-covalent interactions are described mediated via a SUMO-Interaction Motif (SIM) [63,64,65].

A SIM consists of a short hydrophobic core which is often flanked by acidic and/or Serine residues [66]. The consensus sequence for the hydrophobic core is identified as Val/Ile-X-Val/Ile-Val/Ile (V/I-X-V/I-V/I). This motif can bind SUMO either in a parallel or anti-parallel orientation [67]. The acidic residues at the ends of the SIM interact with the basic residues on the SUMO surface [9], whereas the Serine residues might regulate SUMO binding by phosphorylation, because the

negative charge would strengthen SUMO binding. However, the importance of the Serine residues has been questioned in another study [68].

SIMs have been identified in a wide range of proteins including SUMO substrates, SUMO enzymes and SUMO binding proteins with functions in many cellular processes including DNA repair, transcriptional activation, nuclear body formation and protein turnover. Here a few examples are presented.

SIMs can function as prerequisite for efficient modification. SIM containing SUMO substrates like the base excision repair enzyme thymine DNA glycosylase (TDG), the transcription factor DAXX and the ubiquitin specific protease Usp25 depend on a functional SIM motif for efficient SUMO conjugation [69]. This suggests that the SIM helps to recruit and/or positions the Ubc9~SUMO thioester for efficient conjugation.

In case of TDG the SIM also contributes to its conformational changes. TDG binds and hydrolyses mismatched Thymine and Uracil bases and remains tightly bound to abasic sites. Sumoylation of TDG and the subsequent intramolecular SIM interaction leads to a conformational change which releases TDG from DNA[70].

The most common function of SIM containing proteins is to establish interactions with SUMO conjugated proteins. One example is represented by the SUMO targeted ubiquitin E3 ligase RNF4 that recognizes polysumoylated substrates with its four SIMs [71,72].

Another prominent example is PML which is a SUMO substrate but also contains SIMs that non-covalently interact with SUMO. PML sumoylation is a prerequisite for nuclear PML body formation and it is thought that sumoylated PML functions as a molecular scaffold to recruit its binding proteins via covalent and non-covalent SUMO interactions [11].

Several SUMO substrates including proteins which are important for transcription, DNA repair and chromatin remodelling were found to have SIMs including e.g. Sp100, PML, TDG, Daxx, and all SUMO E3 ligases [65,73].

The identified functions of SIMs are very diverse as they can be important for substrate modification, conformational changes and interation with specific binding partners.

In the case of TDG the SIM is not only required for efficient SUMO modification by recruiting or positioning the Ubc9~SUMO thioester [42], it is also involved in the intramolecular interaction with the SUMO covalently attached to TDG leading to a conformational change [74,70]. A prominent

example for the involvement of SIMs in recruitment of binding partners is PML body formation [75,76].

SIMs were found to be regulated by phosphorylation which is performed by the Serine/Threonine Kinase CK2. This phospho-regulated SIM module was found in the E3 ligase PIAS1, in PML and in PMSCL1. *The phosphor-dependent SUMO does not impair the ligase activity but affects the transcriptional coregulatory role of PIAS1* [77].

Ubc9 itself has no classical SIM but also interacts noncovalently with SUMO in a SIM independent manner. It was shown that SUMO interacts far from the E2 active site, with the N-terminal helix, the first β-strand and the intervening loop of Ubc9 [78].

1.4. CONSEQUENCES OF SUMOYLATION

Sumoylation can influence diverse aspects of a target protein including stability, intracellular localization and/or activity. This large variety of consequences can be explained by the fact that conjugation with SUMO locally alters the surfaces of its substrates and therefore it can interfere with existing /or provide novel intra- and extramolecular binding interfaces.

Sumoylation creates novel extramolecular binding interfaces

One prime example comes from studies with RanGAP1. Unmodified RanGAP1 is soluble in the cytosol. Upon sumoylation RanGAP1 changes its cellular localization by recruiting it to the outer nuclear membrane where it interacts with the nucleoporin RanBP2 [4,39].

As already mentioned above PML has a prime function in nuclear body formation: Promyelotic leukaemia nuclear bodies are dynamic intranuclear structures which lack a membrane. They are regulated in response to genomic stress and during the cell cycle and possess crucial tumor-suppressive functions [76]. PML acts as the structural scaffold of these nuclear bodies and is able to recruit other proteins which are important for transcription and DNA-damage response [76]. PML is a SUMO substrate with several SIMs and this provides binding interfaces for both sumoylated substrates and SIM containing proteins [75]. Several PML binding proteins are SIM containing SUMO substrates including DAXX, TDG and Sp100 [65].

The polymerase processing factor PCNA is a SUMO substrate which recruits upon sumoylation the yeast DNA helicase Srs2 during S phase to replication forks to prevent DNA recombination [79,80]. PCNA functions as a messenger platform for different polymerases due to its posttranslational modifications [81].

Sumoylation interferes with an existing binding interface

The ubiquitin-conjugating enzyme E2-25K is sumoylated at its first N-terminal alpha helix, an established binding interface for the ubiquitin E1 enzyme. E2-25K sumoylation impairs its activity in forming ubiquitin thioesters and unanchored ubiquitin chains by interfering with E1 interaction [10].

Sumoylation dependent conformational changes via intramolecular interactions

As mentioned earlier the Thymine DNA Glycosylase is a DNA repair enzyme and an enzyme that removes mismatched thymine and uracil bases by binding and hydrolysing the mismatched base. TDG then remains tightly bound to the abasic site [82]. Sumoylation of TDG is the important switch that triggers a conformational change in TDG [70]. Therein residues which are required for SUMO modification as well as the residues in the SIM are required to induce the conformational change in TDG. A protruding helix in the protein structure of sumoylated TDG interferes sterically with DNA binding [83]. This results in decreased affinity of TDG for DNA and TDG can be releases from the DNA and the subsequent repair of the abasic site can be further promoted.

1.5. REGULATION OF SUMOYLATION

Sumoylation is regulated at diverse levels including the individual substrates, SUMO and its enzymes for conjugation and deconjugation.

Many SUMO substrates are targets for different posttranslational modifications such as acetylation, methylation, phosphorylation and ubiquitination. These modifications can regulate SUMO conjugation in different ways.

Phosphorylation for example can either enhance substrate sumoylation by increasing its affinity for the E2 or the E3 enzyme [60] but also negatively regulate sumoylation by interfering with the SUMO conjugation enzymes as it is described for p53 [84]. In case of IKBα sumoylation protects from proteasomal degradation as ubiquitin and SUMO compete for the same acceptor Lysine [85].

The opposite is true for other substrates in which sumoylation is a prerequisite for ubiquitination and proteasomal degradations (eg. PML etc).
Also the intracellular localization can determine the sumoylation status of a protein as it was found for Sp100 which needs to be recruited to the nucleus for modification [86].

SUMO itself seems to be regulated at different levels. SUMO1-3 are ubiquitously expressed, but there are variations in different tissues [87]. SUMO1 expression was shown to be increased in response to hypoxia [88]. Additionally, SUMO1 itself can be a target for posttranslational modifications like acetylation and phosphorylation [89,90].

Influencing the regulation of the E1 enzyme has a huge impact on global sumoylation [12]. The protein Gam1 of the CELO virus induces proteasomal degradation of SUMO E1 enzyme [91]. During oxidative stress, low H_2O_2 levels induce reversible disulphide bond formation between the catalytic Cysteines of the E1 subunit Uba2 and Ubc9. This results in desumolation of most cellular SUMO substrates [92].

Ubc9 is the only known SUMO E2 enzyme. Ubc9 can get modified by S-nitrosylation after treatment with the nitric oxide donor GSNO [46]. Additional mechanisms of Ubc9 regulation will be discussed in the next section.

SUMO E3 ligases can also be regulated by posttranslational modifications. As for example phosphorylation of Topors negatively influences its E3 function [48].The opposite was shown for the polycomb group E3 ligase Pc2 which is activated upon phosphorylation [47]. S-nitrosylation negatively influences the E3 ligase Pias3 by marking it for ubiquitin dependent degradation [46].

SUMO proteases seem to be regulated at the transcriptional level since they are differentially expressed in different tissues [87].
The regulated intracellular localization of substrates, E3 enzymes and SUMO proteases determines the sumoylation status of a substrate and the activity of the enzymes, respectively [52,55,93,94,95,96,97,98,99,100,101].

1.5.1. UBC9 FUNCTION AND REGULATION

Ubc9 was found to be highly upregulated in many types of different human cancer including lung adenocarcinoma [102,103], melanoma[104], prostate cancer [105], breast cancer [106,107] and a specific form of acute myeloid leukemia (AML) [108]. In addition sumoylation is implicated in the pathogenesis of

type 1 diabetes [109]. In summary these findings indicate that Ubc9 overexpression may be involved in the pathogenesis of different diseases and therefore might be an attractive target for drug development[110]. Due to the fact that Ubc9 dependent sumoylation is involved in diverse cellular processes as cell cycle regulation [26], DNA repair [111] and apoptosis [112,113], alterations in sumoylation could have a major impact on cell homeostasis. However, the molecular mechanism of how Ubc9 would mediate pathogenesis remains elusive since most SUMO substrates are expected to be regulated by E3 ligases and would be restricted to their regulation.

Beside upregulation different other mechanism regulating Ubc9 were described including nitrosylation, oxidation and sumoylation [46,92,114]. While oxidation reversibly inactivates Ubc9 the consequences of its nitrosylation are unknown [46].

Our lab recently analysed the consequences of Ubc9 sumoylation. Ubc9 is sumoylated at Lysine 14 in mammalian cells (S*Ubc9) [42]. Detailed biochemical analysis indicates that this modification does not significantly impair its catalytic activity and in this line sumoylation of most substrates including HDAC4, E2-25K, PML or TDG is unaffected [42]. However, turning to two other well known SUMO substrates: RanGAP1 and Sp100 led to different scenarios: In the presence of S*Ubc9 RanGAP1 sumoylation is significantly impaired whereas Sp100 modification is strongly enhanced as otherwise only obtained in the presence of an E3 ligase. This enhancement depends on the non covalent interaction via a SIM motif in Sp100 which recognizes the SUMO conjugated to Ubc9. Interestingly, other SIM containing SUMO substrates (Daxx, PML and TDG) show only minor or no increase with S*Ubc9. Structural analyses of S*Ubc9 indicates that SUMO binds to a hairpin loop in Ubc9 and is therefore forced in a specific orientation. This finding suggests the requirement of a defined distance between the SIM and the site of modification in the substrate [42].

In our current working model Ubc9 itself significantly contributes to target selection at least for selected SUMO substrates (Figure 3):

In all known sumoylation events SUMO is directly transferred from the charged Ubc9 to the substrate. The catalytic cleft of Ubc9 directly interacts with a SUMO concensus motif in the substrate but usually this interaction is not sufficient for efficient modification. RING type E3 ligases recognize both the charged E2 and the substrates which lead to an increase in affinity and an enhanced SUMO transfer to the substrate. Alternativly different E3 independent mechanisms are described: RanGAP1 is very efficiently modified in the absence of an E3 ligase and this depends on an additional binding interface with Ubc9 [39]. Additional Ubc9 binding can

also be achieved by a negatively charged binding interface as it is shown for Elk-1 [61]. Two other mechanisms involving non covalent SUMO binding via SIMs are described: In one scenario the SIM containing substrate positions the SUMO thioester loaded Ubc9 [115,116]; in another scenario recruitment is via the sumoylated (isopeptide linked) Ubc9 [42].

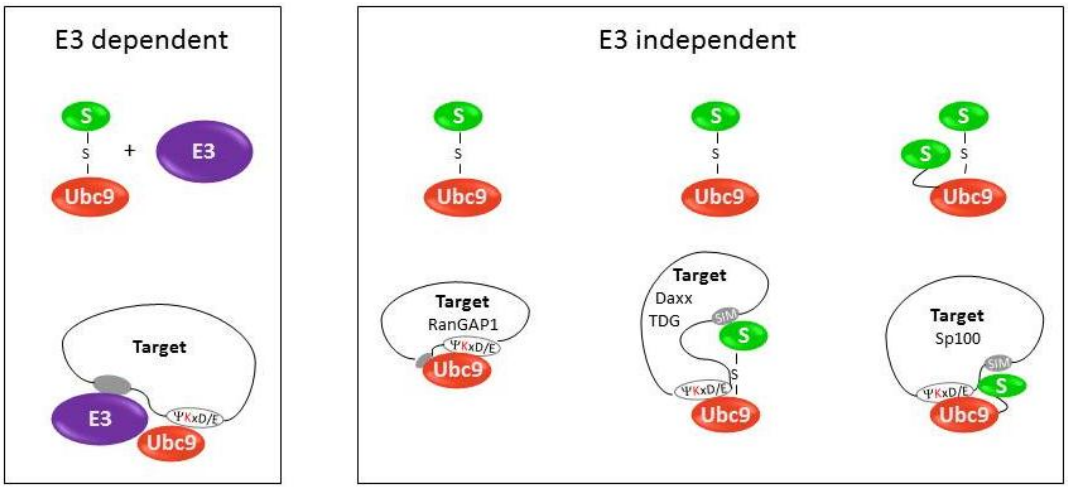

Figure 3 Model of Ubc9´s role in target discrimination via different modes of increasing substrate affinity. Ubc9`s catalytic cleft directly recognizes a SUMO consensus motif in the substrate. For efficient modification this interaction is not sufficient and depends on additional binding interfaces which can be E3 dependent (left panel) or E3 independent (right panel). Classical E3 ligases bind both the substrate and the E2 enzyme to bring them in close proximity for efficient modification. E3 independent interactions involve additional binding interfaces with either Ubc9´s surface (e.g. RanGAP1) or a covalently Ubc9-linked SUMO (Ubc9 thioester linked SUMO: Daxx, TDG, BML, Usp25 or isopeptide linked sumoylated Ubc9: Sp100) and a SIM in the substrate. Model adapted from *Ubc9 Sumoylation regulates target discrimination by Knipscheer et al.*

In conclusion different substrates interact via diverse surfaces directly or indirectly with Ubc9 aside from the consensus motif interaction with Ubc9´s catalytic cleft. These additional binding interfaces (E3 dependent and independent) enhance the affinity to the substrate leading to an increase in modification. Together these findings indicate that Ubc9 regulation significantly contributes to substrate selection and its deregulation (e.g. Ubc9 overexpression) may be a key event in disease onset. To clarify the role of Ubc9 deregulation additional substrates directly regulated by Ubc9 need to be discovered.

1.6. AIM OF MY THESIS

Although most SUMO substrates require E3 ligating enzymes for efficient modification, selected targets like RanGAP1 are efficiently modified without this class of enzymes. RanGAP1 stably interacts with Ubc9 via two binding interfaces: one involves the SUMO consensus site in RanGAP1 and the catalytic cleft in Ubc9 which is common for many SUMO substrates; the second binding interface seems to be specific for RanGAP1 and is not described for other substrates. This interface is required for stable interaction with Ubc9 and for efficient modification. Interestingly also some other substrates developed mechanisms to increase their affinity to Ubc9 leading to enhanced modification. In my thesis I was interested to learn about the generality of E3 independent sumoylation which may help to explain how Ubc9 overexpression, frequently found in cancer, contributes to disease onset and/or pathogenesis.

To achieve this goal I have established conditions to perform an E3 independent in vitro sumoylation reaction on a human ProtoArray™ from Invitrogen. This array contains more than 9000 recombinant human proteins, purified from insect cells and spotted on specially coated glass slides. Such an array was recently established for the identification of specific substrates for ubiquitin E3 ligases [117,118,119]. To perform an in vitro sumoylation reaction on this array the purification of bacterially expressed HA-tagged SUMO1, the heterodimeric SUMO E1 enzyme and the E2 enzyme Ubc9 was required. Prior to applying these enzymes to the array, known SUMO substrates were tested, to identify ideal conditions for the discovery of E3 independent substrates. Finally, initial analysis of two identified proteins was performed.

RESULTS

2. PURIFICATION OF SUMO AND ITS E1 AND E2 ENZYMES

An E3 independent in vitro sumoylation assay requires SUMO and its E1 and E2 enzymes as recombinant proteins expressed and purified from bacteria. Subsequently the optimal conditions have to be identified by comparing sumoylation of known SUMO substrates.

2.1. PURIFICATION OF THE SUMO ENZYME E1

The SUMO E1 enzyme is a heterodimer between Aos1 and Uba2. Aos1 has a molecular mass of approximately 40 kDa and Uba2 of approximately 90 kDa. Both subunits were cloned under the IPTG inducible T7 promotor as His tagged versions. While Aos1 was N-terminally tagged, Uba2 was tagged at its C-terminus. The proteins were individually expressed and purified following standard protocols. Subsequently, both components were mixed to form the catalytic active heterodimer which was separated from the single components by gel filtration.

Purification of His-Aos1

In brief, the His-Aos1 containing plasmid was transformed in the bacterial strain E. coli BL21 gold and expression was induced upon addition of 1 mM IPTG for 6 hours at 25 °C. Bacterial lysis was by shock freezing in liquid nitrogen prior addition of Lysosyme. His-tagged proteins were enriched on Ni2+-beads (Invitrogen) and elution was with high concentrations of imidazol which releases the tagged proteins from the beads. Protein containing fractions were pooled, concentrated and further purified by size exclusion chromatography on a gelfiltration column using an ÄKTA FPLC system.

Figure 4-A shows the chromatogram of His-Aos1. On the x-axis the elution volume is plotted in fractions collected and the y-axis demonstrates the protein concentration in mAU (milli absorbence unit) which are measured by light absorbance of aromatic amino acids (Tryptophan, Tyrosin and Phenylalanin) at 280nm. The conductivity of the buffer is measured in millisiemens/cm (mS/cm) and determines the salt concentration. His-Aos1 elutes from the column as broad peak with a sholder. 10 µl of every other peak fraction, together with aliquots of uninduced and induced His-Aos1 transformed cells, were separated on a 5-20% SDS PAGE (Figure 4-B). Protein was stained with Coomassie brilliant blue. Indeed, strong induction of His-

Aos1 migrating at approximately 40kDa was obtained upon IPTG induction. Elution of His-Aos1 peaked at fractions 7 to 17 (5.5 ml) were pooled for further usage.

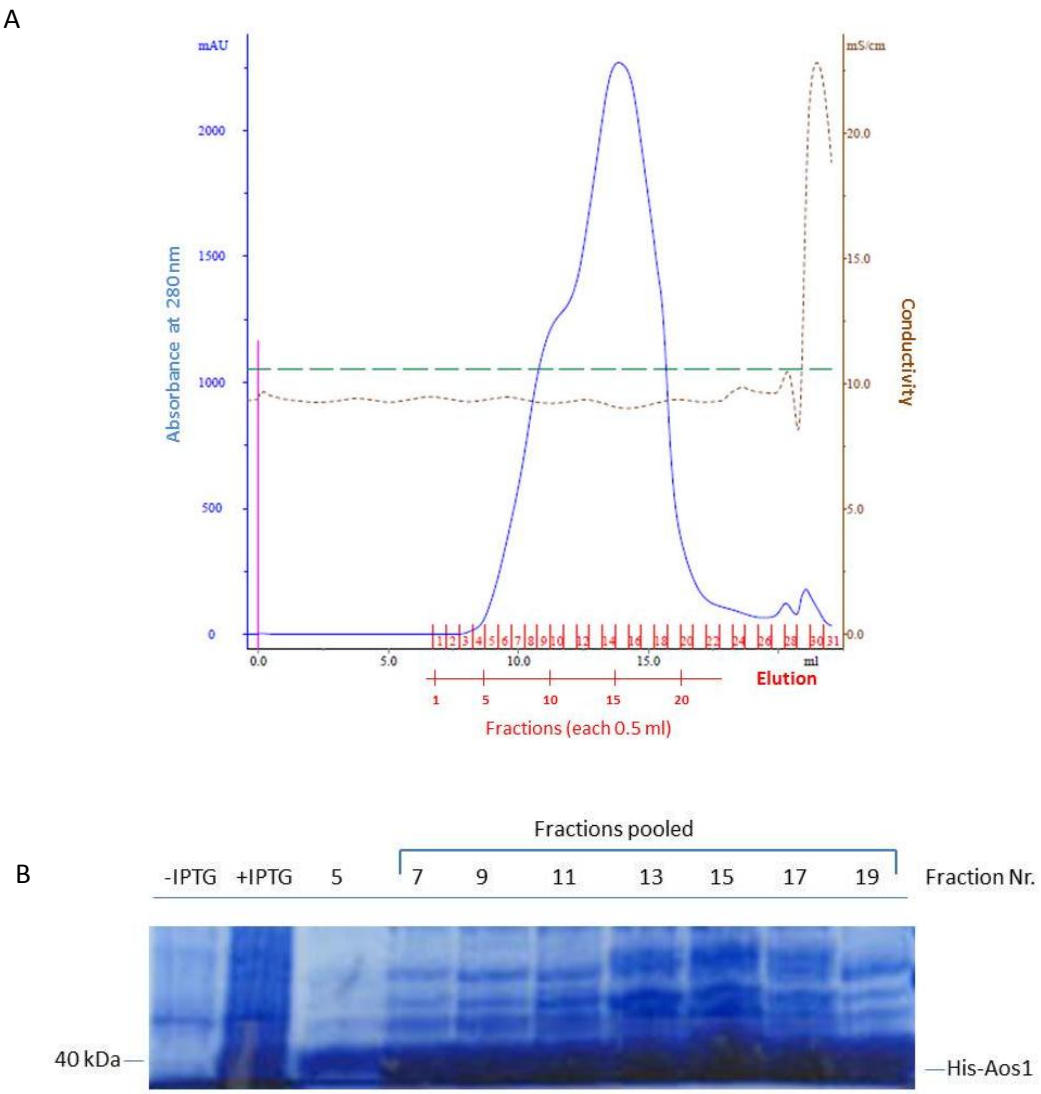

Figure 4. Purification of recombinant His-Aos1. His-Aos1 expression was IPTG induced in E. coli BL21 gold, enriched on Ni2+ beads prior separation by size exclusion chromatography using an ÄKTA FPLC system. (A) Elution chromatogram of His-Aos1 on a S200 gelfiltration column. The x-axis represents the eluted volume in sample fractions (0,5ml) and the y-axis the protein concentration in mAU (milli absorbance unit) at 280nm. The salt concentration is indicated as conductivity in mS/cm. (B) Immunoblot analyisis of uninduced (-IPTG) and induced (+IPTG) cells and eluted peak fractions from (A) were separated on a 5-20% SDS-PAGE. Protein was stained with Coomassie brilliant blue. Fractions 7 to 17 were pooled and used for E1 complex formation.

Purification of Uba2-His

Next, Uba2 which is C-terminally tagged with His-(Uba2-His) was purified following the same procedure. Figure 5-A shows the monitored size exclusion gelfiltration elution chromatogram. Uba2-His eluated in one major peak with several small shoulders. Again protein containing fractions were analysed on SDS-PAGE together with aliquots of uninduced and induced bacteria

samples. As shown is Figure 5-B Uba2-His migrates at 90 kDa. Since all fractions contained fair amout of Uba2 they were all pooled and used for E1 complex formation.

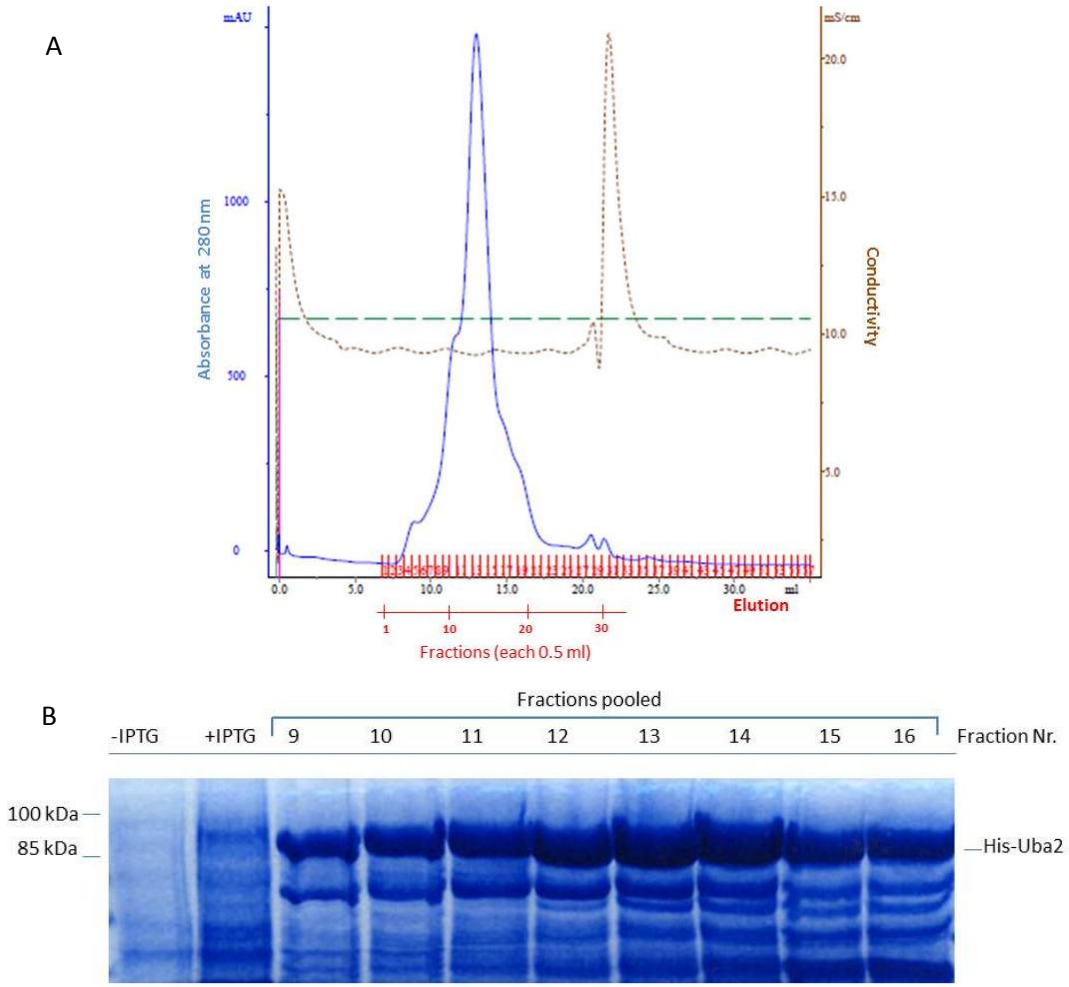

Figure 5 Purification of recombinant His-Uba2. His-Uba2 expression was IPTG induced in E.coli BL21 gold, enriched on Ni2+ beads prior separation by size exclusion chromatography using an ÄKTA FPLC system. (A) Elution chromatogram of His-Uba2 on a S200 gelfiltration column. The x-axis represents the eluted volume in sample fractions (0,5ml) and the y-axis the protein concentration in mAU (milli absorbance unit) at 280nm. The salt concentration is indicated as conductivity in mS/cm. (B) Immunoblot analyisis of uninduced (-IPTG) and induced (+IPTG) cells and eluted peak fractions from (A) were separated on a 5-20% SDS-PAGE . Protein was stained with Coomassie brilliant blue. Fractions 9 to 16 were pooled and used for E1 complex formation.

E1 complex formation

For forming an acive E1 complex, comparable molar amounts of His-Aos1 and Uba2-His have to be used. Therefore the concentrations of both purified proteins were estimated on a gradient gel (Figure 6-A) to use by a dilution series. Samples were separated on an SDS PAGE and stained with Coomassie brilliant blue. As demonstrated in Figure 6-A His-Aos1 was approximately twice as concentrated as Uba2-His and therefore they were mixed in a 1:2 ratio to obtain equimolar amounts for complex formation. After one hour incubation on ice the reaction was subjected to

size exclusion chromatography to separate the complex from its single components. The elution chromatogram is shown in Figure 6-B and demonstrates three peaks. The first and largest peak corresponds to the complex, the second represents Uba2-His and the third the smallest component His-Aos1. Fractions of the first peak were separated on SDS-PAGE and proteins were stained with Comassie brilliant blue as depicted in Figure 6-C. Fractions containing equimolar amounts of His-Aos1 and Uba2-His (35-37) were pooled and concentration was measured by comparison to defined concentrations of a mixture of two reference proteins, Ovalbumine (44.3kDa) and Phosphorylase (97.2.kDa) (Figure 6-D). The concentration of the purified E1 was estimated to 0.5 µg/µl.

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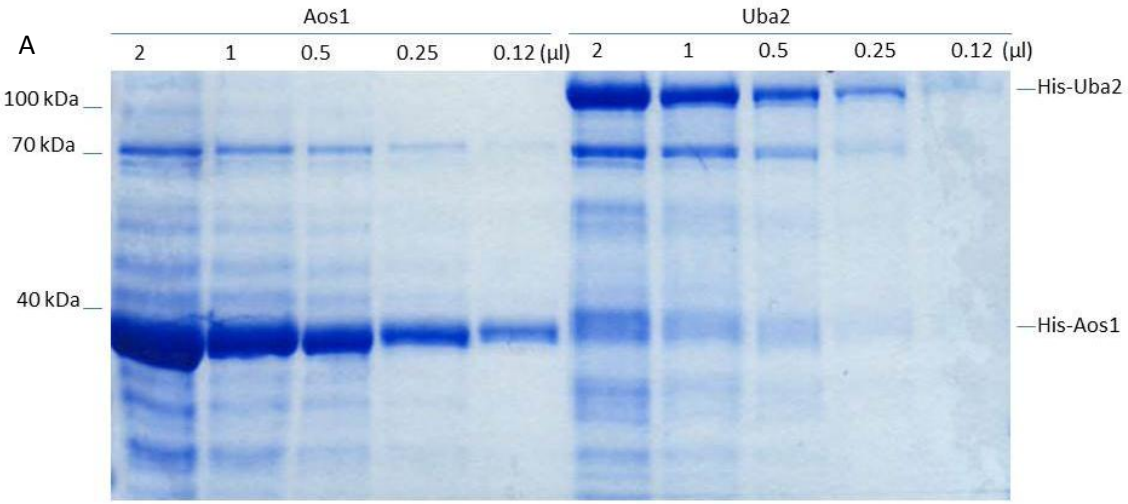

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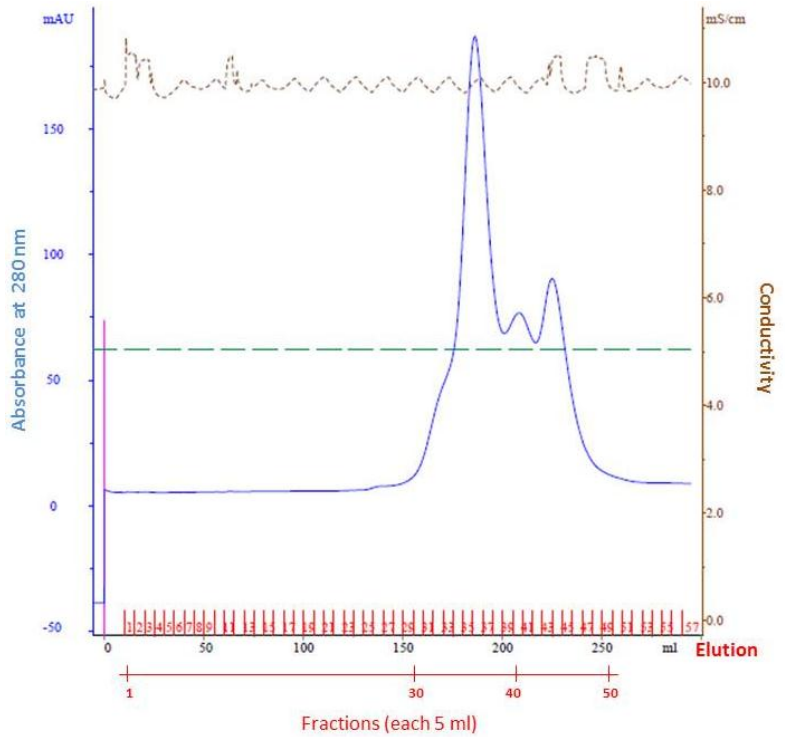

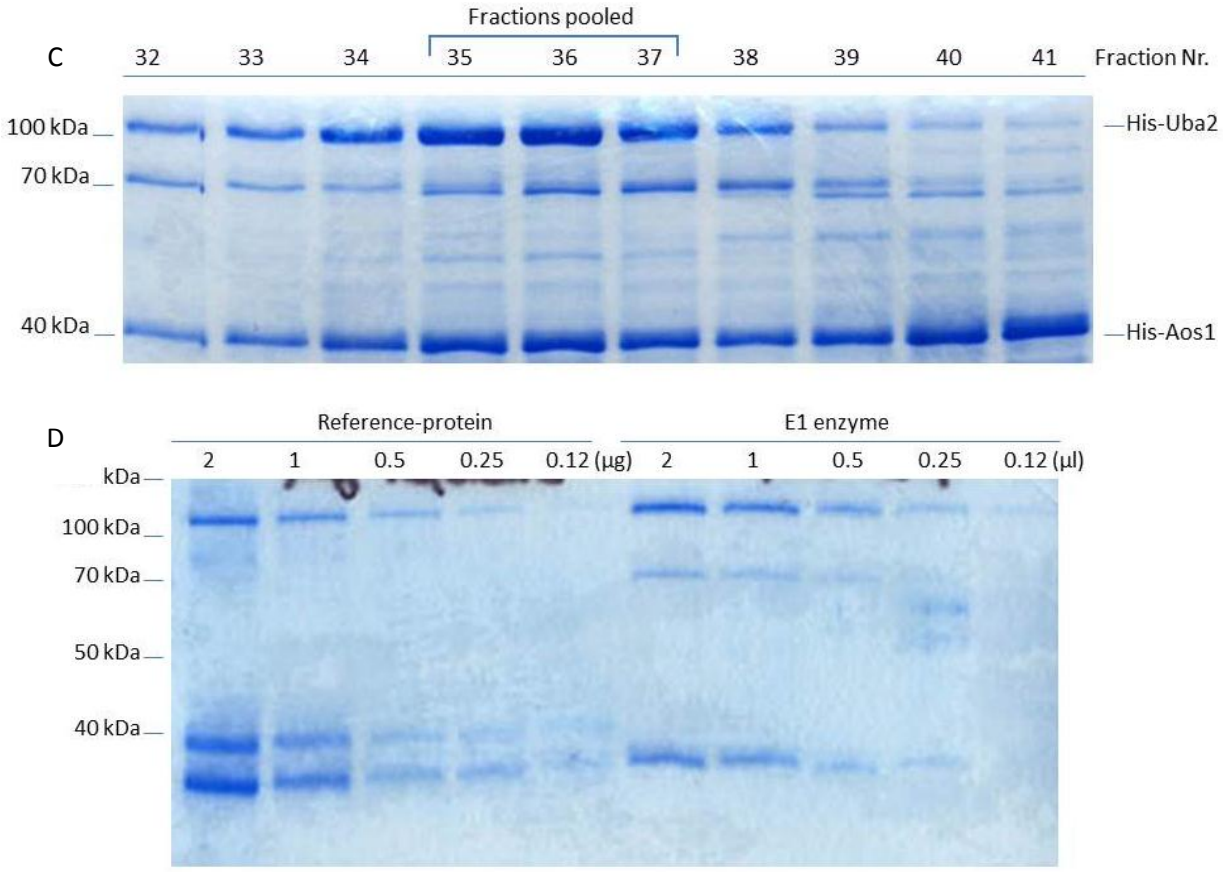

Figure 6 E1 complex formation. (A) Identification of equivalent molar concentrations of purified His-Aos1 and His-Uba2. Protein amounts of 2, 1, 0.5, 0.25 and 0.12 µl were determined on a 5-20% SDS PAG, stained with Coomassie brilliant blue. (B) Elution chromatogram of the E1 complex on a S200 gelfiltration column. The x-axis represents the eluted volume in sample fractions (0,5ml) and the y-axis the protein concentration in mAU (milli absorbance unit) at 280nm. The salt concentration is indicated as conductivity in mS/cm. (C) Immunoblot analyisis of eluted peak fractions from (B) were separated on a 5-20% SDS-PAGE. Protein was stained with Coomassie brilliant blue.. (D) Concentration determination of the E1 complex with two reference proteins Ovalbumine and Phosphorylase. E1 protein concentration of 2, 1, 0.5, 0.25 and 0.12 µl was compared to 2, 1, 0.5, 0.25 and 0.12 µg Ovalbumine/Phosphorylase. The concentration was determined as 0.5 µg/µl.

2.2. PURIFICATION OF THE SUMO E2 ENZYME UBC9

The sole SUMO E2 enzyme Ubc9 has a molecular mass of 18 kDa and was purified as untagged protein. It was cloned into the pET23a vector were its expression is driven by the T7 promotor. The plasmid was transformed into E. coli strain BL21 gold and induction was with 1 mM IPTG for 4 hours at 37°C. Because of its small size Ubc9 easily leaks out by lysing the bacteria in a freeze/thaw step and no further Lysozmye treatment is required. The Ubc9 containing supernatant was incubated with SP Sepharose beads (GE Healthcare) which are strong cation exchangers and efficiently bind to the positively charged Ubc9 by incubation for 1.5 hours at 4°C. After extensive washing steps, Ubc9 was eluted in 1ml fractions with high salt concentration (300 mM NaCl). Protein containing fractions, determined by spotting on a nitrocellulose

membrane and staining with Ponceau, were collected and pooled. Next, the pooled sample was applied to size exclusion chromatiography on a FPLC Äkta system. Ubc9 eluated in one clean peak as monitored via a protein density profile shown in Figure 7-A. Peak fractions were analysed on a 12.5% SDS PAGE and protein was stained with Coomassie brilliant blue (Figure 7-B). Fraktion 50 to 54 contained highly concentrated Ubc9 and were pooled before determining the protein concentration in comparison to defined concentrations of Trypsin Inhibitor (20.1 kDa) (Figure 7-C). The estimated concentration of Ubc9 was 2 µg/µl.

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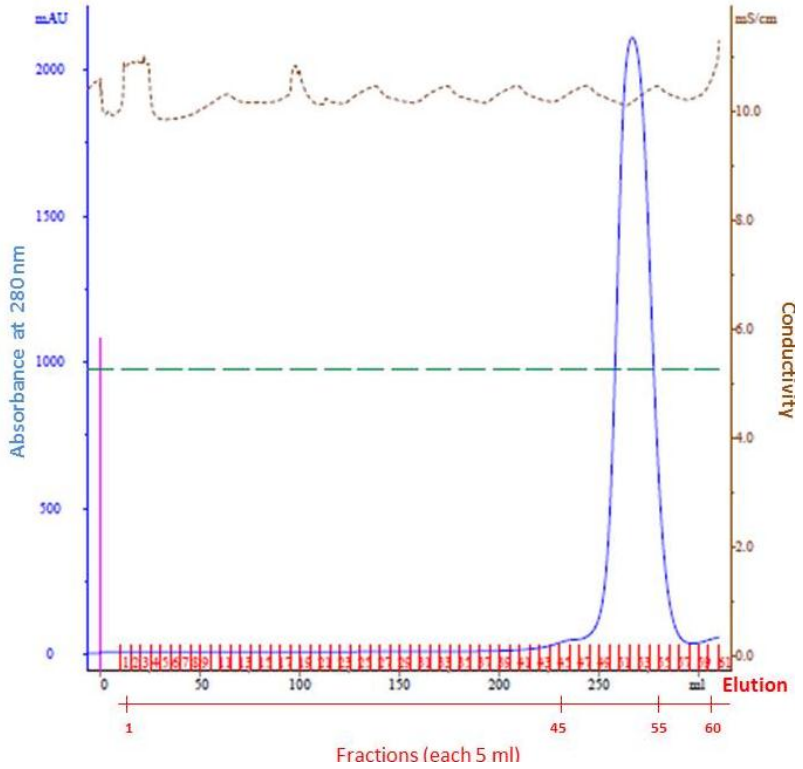

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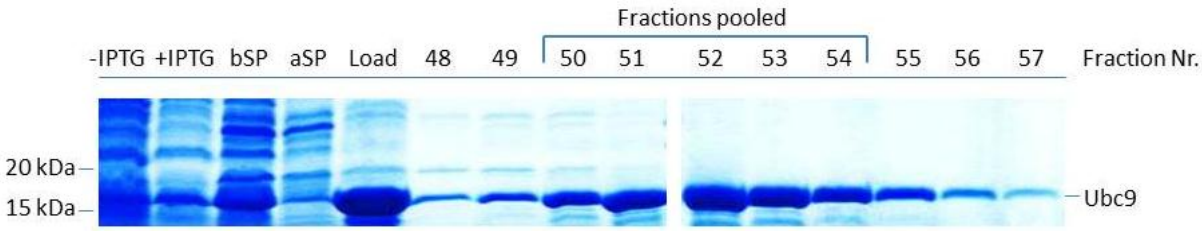

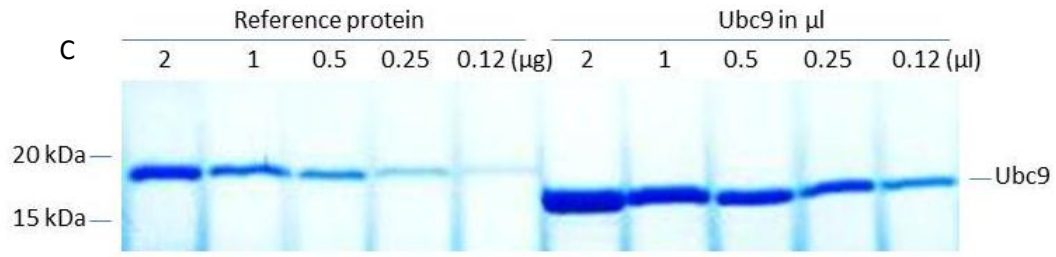

Figure 7 Purification of recombinant Ubc9. Ubc9 expression was induced by IPTG in BL21 gold. Purification involved binding to SP Sepharose and separation on size exclusion chromatography using an ÄKTA FPLC system. (A) Elution chromatogram indicated a single peak containing recombinant Ubc9. (B) Aliquotes of uninduced (-IPTG) and induced (+IPTG) bacteria cells, extracts before (bSP) and after (aSP) incubation with SP beades, sample injected to gelfiltration (Load) and eluted peak fractions from (A) were separated on 15% SDS PAGE and protein was stained with Coomassie brilliant blue. Fractions 50 to 54 were pooled and used for further usage. (C) Determination of the protein concentration by comparison to the reference protein Trypsin Inhibitor. Ubc9 concetration in 2, 1, 0.5, 0.25 and 0.12 µl was compared to 2, 1, 0.5, 0.25 and 0.12 µg of Trypsin Inhibitor. Concentration was determined as 2 µg/µl.

2.3. PURIFICATION OF SUMO1 PROTEIN

SUMO1 with a predicted size of 11kDa was cloned in its active form (SUMO1ΔC4) in pET11a under the control of the T7 promoter. IPTG induced expression in E.coli BL21 gold was at 37 °C for 4 hours. The cells were lysed by a freeze and thaw cycle followed by sonification. SUMO1 has a negative charge and can therefore be bound to the strong anion exchange matrix of Q Sepharose (GE Healthcare). The bound SUMO protein was eluted with high salt concentration (500 mM NaCl). Protein containing fractions were identified by spotting small aliquots of each fraction on a nitrocellulose membrane and staining with Ponceau.

The peak fractions were pooled and separated on a size exclusion chromatography using an ÄKTA FPLC system. Protein elution in 5 ml fractions was monitored via a protein density chromatogram as depicted in Figure 8-A. The elution profile shows three peaks of which the middle peak represents SUMO1 as it was determinded by earlier purifications. Fraction 33 to 38 of this peak were analysed on a 12.5% SDS-PAGE (Figure 8-B) followed by Coomassie brilliant blue staining. Importantly, in SDS-PAGE SUMO1 migrates at approximately 20 kDa because of its long flexible N-terminus. Fractions 33 to 37 containig highly concentrated SUMO1 were pooled and protein concentration was determined to 2 µg/µl by comparison to defined concentration of α-Lactalbumine (14.2 kDa) (Figure 8-C).

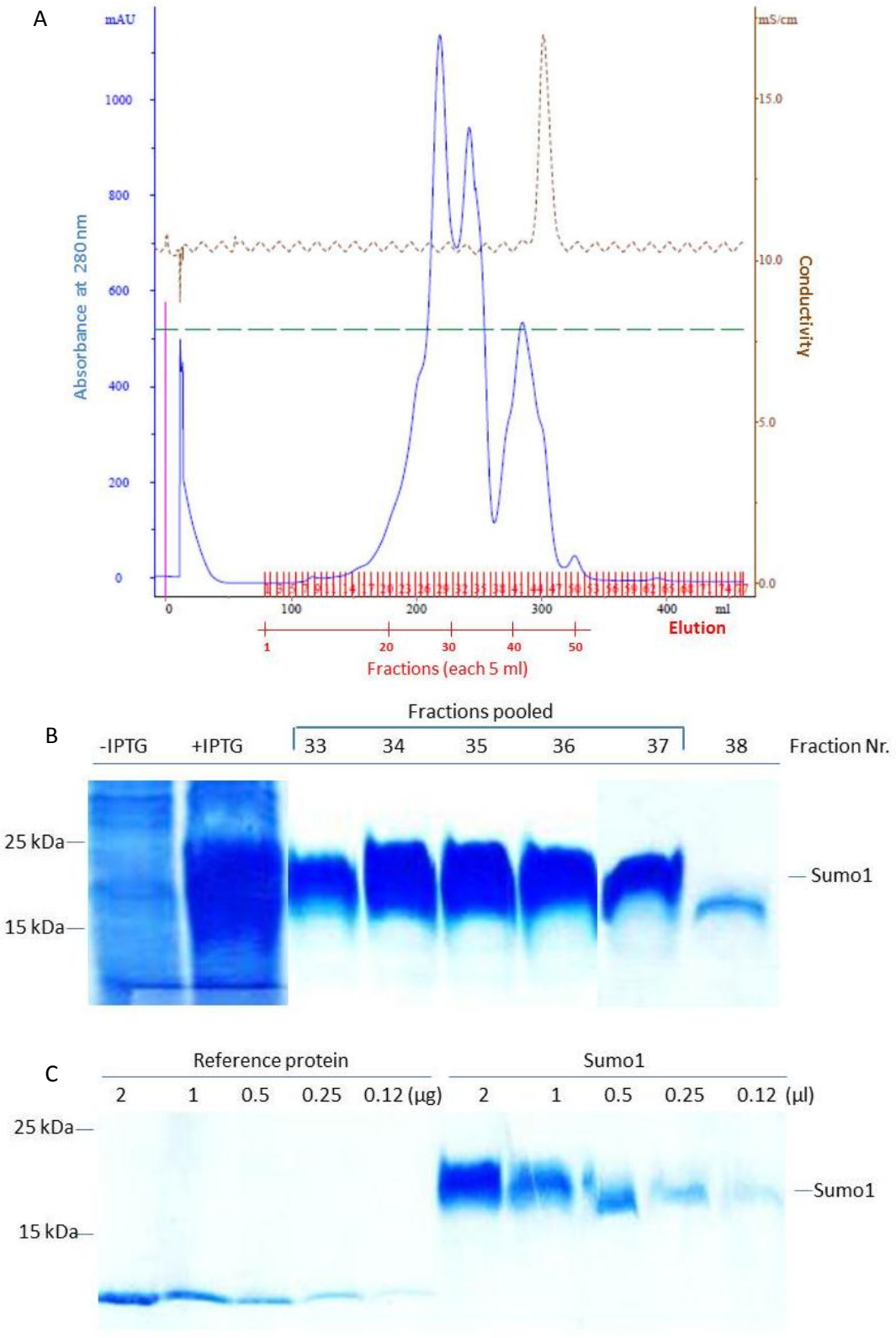

Figure 8 Purification of recombinant SUMO1. SUMO1 expression was IPTG induced in BL21 gold, enriched on Q sepharose beads prior separation by size exclusion chromatography using an ÄKTA FPLC system. (A) Elution chromatogram of SUMO1 on a S200 gelfiltration column. The x-axis represents the eluted volume in sample fractions (5ml) and the y-axis the protein concentration in mAU (milli absorbance unit) at 280nm. The salt concentration is indicated as conductivity in mS/cm. (B) Immunoblot analysis of uninduced (-IPTG) and induced (+IPTG) cells and eluted peak fractions from (A) were separated on a 12.5% SDS-PAGE. Protein was stained with Coomassie brilliant blue. Fractions 33 to 38 were pooled and for further usage. (C) Concentration determination of

purified SUMO1 with the reference protein α-Lactalbumine. SUMO1 protein content in 2, 1, 0.5, 0.25 and 0.12 µl was compared to 2, 1, 0.5, 0.25 and 0.12 µg α-Lactalbumine. Concentration was determined as 2 µg/µl.

2.4. UBC9 ACTIVITY IN COMPARISON TO AN EARLIER PREPARATION

Ubc9´s enzymatic activities can significantly differ between various preparations. Therfore the newly purified Ubc9 was compared in substrate modification to an earlier Ubc9 preparation in a concentration dependent manner (Figure 9). As substrate E2-25K was used, which is efficiently modified in the absence of an E3 ligase and was available in the Pichler laboratory.

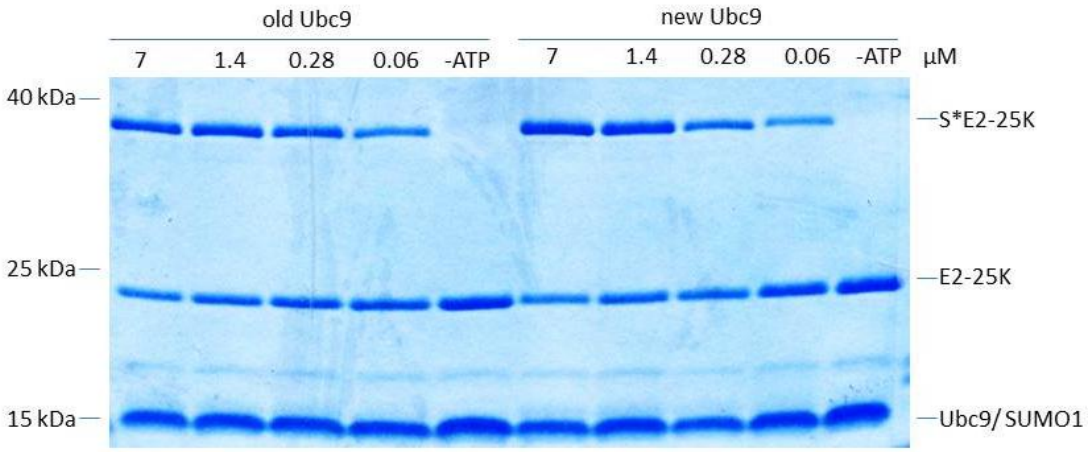

Figure 9 Comparison of two different Ubc9 preparations on substrate modification. In vitro sumoylation assay with 3.5 µg E2-25K, 4.4 µg SUMO1, 150 ng E1 and increasing concentrations of Ubc9 from two purifications was performed for 1 hour at 30 °C in the presence of ATP. Reactions were stopped by addition of sample buffer. Separation was on 5-20% SDS-PAGE and detection was by Coomassie staining.

The 20 µl in vitro sumoylation reaction contained 4 µg of SUMO1, 150 ng E1, 3.5 µg E2-25K and increasing concentrations (60 nM to 7 µM) of Ubc9 and ATP . E2-25K was efficiently sumoylated at all concentrations tested and showed comparable activity to the older preparation.

PURIFICATION OF SUBSTRATES

E3 independent in vitro sumoyltion assays significantly differ in substrates modification efficiency. Substrates like RanGAP1 or TDG require traces of Ubc9 for efficient modification. Most other substrates (e.g.DAXX, Sp100, p53 [42,45]) are only weakly modified even at high levels of Ubc9 and only get efficiently modified in the presence of an E3. To establish the conditions for an E3 independent in vitro sumoylation assay I purified various well known SUMO substates to compare their sumoylation efficiency at various Ubc9 concentrations.

2.5. PURIFICATION OF RANGAP1

RanGAP1 has a predicted size of 63 kDa and was cloned in the plasmid pET11d under the control of the T7 promoter. IPTG induced expression in E.coli BL21 gold was performed at 37 °C for 4 hours. The cells were lysed by a freeze and thaw cycle followed by subsequent Lysozyme treatment. Since RanGAP1 was expressed in inclusion bodies it was recovered from pellet fraction by denaturing conditions. The negatively charged RanGAP1 was incubated with Q Sepharose beads (GE Healthcare) and eluted in 1 ml fractions with RanGAP1 Elutionbuffer. Protein containing fractions were pooled, concentrated and applied to size exclusion chromatiography on a FPLC Äkta system. Protein elution was monitored via a protein density profile, a chromatogram which is shown in Figure 10-A. Peak fractions were analysed on a 7% SDS PAGE (Figure 10-B) and protein was stained with Coomassie brilliant blue. Fractions 21 to 30 of the second peak contained high amounts of RanGAP1 and were pooled for further usage. The protein concentration was determined by comparison of serial dilutions of RanGAP1 with definded concentrations of the reference protein BSA (66 kDa). Concentration was 0.7 µg/µl (Figure 10-C).

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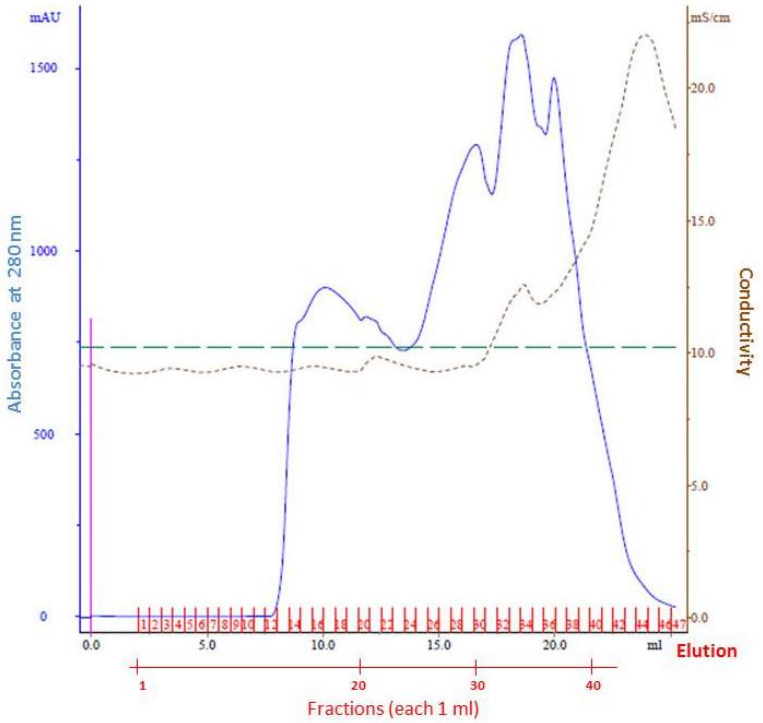

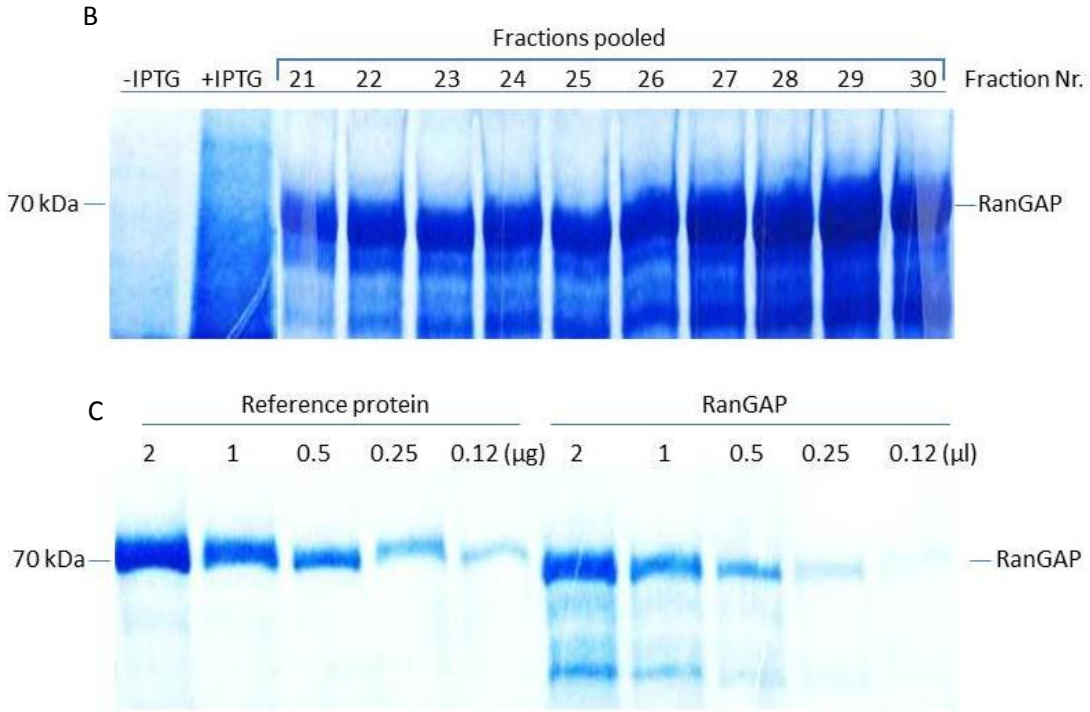

Figure 10 Purification of recombinant RanGAP1. RanGAP1 expression was IPTG induced in E.coli BL21 gold, denaturated in urea, dialysed to renaturate and enriched on Q Sepharose beads prior separation by size exclusion chromatography using an ÄKTA FPLC system. (A) Elution chromatogram of RanGAP1 on a S200 gelfiltration column. The x-axis represents the eluted volume in sample fractions (1 ml) and the y-axis the protein concentration in mAU (milli absorbance unit) at 280nm. The salt concentration is indicated as conductivity in mS/cm. (B) Immunoblot analyisis of uninduced (-IPTG) and induced (+IPTG) cells and eluted peak fractions from (A) were separated on a 7% SDS-PAGE. Protein was stained with Coomassie. Fractions 21 to 30 were pooled and stored for further usage. (C) Concentration determination of purified RanGAP1 with the reference protein BSA. A serial dilution of RanGAP1 (2, 1, 0.5, 0.25 and 0.12 µl) was compared to defined concentrations of BSA (2, 1, 0.5, 0.25 and 0.12 µg BSA). RanGAP1 concentration was determined as 0.7 µg/µl.

2.6. PURIFICATION OF GST-DAXX

DAXX, with a predicted size of 53 kDa, was cloned in the plasmid pGEX4T under the control of the T7 promoter and IPTG induced expression in E.coli BL21 gold was performed at 16 °C for 5 hours. The cells were lysed by a freeze and thaw cycle followed by subsequent Lysozyme treatment. GST-DAXX was enriched on GST-sepharose (GE Healthcare) and subsequently eluted with a Gluthatione containing GST-Elutionbuffer.

Protein containing fractions were pooled and applied to size exclusion chromatiography on a FPLC Äkta system (Figure 11-A). Fractions of the major peak were analysed on a 5-20% SDS-PAGE. GST-DAXX containing fractions 17 to 21 were pooled (Figure 11-B) and protein content was determined by serial dilutions of GST-DAXX compared to definded concentrations of the reference protein Ovalbumin (44.3 kDa). Concentration was estimated to 2 µg/µl (Figure 11-C).

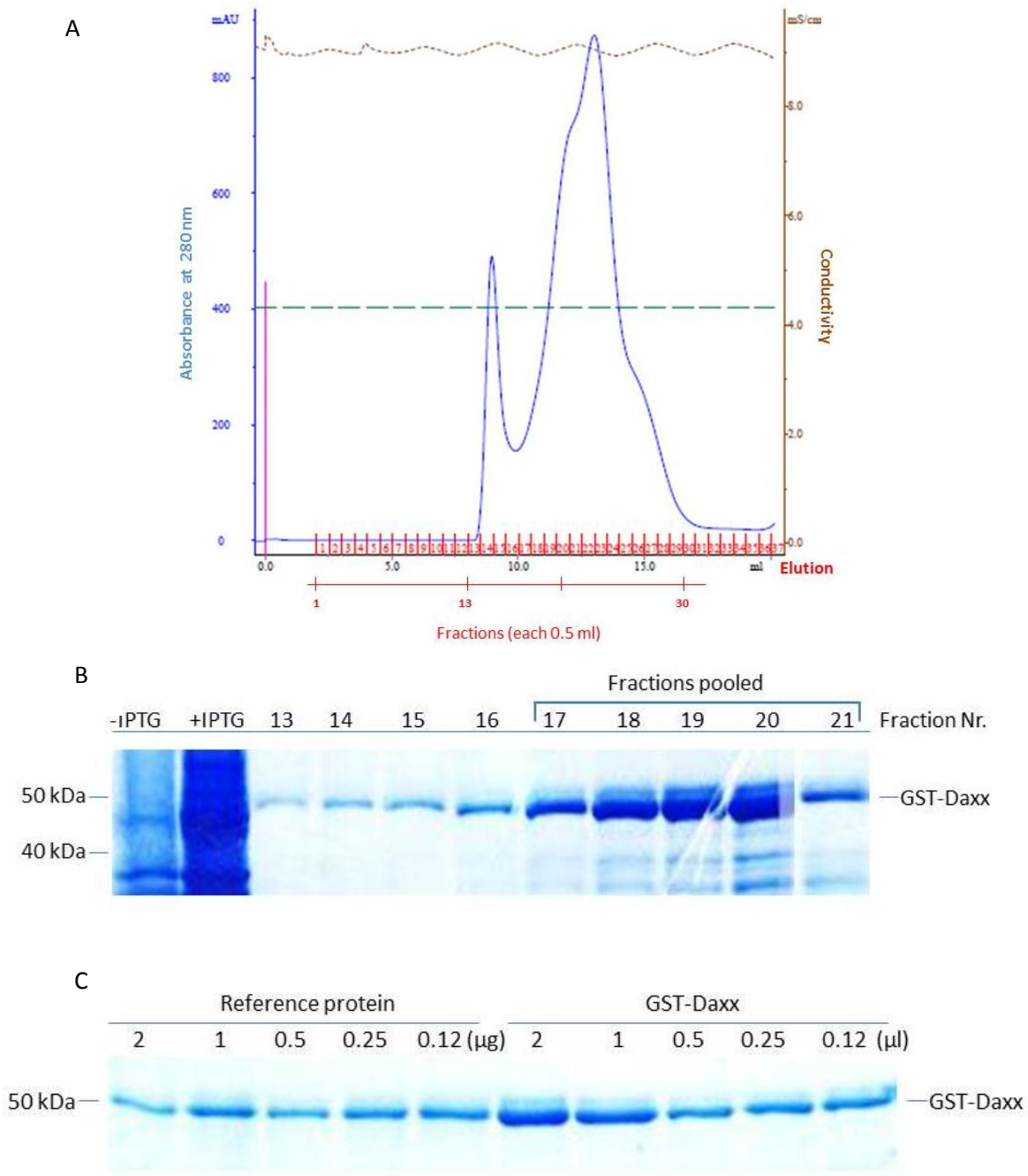

Figure 11 Purification of recombinant GST-DAXX. GST-DAXX expression was IPTG induced in E.coli BL21 gold, enriched on GST sepharose prior separation by size exclusion chromatography using an ÄKTA FPLC system. (A) Elution chromatogram of GST-DAXX on a S200 gelfiltration column. The x-axis represents the eluted volume in sample fractions (0.5 ml) and the y-axis the protein concentration in mAU (milli absorbance unit) at 280nm. The salt concentration is indicated as conductivity in mS/cm. (B) Immunoblot analyisis of uninduced (-IPTG) and induced (+IPTG) cells and eluted peak fractions from (A) were separated on a 5-20% SDS-PAGE. Protein was stained with Coomassie brilliant blue. Fractions 17 to 21 were pooled for further usage. (C) Concentration determination of purified GST-DAXX. Serial dilutions (2, 1, 0.5, 0.25 and 0.12 µl) of GST-DAXX protein were compared to 2, 1, 0.5, 0.25 and 0.12 µg Ovalbumin. Concentration was determined as 2 µg/µl.

2.7. PURIFICATION OF HIS-MYC-TDG

TDG, with a predicted size of 71 kDa, was cloned in the plasmid pET30 (His myc) under the control of the T7 promoter. IPTG induced expression in E.coli BL21 gold was performed at 16 °C for 5 hours. The cells were lysed by a freeze and thaw cycle followed by subsequent Lysozyme treatment. His-myc-TDG was enriched on Ni2+-beads (Invitrogen) which bind the His- tag in TDG. The bound His-myc-TDG protein was eluted with imidazole containing His-Elutionbuffer and protein containing fractions were pooled and applied to size exclusion chromatiography on a FPLC Äkta system (Figure 12-A). Factions of the first peaks were analysed on a 5-20% SDS-PAGE (Figure 12-B). Fractions 7-10 and 11-13 were pooled seperatly. Protein concentration (pooled fraction 11-13) was determined by comparison of serial dilution of His-myc-TDG to defined concentrations of the reference protein Phosphorylase (97.2 kDa). Concentration was estimated to 2 µg/µl (Figure 12-C).

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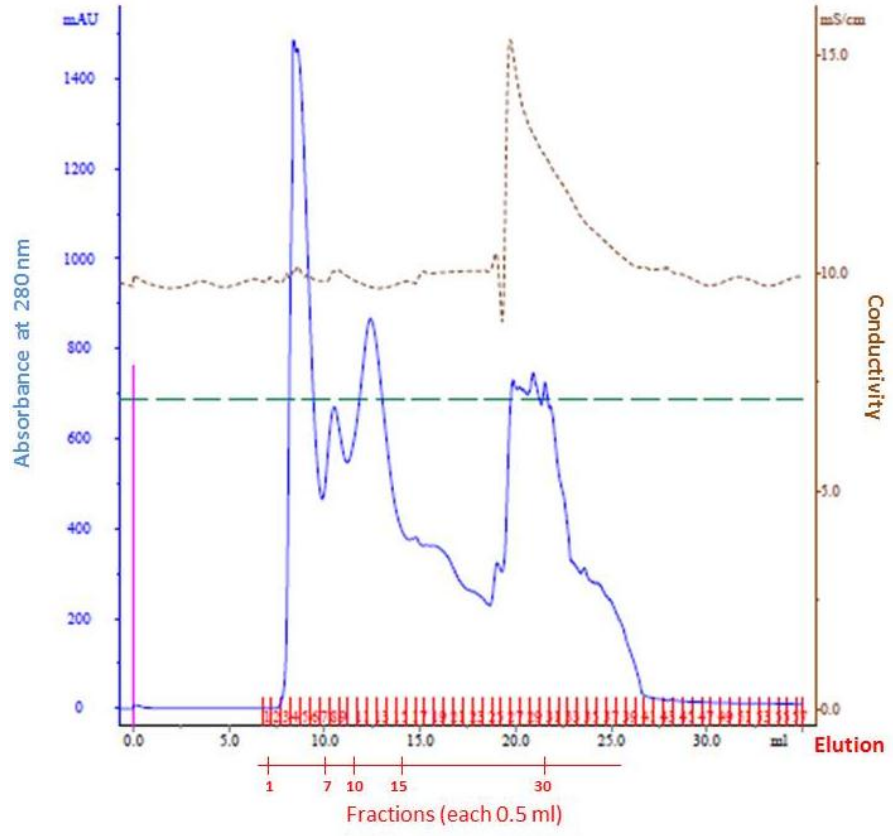

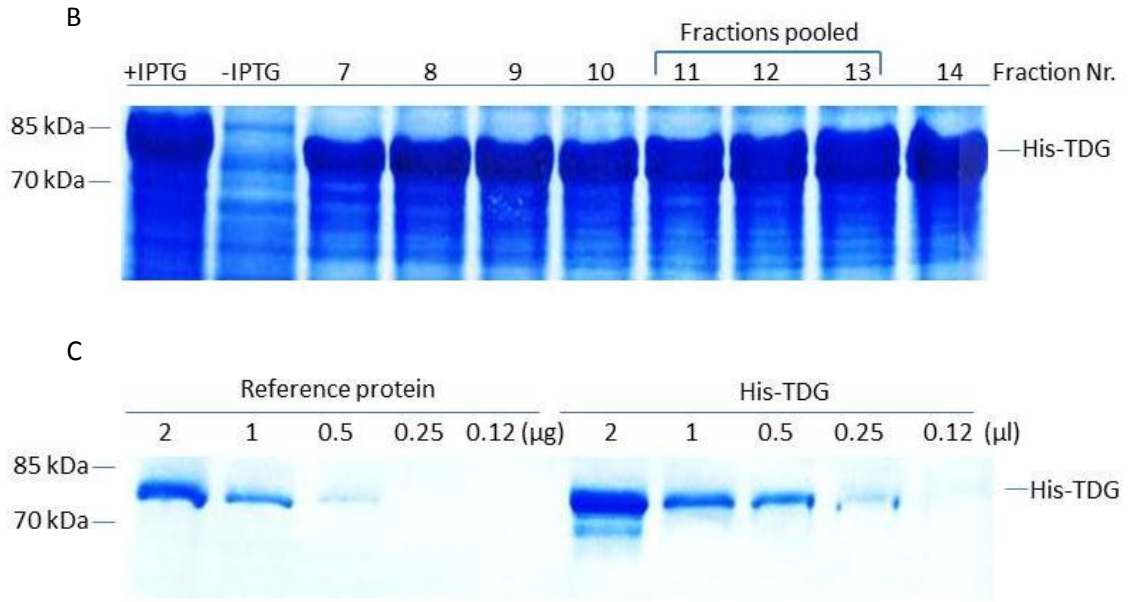

Figure 12 Purification of recombinant His-myc-TDG. His-myc-TDG expression was IPTG induced in E.coli BL21 gold, enriched on Ni2+ beads prior separation by size exclusion chromatography using an ÄKTA FPLC system. (A) Elution chromatogram of His-myc-TDG on a S200 gelfiltration column. The x-axis represents the eluted volume in sample fractions (0.5 ml) and the y-axis the protein concentration in mAU (milli absorbance unit) at 280nm. The salt concentration is indicated as conductivity in mS/cm. (B) Immunoblot analyisis of uninduced (-IPTG) and induced (+IPTG) cells and eluted peak fractions from (A) were separated on a 5-20% SDS-PAGE. Protein was stained with Coomassie brilliant blue. Fractions 11 to 13 were pooled for further usage. (C) Concentration determination of purified His-myc-TDG with the reference protein Phosphorylase. Concentration was determined as 2 µg/µl.

3. IN-VITRO SUMOYLATION ASSAYS OF DIFFERENT SUMO SUBSTRATES

Different in-vitro sumoylation assays with known SUMO substrates were performed to identify optimal conditions for the ProtoArray and to verify the activity of the purified SUMO enzymes.

3.1. RANGAP1

RanGAP1, the Ran GTPase activating protein, was the first mammalian SUMO substrate identified. It was shown to be very efficiently sumoylated in the absence of an E3 Ligase [3,4] and it only requires little amounts of the E2 enzyme Ubc9 for efficient RanGAP1 sumoylation [39].

2.2 µM RanGAP1 was modified in the presence of 4 µM HA-SUMO, 100 nM E1 and decreasing concentrations (50 nM to 0.4 nM) of Ubc9 or S*Ubc9 at 30 °C for 30 minutes. The reaction was stopped with Leammli buffer and samples were resolved on 5-20% SDS PAGE followed by immunoblotting was with HA (Figure 13-A) and RanGAP1 (Figure 13-B) antibodies. Whereas the HA antibody only recognizes the SUMO modified RanGAP1 species the RanGAP1 antibody detects both, modified and unmodified RanGAP1. Under these conditions, RanGAP1 was sumoylated at already the lowest Ubc9 concentration (0.4 nM).

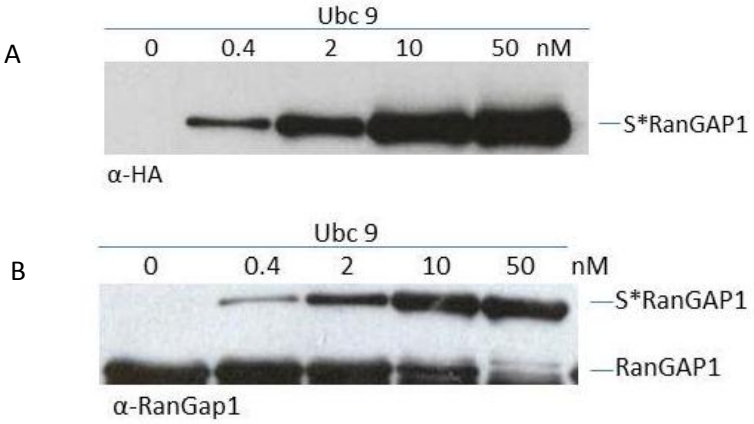

Figure 13 In vitro sumoyation of RanGAP1. RanGAP1 (2.2 µM) was sumoylated in the the presence of 4 µM HA-SUMO1, 100 nM E1 and indicated concentrations of of Ubc9 at 30 °C for 30 minutes. Reaction was stopped with Laemmli buffer and samples were separated on 5-20% SDS PAGE (A) Immunostaining with HA antibodies (1:1500). (B) Immunostaining with RanGAP1 antibodies (1:5000).

3.2. E2-25K

Next, E2-25K, a ubiquitin E2 conjugating enzyme was investigated. It is another known SUMO substrates which gets modified without the help of an E3 ligase in vitro [10].

2.2 µM E2-25K were incubated with 4 µM HA-SUMO1, 100 nM E1 and decreasing concentrations of Ubc9 (500 to 20 nM). The reaction was stopped with Leammli buffer and samples were separated on 5-20% SDS PAGE followed by immunoblotting with anti-HA antibodies as shown in Figure 14-A and with anti-E2-25K antibodies as shown in Figure 14-B. Compared to RanGAP1, E2-25K sumoylation depended on much higher Ubc9 concentrations for efficient modification.

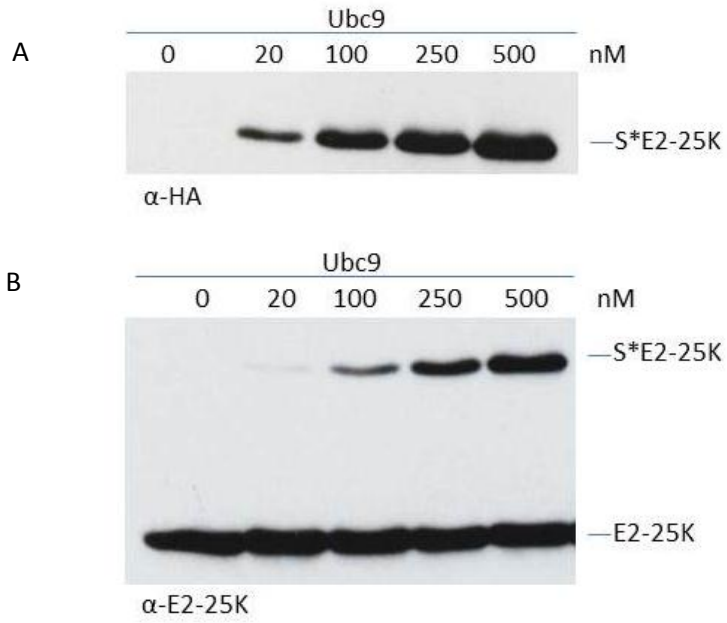

Figure 14 In vitro sumoylation of E2-25K. E2-25K (2.2 µM) was sumoylated in the presence of 4 µM HA-SUMO1, 100 nM E1 and indicated concentrations of Ubc9 at 30 °C for 60 minutes. (A) Immunostaining with HA antibodies (1:1500). (B) Immunostaining with E2-25K antibodies (1:2000).

3.3. GST-DAXX

Daxx is a SUMO substrate which depends on an E3 ligase for efficient SUMO modification.

625 nM of Gst-Daxx were incubated with 4 µM HA-SUMO1, 100 nM E1 and decreasing concentrations of Ubc9 (1000 to 100 nM). Reaction was stopped with Leammli buffer and samples were separated on 5-20% SDS PAGE followed by detection with anti-HA antibodies (Figure 15-A) and anti-GST antibodies (Figure 15-B). Although extremely high concentrations of Ubc9 were used only traces of Gst-Daxx was sumoylated suggesting the requirement of an E3 ligase for efficient modification.

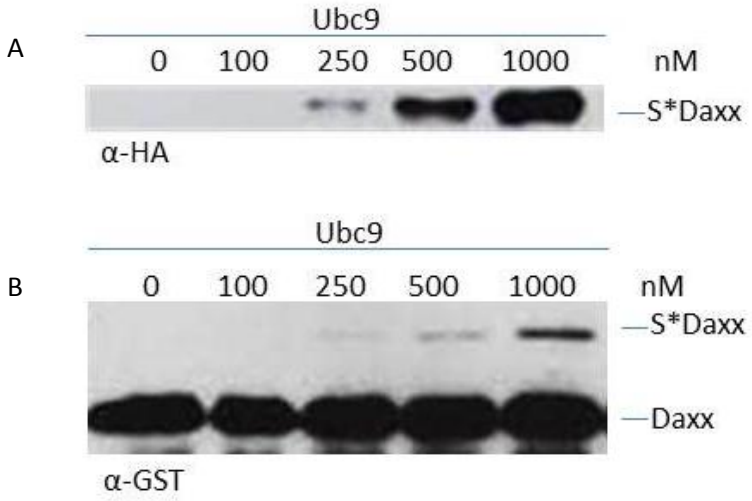

Figure 15 In vitro sumoylation of GST-Daxx. GST-Daxx (625 nM) was sumoylated in the presence of 4 µM HA-SUMO1, 100 nM E1 and with indicated concentrations of Ubc9 at 30 °C for 60 minutes. (A) Immunostaining with HA antibodies (1:1500). (B) Immunostaining with GST antibodies (1:7000).

3.4. HIS-MYC-TDG

The SUMO substrate TDG was modified in the absence of an E3 ligase in vitro.

357 nM of His-myc-TDG were incubated with 4 µM HA-SUMO1, 100 nM E1 and decreasing concentrations of Ubc9 (500 to 20 nM). The reaction was stopped with Leammli buffer and samples were separated on 5-20% SDS PAGE. Signals were detected with anti-HA antibodies (Figure 16-A) and anti-Myc antibodies (Figure 16-B).

As shown in Figure 16-B already 100 nM Ubc9 converted more than half of His Myc-TDG to its sumoylated form and at 250 nM Ubc9 nearly 100% TDG sumyolation was achieved.

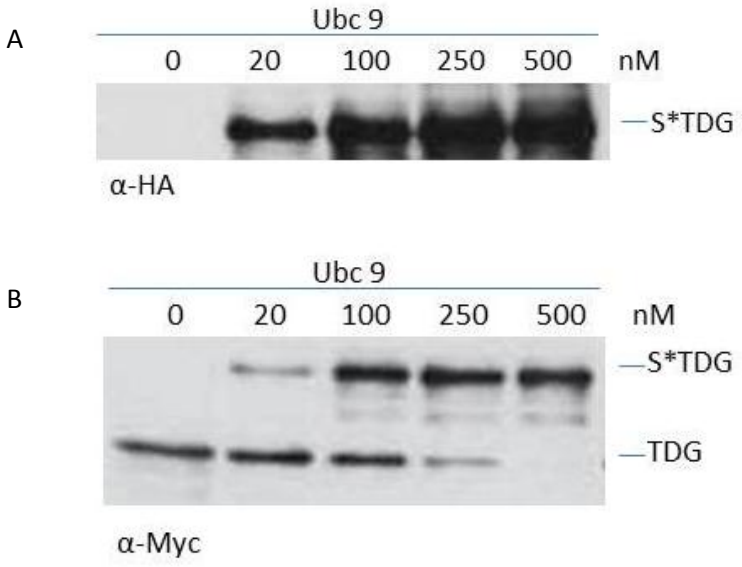

Figure 16 In vitro sumoylation of His myc TDG. His-myc-TDG (357 nM) was sumoylated in the presence of 4 µM HA-SUMO1, 100 nM E1 with indicated concentrations of Ubc9 at 30 °C for 60 minutes. (A) Immunostaining with HA antibodies (1:1500). (B) Immunostaining with Myc antibodies (1:10000).

The analysis of several known SUMO substrates in this study but also in former studies indicated that only a few substrates are efficiently modied in the presence of 100 nM Ubc9 (RanGAP1, TDG, E2-25K) whereas most other substrates depend on either higher E2 concentrations or the presence of an E3 ligase to obtain detectable modification (Sp100, DAXX, PML, HDAC4 [42]).
Therefore the concentrations of 100 nM Ubc9, 100 nM E1 and an excess of 4 µM HA-SUMO1 were chosen as the optimal condition for performing the SUMO in-vitro reaction on the ProtoArray™ to detect putative E3 independent SUMO substrates.

4. PROTOARRAY

To gain novel insights into E3 independent substrate sumoylation it is of great interest to identify additional substrates which are modified in an E1/E2 dependent manner. Performing an in vitro sumoylation on a human protein microarray (ProtoArray) offers a novel and rapid tool to identify such substrates: purified enzymes are applied on the arrays and candidate substrates can be quickly identified [120]. Thousands of human proteins are spotted in duplicates on the array. Multiple gene families are represented such as kinases, membrane-associated proteins, cell-signalling proteins, metabolic proteins and many more.

For my project the ProtoArray Human Protein Microarrays v5.0 was used. The human protein collections on the protoarray are human clones which were obtained either from the Invitrogen Ultimate™ ORF (open reading frame) collection or from a Gateway® collection of kinase clones (by Protometrix). Each clone's nucleotide sequence was verified by full length sequencing. All clones were then transferred into a system for expressing recombinant proteins in insect cells via baculovirus infection. Proteins are expressed at high levels in insect cells which are related to mammalian cells with respect to protein folding and post-translational modifications such as phosphorylation and glycosylation. This is a great adventage compared to proteins expressed in E.coli.

An in-vitro sumoylation reaction was performed on such an array and upon extensive washing steps HA-SUMO1 modified proteins were detected with mouse anti-HA antibodies (Figure 17). Using a fluorescent (Alexa Fluor 647) conjugated secondary rabbit anti-mouse antibody allowed the readout in a fluorescent microplate reader.

The enzyme concentration dependent increase in substrate modification is a hallmark of an enzymatic reaction and therefore two different Ubc9 concentrations were used instead of reactions in duplets. Since no reference assays comparing in vitro sumoylation "in solution" to such assays "on slides" were available, we decided to use 100 nM Ubc9 as estimated (see above) but also a five times higher concentration Ubc9 (500 nM) in case the assay is less sensitive. The assay buffer and the concentrations of HA-SUMO1 (4 µM), the E1 (100 nM) and ATP remained unchanged to the "in solution" sumoylation assays. As control we used a reaction lacking Ubc9 to confirm E2 dependence and also to distinguish proteins that are covalently modified with SUMO from putative SUMO binding proteins. As an additional negative control the detection reagent alone was used to exclude unspecific background signals. The reactions performed are summarized in Table1.

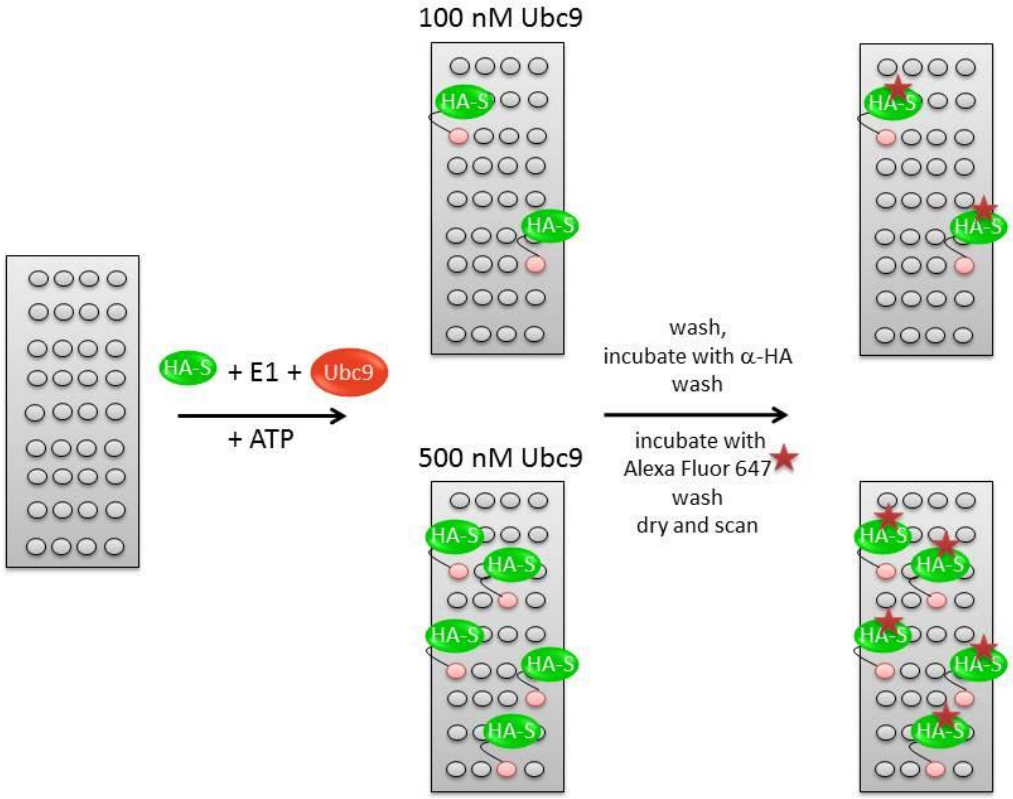

Figure 17 Model of the ProtoArray™ for the Ubc9 assays. The ProtoArray™ which contained more than 9000 recombinant human proteins spotted in duplicates on glass slides was used for performing an in-vitro sumoylation reaction with HA-SUMO1(4 µM), E1 (100 nM) and two different Ubc9 concentrations (100 nM and 500 nM). After Incubation for 1 hour at 30 °C, slides were extensively washed and incubated with anti-HA antibodies for 90 minutes at 4 °C. Follwing additional washing steps the glass sides wereincubated with a secondary Alexa Fluor 647 labeled anti-mouse antibodies for 90 minutes at 4 °C .Before drying and scanning addional washing steps were included.

Table 1 Assay conditions for the different assays on the ProtoArray

Assay	E1	Ubc9	HA-SUMO1
Negative control	-	-	-
E1 control	100 nM	-	4 µM
100 nM Ubc9	100 nM	100 nM	4 µM
500 nM Ubc9	100 nM	500 nM	4 µM

A small percentage (less than 0.5%) of the proteins exhibited significant signals in the negative control (detection reagent only) assay due to interaction with the detection reagents and were therefore eliminated from the analysis.

Maximal signals were observed at >65 000 Relative Fluorescence Units (RFU) on all arrays probed with the sumoylation enzymes. Scanner settings were selected such that the saturated signals were observed for a small number of protein spots on the array probed with the highest concentration of E2. The maximum average background signal was 232 RFU and this was measured on the array which was probed with 500 nM Ubc9. The values indicate good-signal-to-background levels from the samples.

The average background values were calculated from local background pixel intensity values measured by the program GenePix 6.0 for all protein features on the array and reported in relative fluorescence units.

Out of 9000 proteins spotted on the array approximately 800 proteins showed a positive signal in an E2 dependent manner. Many of these proteins are known SUMO substrates which confirmes the quality of this assay. We also detected several novel SUMO substrates which will be of interest for further analysis in the Pichler laboratory. From this assay alone we are not able to conclude if the identified substrates are modified in an E3 independent manner although the difference in signal intensities may indicate if a substrate rather depends on an E3 ligase or not. Unexpectedly, two known SUMO substrates, Mef2A and Hsf1 were identified with nearly maximum scores at 100 nM Ubc9 and with saturated maximal scores with 500 nM Ubc9. Both substrates have a PSDM and were described to depend on phosphorylation prior their sumoylation [58,59]. This may indicate that some proteins kept posttranslation modifications from their expression in insect cells.

I also analysed the proteins I have tested earlier for sumoylation efficiency. We could not draw any conclusions for Daxx since it was not presented on the screen and RanGAP1 showed unspecific signals in the E1 dependent control reactions. Sp100 which is only efficiently sumoylated in the presence of an E3 ligase or the sumoylated Ubc9 in “in solution” sumoylation assays [42], was obtained with maximal scores at both Ubc9 concentrations. TDG, on the other side, was very efficiently modified “in solution” but showed rather low signals “on both slides”. This could indicate that the proteins purified in E.coli used in our “in solution” assays are not proberly folded or lack modifications important for sumoylation.

To learn more about these differences I aimed to investige the two phosphorylation dependent SUMO substrates Mef2A and Hsf1 in “in solution” sumoylation assays.

4.1. PURIFICATION OF SUBSTRATES IDENTIFIED IN THE PROTOARRAY

Two candidate substrates, Hsf1 and Mef2A, have been chosen to be cloned into bacterial expression vectors and purified in E.coli. The purified proteins were then tested in “in solution” sumoylation assays with different Ubc9 concentrations.

4.1.1. HSF1

A heat shock response is characterized by synthesis of heat shock proteins, which are molecular chaperons. The transcription of heat shock proteins is regulated by a family of heat shock transcription factors (HSF) which bind to the heat shock element (HSE) in the promoter regions of heat shock genes and thereby activating their expression. The phosphorylation of Hsf1 is strongly induced during heat shock. When Hsf1 is activated, it trimerizes, binds to DNA and localizes in nuclear stress granules. In these granules Hsf1 undergoes multiple marked phosphorylations. This is known to correlate with its transcription activity. Hsf1 contains three SUMO consensus sites and is known to be sumoylated at Lysine 298 which is located in the regulatory domain of Hsf1. This site is adjacent to several critical phosphorylation sites. It was shown by the Sistonen laboratory that Hsf1 S303 phosphorylation is critical for its sumoylation [58].

As shown in Figure 18 Hsf1 was identified as high score substrate in an E2 concentration dependent sumoylation reaction which implicates it as an E3 independent SUMO substrate. To analyse Hsf1 in solution sumoylation assays it needed to be recloned, expressed and purified.

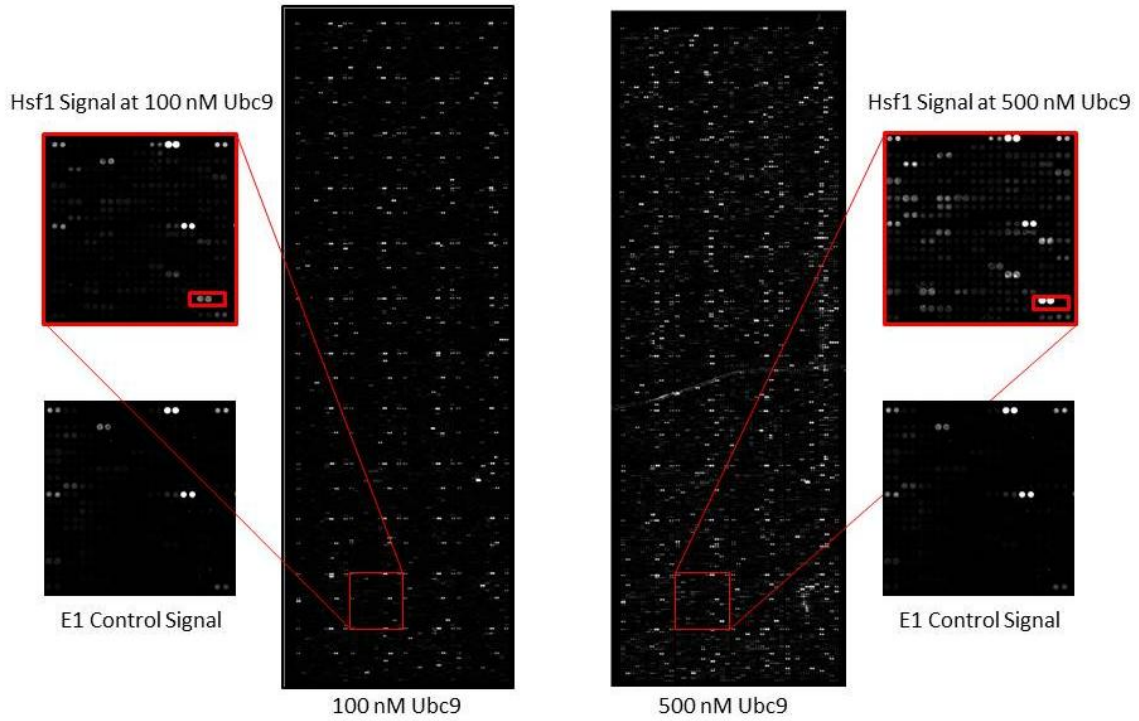

Figure 18 Hsf1 Signal on the two Ubc9 ProtoArrays. Detection signal of Hsf1 on the ProtoArray incubated with the reaction mixture with 100 nM Ubc9 and with 500 nM Ubc9 in comparison to the E1 control assays.

We obtained Hsf1 in the mammalian expression vector pcDNA3.1A and I have recloned it into the bacterial expression vector pET23a by using EcoRI and HindIII restriction sites. The correct sequence was verified by DNA sequencing.

4.1.1.1. PURIFICATION OF HIS-HSF1

Hsf1 was cloned as a His fusion in the plasmid pET23a (His) under the control of the T7 promoter and has a predicted size of 79 kDa. IPTG induced expression in E.coli BL21 gold was performed at 16 °C for 6 hours. The cells were lysed by a freeze and thaw cycle followed by subsequent Lysozyme treatment. His-Hsf1 was enriched on Ni2+-beads (Invitrogen) which bind to the His tag in Hsf1.

The bound His-Hsf1 protein was eluted with imidazole containing His-Elutionbuffer and protein containing fractions were applied to size exclusion chromatiography (S200 column) on a FPLC Äkta system (Figure 19-A). Peak fractions were analysed on a 10 % SDS PAGE (Figure 19-B) and fractions 12 to 16 were pooled. His-Hsf1 concentrations were determined by serial dilutions in comparison to defined concentration of the reference protein BSA (66 kDa) on a 5-20% SDS-PAGE (Figure 19-C). The concentration of His-Hsf1 was estimated to 2 µg/µl.

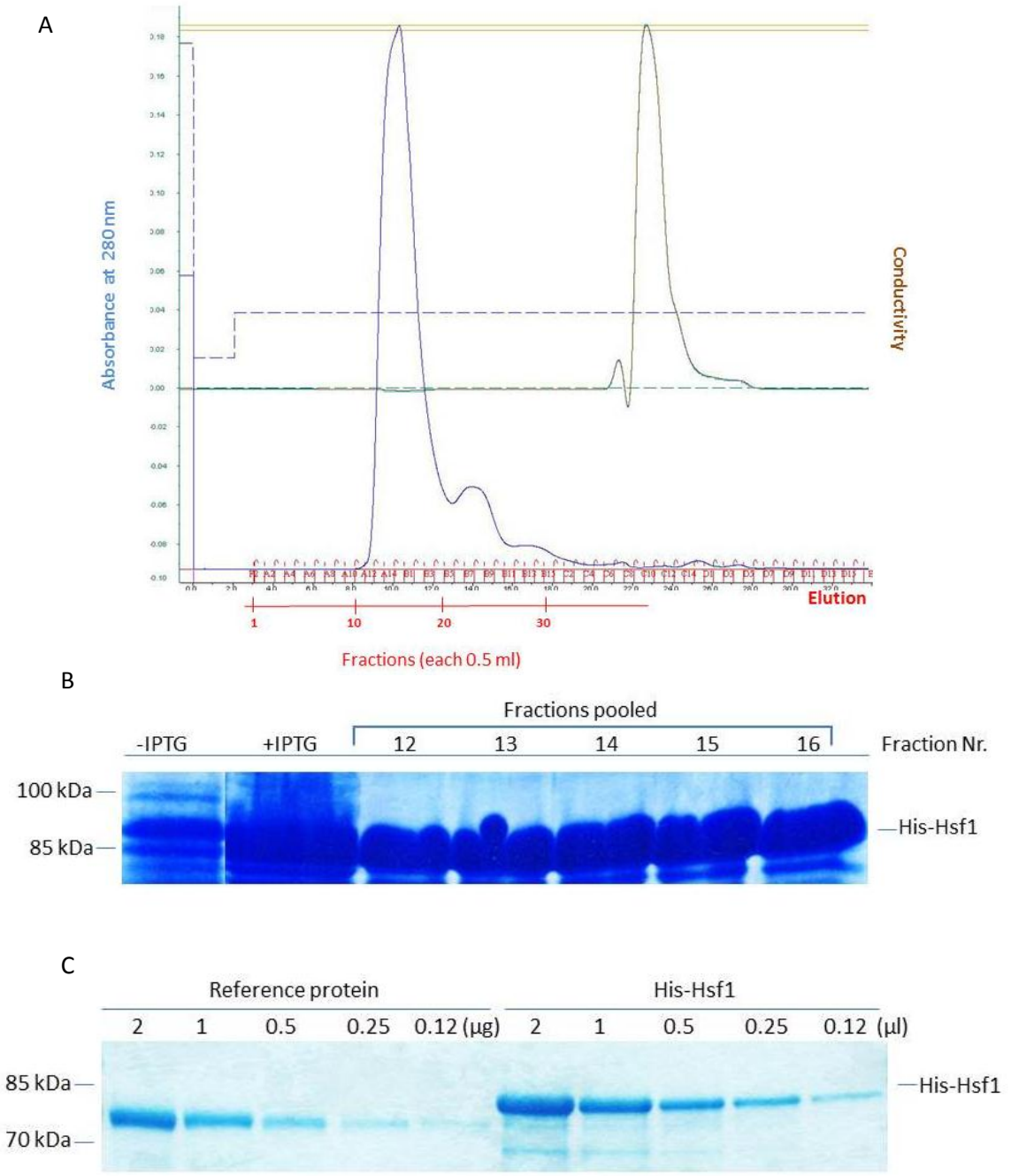

Figure 19 Purification of recombinant His-Hsf1. His-Hsf1 expression was IPTG induced in E.coli BL21 gold, enriched on Ni2+ beads prior separation by size exclusion chromatography using an ÄKTA FPLC system. (A) Elution chromatogram of His-Hsf1 on a S200 gelfiltration. The x-axis represents the eluted volume in sample fractions (0.5 ml) and the y-axis the protein concentration in mAU (milli absorbance unit) at 280nm. The salt concentration is indicated as conductivity in mS/cm. (B) Immunoblot analyisis of uninduced (-IPTG) and induced (+IPTG) cells and eluted peak fractions from (A) were separated by 10% SDS-PAGE. Protein was stained with Coomassie brilliant blue. Fractions 12 to 16 were pooled and stored for further usage. (C) Protein concentration was determination by comparison of different His-Hsf1 dilution to defined concentratios of the reference protein BSA on a 5-20% SDS-PAGE. Concentration was determined to 2 µg/µl.

4.1.1.2. IN SOLUTION SUMOYLATION ASSAY WITH HIS-HSF1

In-vitro SUMO assays with purified His-Hsf1 was performed at the same conditions as used on the ProtoArray™ which included 4 µM HA-SUMO1, 100 nM E1 and varying concentrations of Ubc9 from 100 nM to 1 µM. Assays were prepared in 20 µl reaction and incubated at 30 °C for one hour. The reactions were stopped with 2x SDS-Laemmli buffer and resolved via 5-20% SDS-PAGE followed by immunoblotting with anti-HA (Figure 20-A) and anti-His (Figure 20-B) antibodies.

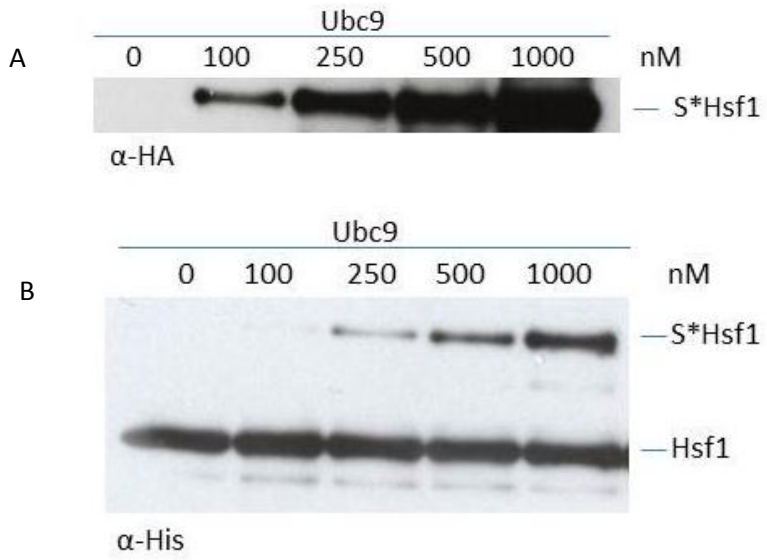

Figure 20 In vitro sumoylation of His. Hsf1.His-Hsf1 (333 nM) was sumoylated in the presence of 4 µM HA-SUMO1, 100 nM E1 and indicated concentrations of Ubc9 at 30°C for 60 minutes. (A) Immunostaining with HA antibodies (1:1500). (B) Immunostaining with His antibodies (1:1000).

The HA immunoblot indicates sumoylation of Hsf1 already at 100 nM Ubc9. Hsf1 modified with SUMO at 100 nM Ubc9 could not be detected with the less sensitive His-antibody (Figure 20-B).

Hsf1 contains a PDSM and was shown to get sumoylated at Lysine 298 after phosphorylation at Serine 303. The research group of Lea Sistonen observed that a S303 phosphorylation deficient mutant could not be sumoylated whereas a mutant which mimics S303 phosphorylation significantly promotes Hsf1 sumoylation in vivo [58]. This indicates that Serine 303 phosphorylation is required for efficient K298 sumoylation in vivo.

However the His-Hsf1 I have purified and used for my in-vitro assays is not phosphorylated because it was purified from bacteria. Therefore it is expected that Hsf1 sumoylation is even more enhanced if Hsf1 would be prior phosphorylated.

4.1.2. MEF2A

Myocyte-enhancement factor 2 (MEF2) is a transcription factor and another substrate for phosphorylation dependent SUMO modification [121]. It is expressed in the mammalian brain during the synaptogenesis. When Mef2 is sumoylated, it promotes in postsynaptic dendritic differentiation. SUMO modification in Mef2A occurs primarily on a lysine residue that is part of the PDSM [60].

Mef2A was characterized as a potential E3 independent substrate on the ProtoArray™ by exhibiting high signals at both concentrations of Ubc9 (Figure 21).

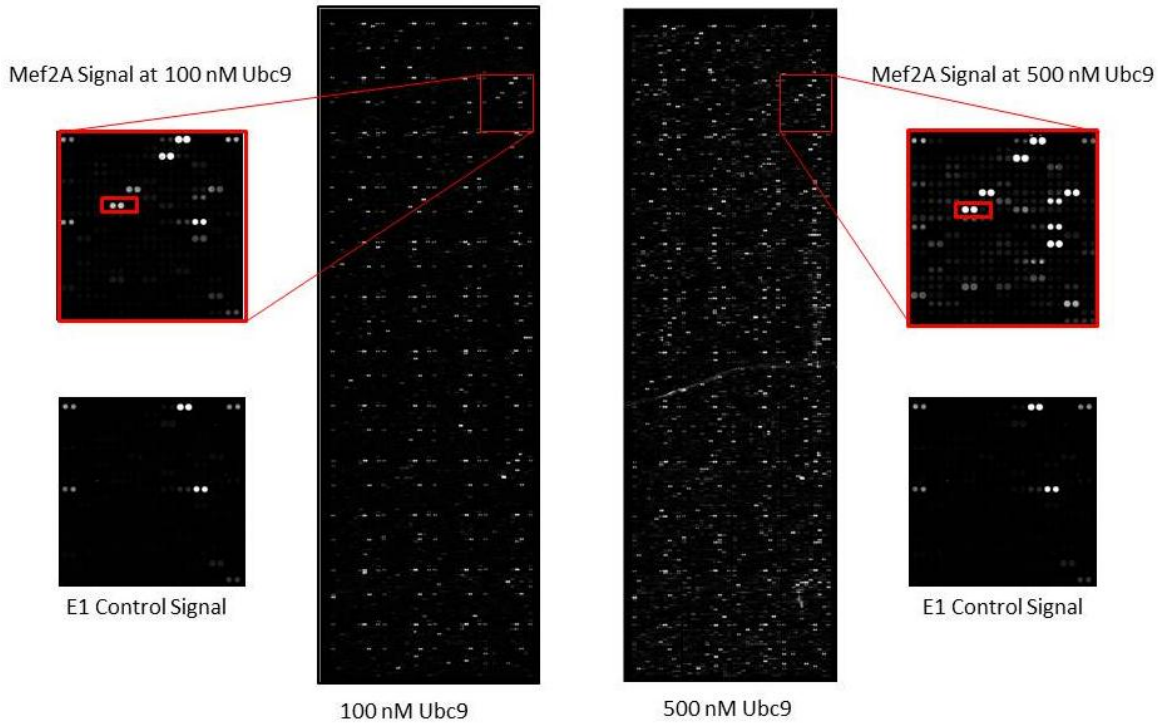

Figure 21 Mef2A Signal on the two Ubc9 ProtoArrays. Detection signal of Mef2A on the ProtoArray incubated with the reaction mixture with 100 nM Ubc9 and with 500 nM Ubc9 in comparison to the E1 control assay.

Mef2A was cloned from the pcDNA3.1A mammalian expression vector by amplifying with primes containing EcoRI and XhoI restriction sites. The amplicon from the PCR was digested with these restriction enzymes and ligated into the bacterial expression vector pGEX6p.1 which encodes a GST tag.

4.1.2.1. PURIFICATION OF GST-MEF2A

Mef2A was cloned in the plasmid pGEX 6p.1 (GST) under the control of the T7 promoter and with a predicted size of 75 kDa IPTG induced expression in E.coli BL21 gold was performed at 16 °C for 6 hours. The cells were lysed by a freeze and thaw cycle followed by subsequent

Lysozyme treatment. Upon enrichment on gluthatione Sepharose (GE Healthcare) GST-Mef2A was eluted with Gluthatione containing GST-Elutionbuffer.

Protein containing fractions were pooled and applied to size exclusion chromatiography (S200) on a FPLC Äkta system (Figure 22-A). Peak fractions were analysed on a 10% SDS-PAGE (Figure 22-B) and fractions 17 to 20 from the major peak were collected. Protein content was determined by serial dilutions of the reference protein BSA (66 kDa) and GST-Mef2A on a 5-20% SDS-PAGE (Figure 22-C). The concentration of GST-Mef2A was estimated to 0.125 µg/µl.

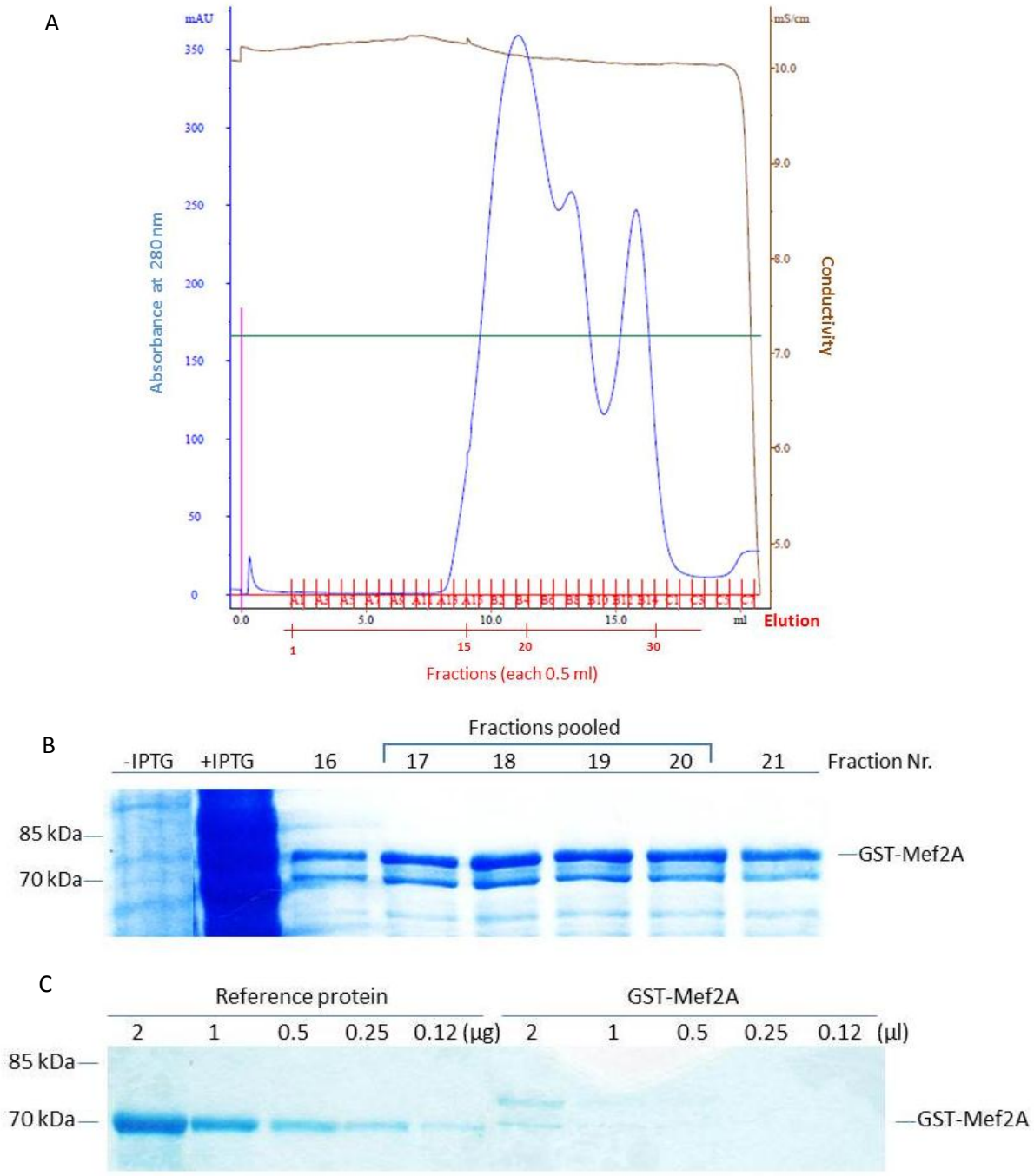

Figure 22 Purification of recombinant GST-Mef2A. GST-Mef2A expression was IPTG induced in BL21 gold, dialysed, enriched on GST beads prior separation by size exclusion chromatography using an ÄKTA FPLC system. (A) Elution chromatogram of GST-Mef2A on a

S200 gelfiltration. The x-axis represents the eluted volume in sample fractions (0.5 ml) and the y-axis the protein concentration in mAU (milli absorbance unit) at 280nm. The salt concentration is indicated as conductivity in mS/cm. (B) Immunoblot analyisis of uninduced (-IPTG) and induced (+IPTG) cells and eluted peak fractions from (A) were separated by 10% SDS-PAGE. Protein was stained with Coomassie brilliant blue. Fractions 17 to 21 were pooled and stored for further usage. (C) Concentration determination of purified GST-Mef2A with the reference protein BSA on a 5-20% SDS-PAGE. GST-Mef2A1 protein content in 2, 1, 0.5, 0.25 and 0.12 µl was compared to 2, 1, 0.5, 0.25 and 0.12 µg BSA. Concentration was determined as 0.125 µg/µl.

4.1.2.2. IN SOLUTION SUMOYLATION ASSAY WITH GST-MEF2A

In-vitro SUMO assays with the purified GST-Mef2A was performed at the same conditions as the ProtoArray™ which included 4 µM HA-SUMO1, 100 nM E1 and varying concentrations of Ubc9 from 100 nM to 1 µM. Assays were prepared in 20 µl and incubated at 30 °C for one hour. Then the reactions were stopped with 2x SDS-Laemmli buffer and analysed on a 5-20% SDS-gradient gel followed by immunostaining with anti-HA- (Figure 23-A) and anti-GST- (Figure 23-B) antibodies.

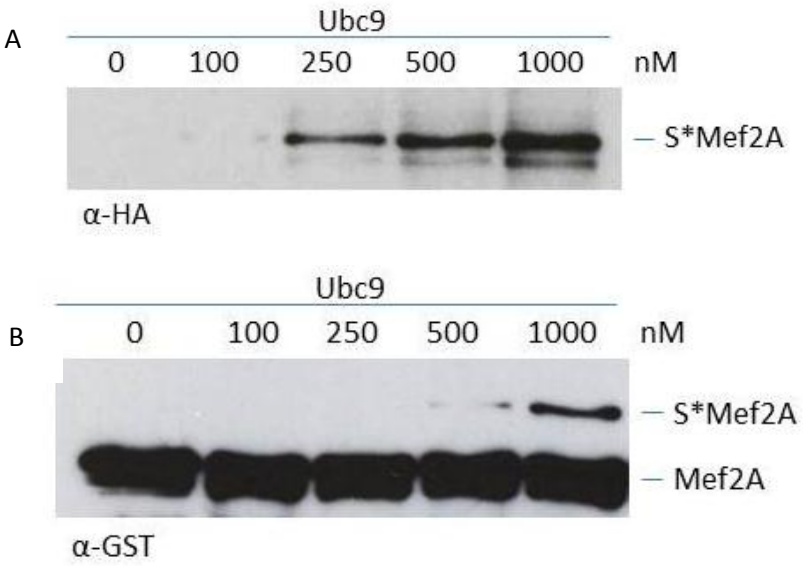

Figure 23 In vitro sumoylation of GST-Mef2A. GST-Mef2A (641 nM) was sumoylated in the presence of 4 µM HA-SUMO1, 100 nM E1 and indicated concentrations of Ubc9 at 30 °C for 60 minutes. (A) Immunostaining with HA antibodies (1:1500). (B) .Immunostaining with GST antibodies (1:7000).

GST-Mef2A was not as efficient sumoylated as His-Hsf1 under the tested conditions. At the highest E2 concentration (1 µM) only a small fraction of GST Mef2A was sumoylated which is not suprising since it also depends on phosphorylation prior to efficient sumoylation.

4.2. AURORA A

Aurora A was identified as a potential substrate for Ubc9 with moderate but significant signal intensities increasing with Ubc9 concentrations (3831 RFU with 100 nM Ubc9 and 12148 RFU with 500 nM Ubc9- maximum value 65000 RFU).

Aurora A is a Serine/Threonin Kinase and has important functions in centrosome maturation, spindle formation, chromosome orientation. In addition it is frequently upregulated in human cancers. Aurora A was shown to interact with Ubc9 and can be sumoylated in vivo and in vitro. Defects in its sumoylation pattern might be implicated in tumor development [122]. Figure 24 indicates the measured signal on both ProtoArrays.

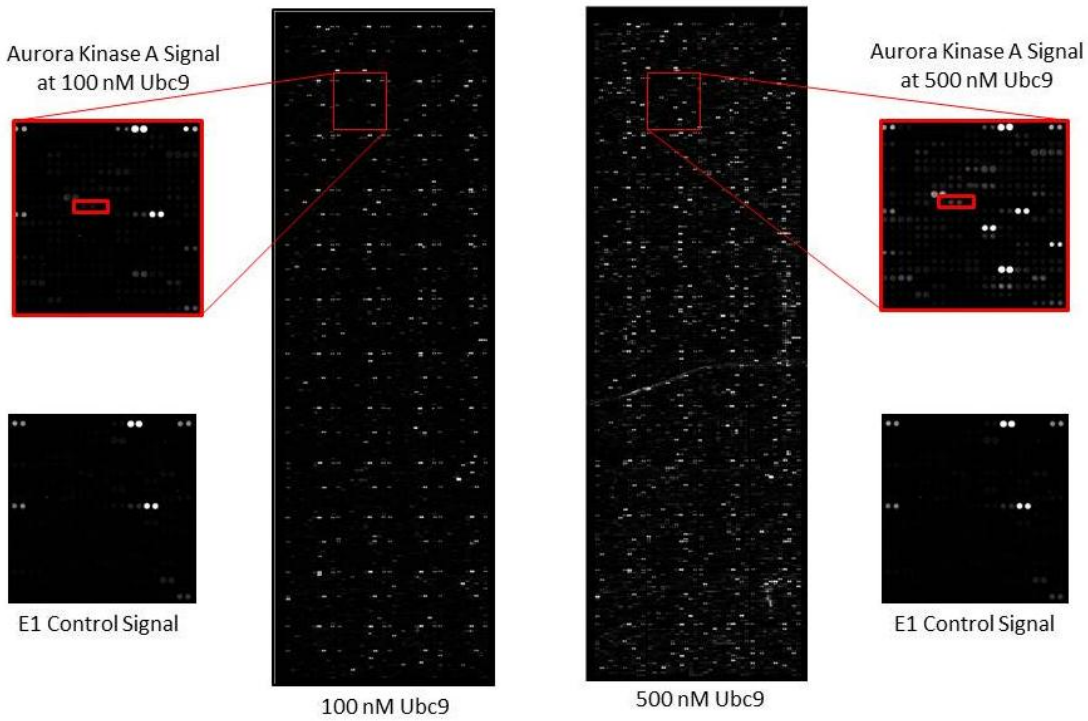

Figure 24 AuroraA Signal on the two Ubc9 ProtoArrays. Detection signal of AuroraA on the ProtoArray incubated with the reaction mixture with 100 nM Ubc9 and with 500 nM Ubc9.

Due to the limited time during my master thesis, the cloning of AuroraA could not be completed.

DISCUSSION

Upregulation of the sole SUMO E2 enzyme Ubc9 frequently correlates with different types of cancer [102,104,105,107,109]. How Ubc9 could contribute to disease onset or pathogenesis in unknown since most SUMO substrates are assumed to be regulated at the step of E3 ligases. Although Ubc9 can directly interact with a SUMO consensus motif in substrates, this binding interface is usually not sufficient for efficient modification and requires the help of an E3 ligase.
In the recent years different studies indicate that selected SUMO substrates do not depend on E3 ligases, like RanGAP1 which is the most abundant SUMO substrate in mammalian cells. Biochemically RanGAP1 stably interacts with Ubc9 and a second binding interface, in addition to the SUMO consensus site interaction, was identified. This additional interaction is not only required for stable Ubc9 binding (Andrea Pichler, unpublished results) but also for efficient modification [39]. Analog mechanisms including phosphorylation and negatively charged binding interfaces in different substrates were identified to enhance Ubc9 interaction and SUMO modification. The goal of my thesis was to learn about the generality of E3 independent sumoylation which may explain consequences of Ubc9 overexpression.

To obtain this goal I performed an E3 independent in vitro sumoylation reaction on a human Protein Array containing more than 9000 recombinant proteins. As negative control we used the same reaction without the E2 enzyme. One advantage of our screen, in comparison to other methods identifiying new SUMO substrates from cells by pull downs or immunopreciptiation, is that our assay does not depend on the expression levels of the individual proteins. This may allow the identification of highly regulated substrates which are only expressed upon certain stimuli or in specific phases of the cell cycle. One disadvantage is that recombinant proteins on the screen may be partially unfolded or in some cases do only represent protein domains which can cause false positive results.

Our assay identified more than 800 putative SUMO substrates of which many were identified in earlier screens or described as SUMO substrates. In my thesis I concentrated on two of such substrates: Hsf1 and Mef2A which showed extremely high values in our screen. I expressed these substrates in bacteria and tested them in our in vitro sumyolation assay. The bacterially expressed proteins depend on relatively high E2 concentrations for in vitro sumoylation which is not surprising because both proteins depend on phosphorylation prior to sumoylation in vivo

[58,59]. Since the proteins on the array are isolated from insect cells they could still be phosphorylated which would explain the discrepancy in sumoylation efficiency.

During the practical work of this thesis the Lima lab mapped a basic batch on Ubc9 (Lysines 65, 74 and 76) which recognizes the negatively charged phosphorylated Serine in MEF2A and is required to mediate enhanced sumoylation of phosphorylation dependent sumoylation motifs in vitro and in vivo [60]. In addition they could show that this phosphorylation dependent enhancement is E3 independent. These findings support that at least some of the substrates identified in my thesis are modified indeed in an E3 independent manner. Our screen also identified rather novel, highly regulated SUMO substrates like Aurora B, which showed weak but significant modification values and was published while I was writing the manuscript. The substrates with low values in our screen are indeed regulated in an E3 independent manner is rather unlikely since Ubc9 can also modify E3 dependent substrates but not very efficiently even at high concentrations. Additional studies on several identified substrates, their interaction with Ubc9 and their efficiency in vitro sumoylation will be required to make general conclusions about E3 independent sumoylation.

MATERIALS AND METHODS

5. MATERIALS

5.1. REAGENTS& BUFFERS

Ampicillin stock solution (100 mg/ml)

Dissolve 5 gram of ampicillin in 50 millilitres of double distilled H_2O. Filtersterilize and store aliquots at -20°C.

Ammoniumpersulfate (APS) (10%)

Dissolve 100 mg of APS (Roth) in 1 ml of double distilled H_2O. Store aliquots at -20°C.

ATP (100 mM)

Mix 100 mM ATP, 100 mM $Mg(OAc)_2$ and 20 mM HEPES pH 7.4. Titrate pH with 10 N NaOH and check with a pH paper. Freeze 50µl aliquots in liquid nitrogen and store at -20°C.

Aprotinin (AP) (1 mg/ml stock solution)

Dissolve 1 mg Aprotinin (ROTH 100 mg 4°C) in 1 ml 20 mM HEPES pH 7.4. Freeze aliquots in liquid nitrogen and store at -20°C.

1% acetid acid

Mix 10 ml of 100% acetic acid with 990 ml of double distilled H_2O. Store at room temperature.

Colloidal Coomassie stock solution

Dissolve 100 g $(NH_4)2SO_4$ in 800 ml H_2O. Add 20 g ortho-phosphoric acid and 1 g Coomassie Brilliant Blue G250 and fill to 1 l with H_2O. Stirr at least 24 hours at RT before use.

To prepare a working stock solution add 20% methanol.

DTT (1,4-Dithiothreit) (1M stock solution)

Dissolve 0.77 g of DTT powder (MG=154.2) (Roth) in 5 ml of double distilled H_2O. Freeze aliquots in liquid nitrogen. Store at -20°C.

Isopropyl-β-D-thiogalactopyranosid (1M stock solution)

Dissolve 11.92 g of IPTG powder in 50 ml double distilled H_2O. Filtersterilize and freeze aliquots in liquid nitrogen. Store at -20°C.

Kanamycin (60 mg/ml stock solution)

Dissolve 60 mg of kanamycin in 1 ml of double distilled H_2O. Filtersterilize and store aliquots at -20°C.

Laemmli-buffer (Western Blot Runningbuffer) (10x stock solution: 250 mM tris, 1.92 M glycine, 0.5% SDS)

Dissolve 30.28 g tris(base) with 144.13 g glycine and 5 g SDS in H_2O at a total volume of 1 litre. Store at room temperature.

LB medium

Dissolve 10 g of bacterial peptone (tryptone), 5 g of yeast extract, 5 g of NaCl (and 15 g of agar for plates) in 1 litre water. Autoclave and cool down to 50-60°C. For preparation of plates: add the appropriate amount of antibiotics and pour the plates. Plates were stored at 4°C.

Leupeptin/Pepstatin (LP) (1 mg/ml stock solution)

Dissolve 1 mg of leupeptin (Calbiochem) and 1 mg of pepstatin A (Calbiochem) in 1 ml of DMSO. Freeze aliquots in liquid nitrogen. Store at -20°C.

Loading Dye Solution for DNA (6x stock solution)

0.2% Bromphenol Blue, 0.2% Xylene Cyanol FF, 60% glycerol and 60 mM EDTA. Store at room temperature.

Mini Prep Buffer 1

Dissolve 3.03 g tris(base) and 1.86 g $Na_2EDTA.2H_2O$ in 400 ml ddH_2O, adjust pH to 8.0 and fill up to 500 ml. Autoclave and store at room temperature.

Mini Prep Buffer 2

Dissolve 2.4 g of NaOH in 475 ml H_2O and add 25 ml of 20% SDS. Autoclave and store at room temperature.

Mini Prep Buffer 3

Dissolve 147.25 g potassium acetate in 250 ml H_2O. Adjust the pH to 5.5 with glacial acetic acid and fill up with H_2O to 500 ml. Autoclave and store at room temperature.

Ovalbumin (20 mg/ml stock solution)

Dissolve 20 mg Albumin Chicken E66 Grade VI A-2512 (from Sigma) in 1 ml double distilled H_2O. Store aliquots at -20°C.

PBS (10x stock solution)

Dissolve 400.91 g of NaCl, 10.06 g of KCl, 89 g of $Na_2HPO_4x\ 2H_2O$ and 12.25 g of KH_2PO_4 in distilled H_2O at a total volume of 5 litres . Titrate the pH with NaOH to 7.4. Store at room temperature.

PBS Tween (To wash western blots)

Mix 1 litre of the 10x PBS stock and 100 ml of 20% Tween 20. Fill up with distilled H_2O to 10 litres. Store at room temperature.

PMSF (Phenylmethylsufonyl flouride) (100 mM stock solution)

Dissolve 870 mg of PMSF in 50 ml isopropanol. Store aliquots at -20°C.

Ponceau-staining solution (0.5 % Ponceau S)

Dissolve 1 g of Ponceau S in 200 ml 1% acetic acid. Store at room temperature.

Protein Purification Elution buffer

- High Salt Elution Buffer (300 mM NaCl)

  25 mM Na-phosphate pH 6.5, 300 mM NaCl, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP.
- High Salt Elution Buffer (500 mM NaCl)

  50mM tris pH 7.5, 500 mM NaCl, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP.
- His Elution Buffer

  50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 1 mM beta-mercaptoethanol, 1 µg/ml aprotinin and 1 µg/ml LP.
- GST Elution Buffer

50 mM tris, 20 mM glutathione (reduced) pH 8 titrated with HCl, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP.

- RanGAP Elution Buffer
  50 mM tris pH 7.4, 1 M NaCl and 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP.

SUMO Enzyme Transport buffer

20 mM tris pH 8.0, 100 mM NaCl, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml leupeptin/pepstatin.

SDS-Laemmli-buffer (for SDS-PAGE) (2x stock solution)

Mix 20 ml of 0.5 M tris pH 6.8, 20 ml of 20% SDS, 0.2 g of Bromphenolblue, 23 ml of 87% glycerine and 17 ml of double distilled H_2O.,Add 20ml of DTT shortly before usage. Store at room temperature. When DTT is added, the buffer can be used for a maximum of two days.

SUMO Assay Buffer (SAB)

Mix 1 ml of 10x Transportbuffer, 100 µl of ovalbumin (20 µg/µl), 250 µl of 2% Tween and 10 µl of LP (1 mg/ml), 10 µl of aprotinin (1mg/ml) and 10 µl of 1 M DTT with 8520 µl ddH_20. Store aliquots at – 20°C.

Transportbuffer (10x stock solution)

200 mM HEPES, 1.1 M KOAc, 20 mM $Mg(OAc)_2$ and 10 mM EGTA pH 7.3 titrated with KOH in distilled H_2O. Store at 4 °C.

TAE (50x stock solution)

Dissolve 242 g tris(base) (2M), 57.1 ml of 100% acetic acid and 100ml of 0.5 M EDTA pH 8.0 in 1 litre distilled H_2O. Titrate the pH of the solution with HCl to 7.7. Store the buffer at room temperature.

Western blot transfer buffer (10x stock solution)

250 mM tris(base), 1.93 M glycine in distilled H_2O. To prepare one litre of a 1x western blot transfer buffer solution, mix 100ml of the 10x stock, 200m of tech. methanol, 1.8 ml of 20% SDS and 700ml of distilled H_2O. Store the solution at room temperature.

5.2. PLASMIDS

Table 2 List of different Plasmids

Protein and Plasmid	Source	Selection Marker
hSUMO1ΔC4 in pET11a	Pichler et al. 2002[29]	Amp
HA-SUMO1ΔC4 in pET23a	Melchior Lab	Amp
His-Aos1 in pET28a	Pichler et al. 2002[29]	Kan
Uba2-His in pET28a	Melchior Lab	Kan
hUbc9 in pET23a	Pichler et al. 2002[29]	Amp
RanGAP1 in pET11d	Mahajan et al 1997 [4]	Amp
DAXX in pGEX4T (residue 572-740, GST-Tag)	Pichler et al. [42]	Amp
TDG in pET 30 (His Myc-Tag)	Takahashi et al [116]	Kan
hHsf1 in pET23a (His-Tag)	Outlined in 6.8.7	Kan
hMef2A in pGEX6p.1 (GST-Tag)	Outlined in 6.8.7	Amp

5.3. ANTIBODIES

Table 3 List of different Antibodies

Antibody	Made by	Working Dilution	Storage
rabbit α-GST	Ludger Hengst	1:7000	Stored at -20°C
mouse α-His	Qiagen	1:1000	Stored at -20°C
mouse α-Myc	Egon Ogris	1:10000	Stored at 4 °C
mouse α-SUMO1	Zymed	1:1000	Stored at -20 °C
goat α-hUBC9	Andrea Pichler	1:2000	Stored at -20 °C
goat α-RanGAP1	Andrea Pichler	1:5000	Stored at 4 °C
goat α-E2-25K	Andrea Pichler	1:2000	Stored at -20 °C
monoclonal mouse α-HA.11	Covance	1:1500	Stored at -20 °C
HRP-coupled α-mouse, α-goat and α-rabbit antibodies	Jackson Laboratories	1:10 000	Stored at -20 °C
Alexa647 labelled α-mouse antibody	Invitrogen	1:2000	Stored at -20 °C

5.4. REFERENCE PROTEINS

α-Lactalbumine [10mM]	14.2 kD
Trypsine Inhibitor [10mM]	20.1 kD
Ovalbumine [10mM]	44.3 kD

BSA [2mM]	66.0 kD
Phosphorylase A [1mM]	97.2 kD

6. METHODS

6.1. PCR

6.1.1. PRIMER DESIGN

To design a primer several things should be considered: the forward primer should start with 6 random bases upstream of the restriction site to support the restriction enzyme. The restriction enzyme site should be found in the multiple cloning site of the vector but not in the gene of interest. The restriction site is followed by the start ATG of the gene and additional 20 to 25 bases of the gene of interest, which should end with a C or G for better annealing. The length of the primer should not exceed more than 60 bases. The ratio between A/T and C/G should be about 50%. The reverse primer was designed complementary to the forward primer.

6.1.2. PCR REACTION

PCR reactions were performed with Phusion High Fidelity DNA Polymerase (Finnzymes, Thermo Fisher). For a reaction volume of 50 µl following amounts were needed: 1U (0.5 µl) Phusion, 10 mM dNTPs (1 µl), 5x Phusion buffer, 10 pmol/µl forward Primer and 10 pmol/µl reverse Primer (2.5 µl), x µl template DNA (approximately 10 – 20 ng should be sufficient) and autoclaved double distilled H_2O. Cycling conditions for the thermal cycler are illustrated in Table 4:

Table 4 PCR conditions

Cycle Step	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	5-10 sec	35x
Annealing	x°C	30 sec	35x
Extension	72°C	15-30s/kb	35x
Final Extension	72°C	5-10 min	1
Hold	4°C	hold	

The annealing temperature is calculated by counting the bases of the primer (without restriction sites and the additional bases) and multiplying the number of adenines and thymines by 2 and the number of guanines and cytosines by 4.

Alternatively (if the optimal annealing temperature is not certain), a gradient PCR can be performed. Then a gradient ranging from 60°C to 70°C can be set at the annealing step. The PCR product was analysed via agarose gel electrophoresis.

6.2. AGAROSE GEL ELECTROPHORESIS

DNA samples were resolved via 1% or 0.8% agarose gel electrophoresis in TAE buffer at 100 V for 30 min.

Selected DNA bands were excised from the gel using a clean scalpel blade and eluted with the Qiaquick Gel Extraction Kit (Qiagen) following the manufactorer's protocol.

6.3. RESTRICTION DIGESTION AND LIGATION

6.3.1. RESTRICTION DIGESTION

Restriction digestions were performed in a volume of 50 µl and 1.5 µl of each restriction enzyme (100000 units/ml from New England Biolabs) were used. In addition 10x RNAse stock and 10x BSA (if needed) were added to the restriction mixture. The 10x buffer was chosen according to the combination of the restriction enzyme. The digestion mixture was incubated at 37°C for 1 ½ hours. Then the digest was mixed with the 6x loading dye solution, resolved on an agarose gel and the desired DNA bands were eluted.

6.3.2. LIGATION

For ligation the DNA insert and the vector were used at a ratio of 3:1. The amount of DNA was estimated after visualizing on an agarose gel. The ligation mixture with a final volume of 20 µl consisted of the insert and the vector DNA, 1 µl T4 Ligase (400000U/ml, from New England BioLabs), 10x T4 Ligase Buffer (New England Biolabs) and ddH_2O if needed. A negative ligation control without the insert DNA was also performed. The two ligation mixtures were incubated at room temperature for 2 hours. Afterwards the mixtures were transformed into competent bacteria.

6.4. TRANSFORMATION

6.4.1. LIST OF COMPETENT BACTERIA

Table 5 Competent bacteria, their function and genetics

Strain	Usage
E.coli DH5α	Transformation of Plasmids
Rosetta C41	Protein Expression
BL21 gold	Protein Expression

6.4.2. PREPARATION OF COMPETENT BACTERIA (TSS-METHOD)

First, the TSS buffer was prepared: for 50 ml of buffer, 5 g of PEG 8000, 0.3 g of $MgCl_2.6H_2O$ and 2.5 ml DMSO were mixed together and filled up with LB to 50 ml. The solution was filtersterilized and stored at either 4°C or -20°C.

An aliquot of competent bacteria was grown in LB with the appropriate antibiotic overnight. The culture was diluted 1:100 the next day and grown to OD 0.2-0.5. Then the culture was centrifuged for 10 minutes at 4°C (4000 rpm). The medium was removed and the pellet carefully resuspended in 1/10 volume of chilled TSS buffer without leaving cell clumps or aggregates. Finally the bacteria were aliquoted (100µl each) in pre-chilled eppendorf tubes and the aliquots were stored at -80°C.

To test the transformation efficiency one aliquot of competent bacteria was transformed with a 1:10000 dilution of plasmid DNA (approx 0.1 ng). After recovery 200µl of the transformation mix were plated on LB plates supplemented with the appropriate antibiotic where it should result at least in 100 colonies.

6.4.3. TRANSFORMATION OF DNA IN BACTERIA

One aliquot of competent bacteria was thawed on ice; the DNA was added (2µl of plasmid DNA for protein expression in BL21gold or 10µl of ligation mix in DH5α bacteria) and mixed. The mix was incubated on ice for 20 min followed by incubation at 42°C for 1 ½ min and another 5 min incubation on ice. After addition of 1 ml LB without antibiotics the mix was incubated for 1 hour at 37°C with 500 rpm. Then it was either added to LB with antibiotics (5 ml for overnight culture) or plated on a LB plate (containing the appropriate antibiotic).

6.5. PLASMID PREPARATION (MINI PREPARATION)

Preparation of plasmid DNA for sequencing or transformation was performed with the QIAprep DNA Mini Kit from Qiagen following the corresponding protocol.

Plasmids that were used for control restriction digestion were prepared as follows; A single colony was inoculated in 2 ml LB supplemented with the appropriate antibiotic and grown at 37°C over night. The next day the culture was pelleted in a 2 millilitre Eppendorf tube by centrifuging for 5 minutes at 4000 rpm /or at 13 200 rpm. The pellet was resuspended in 300 µl buffer 1. Then 300 µl buffer 2 were added to the suspension, mixed gently and incubated for 5 minutes at room temperature. Then 300 µl of buffer 3 were added to the mixture, it was mixed thoroughly and incubated for 5 minutes on ice.

After the incubation time, the mixture was mixed again and centrifuged for 5 minutes at 14 000 rpm on room temperature. Then the supernatant was transferred to a new 1.5 ml Eppendorf tube and centrifuged again for 5 minutes at 13 200 rpm. 850 µl of the supernatant were transferred to a new Eppendorf tube and 595 µl isopropanol were added. It was mixed well and centrifuged for 15 minutes at 13 200 rpm. The liquid was poured off; the pellet was washed once with 70% EtOH and dried on air. Then the pellet was dissolved in 20 to 50 µl H_2O.

6.6. POLYACRYLAMIDE GEL (SDS-PAGE)

For the polymerisation of SDS Laemmli gels following materials are needed: double distilled H_2O, 2 M tris/HCl pH 8.8, 0.5 M tris/HCl pH 6.8, 30% Acrylamid: Bisacrylamid (30:0.8) Rotiphorese (Carl Roth), 20% SDS, ammonium persulfate (APS) (-20°C), TEMED 4°C. For the preparation of gradient gels (5-20%) a gradient mixer was used which contains two containers. In the container with the direct outlet to the gel chamber, the lower concentrated acrylamide solution is poured.

Recipes for 10 gels

Table 6 SDS-PAGE running gel recipe

SDS running gel		5%	7%	10%	12.5%	20%
dd H_2O	ml	42.9	38.35	31.3	25.5	7.9
2 M Tris/HCl pH 8.8	ml	14	14	14	14	14
30% AA:Bis	ml	11.7	16.3	23.33	29.2	46.7
20% SDS	µl	350	350	350	350	350
			mix			
10% APS	µl	700	700	700	700	700
TEMED	µl	70	70	70	70	70

Table 7 SDS-PAGE stacking gel recipe

SDS stacking gel		2x
dd H_2O	ml	45.4
0.5 M Tris/HCl pH 6.8	ml	6
30% AA:Bis	ml	8
20% SDS	µl	300
	mix	
10% APS	µl	300
TEMED	µl	60

The samples were prepared by adding 2x SDS Laemmli buffer with DTT and boiling at 95 °C for 5 minutes. After boiling the samples were loaded in the slots. Then 3 µl of PageRuler™ prestained Protein Ladder (Fermentas) and 7 µl of PageRuler™ unstained Protein Ladder (Fermentas) were loaded in the first and in the last slot, respectively. The gel run was performed at constant amperage of 25 mA per gel.

Coomassie Staining

Before staining with Coomassie Brilliant Blue, the gel needs to be fixed in 40% ethanol and 10% acetic acid for at least 15 minutes. Then the gel was washed two times with water for 10 minutes each. Afterwards the Coomassie stock was diluted with methanol 4:1 and the gels were incubated with the mix overnight. The next day the gel was washed with 1% acetic acid until all Coomassie particles were removed.

6.7. WESTERN BLOT (SEMI-DRY WESTERN BLOT)

Proteins were transferred from the gel to a nitrocellulose membrane by semi dry blotting (15 to 20 volt per gel for 30 minutes (45 minutes for a gradient gel)). During blotting 0.6 ampere should not be exceeded and a constant voltage should be maintained.

After the transfer, the nitrocellulose membrane was washed in ddH_2O for a minute and then shortly dyed in Ponceau. It was destained in 1% acetic acid and the protein ladder bands were marked with a ball pen. Then the membrane was washed in PBS-Tween until the red dye was removed. Afterwards the nitrocellulose membrane was incubated in 5% milk in PBS-Tween for 30 minutes at room temperature or at 4°C overnight.

The membrane was washed for 10 minutes in PBS-Tween prior to incubation with the primary antibody, which was diluted in 5 to 10 ml of 5% milk/PBS-Tween. The incubation with the antibody was performed for 1 ½ to 2 hours at room temperature. Afterwards the membrane was washed three times with PBS-Tween for 5 minutes each. Finally the membrane was incubated with the secondary antibody dilution (in 5% milk/PBS-Tween) for one hour at room temperature followed by washing with PBS-Tween for 3 times for 10 minutes each.

For the detection of the horseradish peroxidase enzyme activity the SuperSignal West Pico Chemiluminescent Substrate (Pierce/Thermo Fisher) or for further enhancement SuperSignal West Femto Chemiluminescent Substrate (Pierce/Thermo Scientific) was used. The Luminol/Enhancer and the Stable Peroxide Buffer solutions were mixed in a ratio of 1:1 and the membrane was incubated with the mix for one minute. Excess reagent was drained and the membrane was covered with a plastic foil in a western blot film cassette. In the dark room the "treated" membrane was exposed to an Amersham Hyperfilm™ ECL from GE Healthcare. The exposure time was varied to achieve optimal results. Then the films were developed in a developer.

6.8. EXPRESSION OF PROTEINS

6.8.1. EXPRESSION AND PURIFICATION OF HUMAN AOS1/UBA2 (E1)

On the first day one aliquot of BL21 gold competent bacteria was transformed with His-Aos1 in pET28a and one aliquot was transformed with plasmid Uba2-His in pET28a. 5 ml of LB complemented with kanamycin was added to the bacteria and the two cultures were grown during the day at 37°C 200 rpm. In the afternoon the cultures were transferred to 40 ml LB with kanamycin and grown overnight.

The next day each culture was diluted to 2 litres and grown to an OD_{600} of 0.5 to 0.6. Then 1 ml aliquot of each culture was taken, centrifuged at 4000 rpm at room temperature, the LB was removed and the bacterial pellets were resuspended in 50 µl of 2x SDS-Laemmli buffer and boiled at 95°C for 5 minutes. Protein expression was induced by adding IPTG at a final concentration of 1 mM from a 1000x stock. The two cultures were incubated at 25°C 200 rpm for 6 hours. Aliquots of the induced cultures were taken every hour.

After the 6 hours bacteria were harvested by centrifugation for 20 minutes at 4°C and 4000 rpm in a Sorvall RC-5 Superspeed refrigerated centrifuge with a Sorvall GS-3 rotor. The pellets were resuspended in 30 ml (15 ml/litre culture) of icecold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl,

10 mM imidazole, 0.1 mM PMSF, 1 mM beta-mercaptoethanol, 1 µg/ml aprotinin and 1 µg/ml LP). Both pellets were frozen in liquid nitrogen and stored at -80°C.

On day 3 the pellets were thawed and split in ultrazentrifuge tubes for Beckmann rotor 50.2Ti. 1 mg/ml lysozyme was added to the suspension and incubated for one hour on ice. Then they were ultracentrifuged for one hour in the ultracentrifuge Beckman (Optima) XL at 4°C and 100 000g. In the meantime 2x 2.5 ml/litre culture of Ni-Beads (ProBond^{TM} resin from Invitrogen) were equilibrated with icecold lysis buffer by washing 2 to 3 times with at least one column volume.

The supernatant after ultracentrifugation was pooled and added to the equilibrated Ni-beads and incubated while rotating for 1.5 hours at 4°C. Ni-beads were then harvested by centrifugation for 5 minutes at 4°C and 1000 rpm. The beads bound with the protein were transferred into a column and the beads were washed extensively with ice-cold wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 1 mM beta-mercaptoethanol, 1 µg/ml aprotinin and 1 µg/ml LP). Then the proteins were eluted with 15x 0.5 ml icecold His elution buffer. To identify the fractions containing the eluted protein, 1 µl of each fraction was spotted on a nitrocellulose membrane and stained with Ponceau S.

The peak fractions were pooled (3 to 5 ml) and concentrated to 1 ml using Vivaspin columns (Sarstedt) or Centricons (Millipore) and then centrifuged for 10 minutes in the ultracentrifuge. One millilitre was applied to the ÄKTA S200 preparative gelfiltration column. The column was already equilibrated in icecold transport buffer, complemented with 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP. The flow rate of the ÄKTA was to 0.5 ml/minute and 0.5 ml fractions were collected. The fractions were analysed via a chromatogram (a protein density profile) and via SDS-PAGE where 10 µl of each peak fraction was analysed on a 5-20 % gradient gel

For Aos1 peak fractions 7 to 17 were pooled and peak Uba2 fractions 9 to 16 were pooled, frozen in liquid nitrogen and stored at -80°C.

On day 4 the dimeric SUMO E1 was reconstituted and therefore equimolar amounts of His-Aos1 and Uba2-His were combined. The required ratio of Aos1 and Uba2 was determined by comparing serial dilutions of each enzyme side by side on a SDS-polyacrylamid gel. Aos1 and Uba2 were combined at a ratio of 1:2 and incubated on ice for 2 hours. Then the sample was applied to the HiLoad 26/60 S200 ÄKTA column that was equilibrated in SUMO enzyme transport buffer. The flow rate of the ÄKTA was set to 2.5 ml/min and fractions of 5 ml were collected. (Fractions 32 to 47 were tested on a coomassie gel for the dimeric E1). Fractions that contain equal amounts of Aos1 (40kDa) and Uba2 (90kDa) (fractions 35 to 37) were pooled and

subsequently aliquoted into 10 µl, 20 µl, 50 µl, 100 µl, 200 µl, 500 µl and 1ml, frozen in liquid nitrogen and stored at -80°C. The protein concentration was estimated by comparing serial dilutions of the protein to serial dilutions of a reference protein of known concentration via SDS PAGE with subsequent staining with Coomassie Brilliant Blue. The protein concentration was estimated as 0.5 µg/µl= 4.5 µM.

6.8.2. EXPRESSION AND PURIFICATION OF HUMAN SUMO1 AND HA-SUMO1

On the first day one aliquot of BL21 gold competent bacteria was transformed with plasmid hSUMO1ΔC4 in pET11a or with HA-hSUMO1ΔC4 in pET23a. 5 ml of LB complemented with ampicillin was added to the bacteria and the culture was grown during the day at 37°C 200 rpm. In the afternoon the culture was transferred to 40 ml LB with ampicillin and grown overnight. The next day the culture was diluted to 4 litres and grown to an OD_{600} of 0.5 to 0.6. Then 1 ml aliquot of the culture was taken, centrifuged at 4000 rpm at room temperature, the LB was removed and the bacterial pellet was resuspended in 50 µl of 2x SDS-Laemmli buffer and boiled at 95°C for 5 minutes.

Protein expression was induced by adding IPTG to a final concentration of 1 mM from a 1000x stock solution. The SUMO1 culture was incubated at 37°C 200 rpm for 4 hours and the HA-hSUMO1 culture was incubated overnight. Aliquots of the induced cultures were taken every hour. After the incubation time the bacteria were harvested by centrifugation at 4°C, at 4000 rpm for 20 minutes in a Sorvall RC-5 Superspeed refrigerated centrifuge with a Sorvall GS-3 rotor. The pellets were resuspended in 30 ml (15ml/litre culture) of icecold SUMO lysis buffer (50 mM tris-HCl pH 8.8, 25 mM NaCl, 0.1 mM PMSF, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP). The pellet was frozen in liquid nitrogen and stored at -80°C.

On day 3 the pellet was thawed and 0.1 mM PMSF 1 mM DTT and 1 µl/ml of aprotinin and LP, respectively were added. The suspension was sonicated (maximum 15 ml at once) 3 times for 30 seconds at 75% intensity. The suspension was split into ultrazentrifuge tubes for a Beckman rotor 50.2Ti and ultracentrifuged for one hour at 4°C and 100 000g in the ultracentrifuge Beckman (Optima) XL. In the meantime 3-4 ml (=6-8 ml suspension) Q Sepharose™ Fast Flow beads from GE Healthcare were filled into an appropriate column and equilibrated with icecold SUMO lysis buffer (50 mM Tris-HCl pH 8.8, 25 mM NaCl, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP) by washing 2-3 times with at least one column volume. The supernatant after ultracentrifugation was quickly transferred to a column with the equilibrated Q sepharose beads. The column was washed 2 to 3 times with 6x column volume lysis buffer. The protein was

eluted with 8 ml (16x 0.5 ml) icecold high salt elution buffer (500 mM NaCl). Each fraction was tested for protein by spotting 1 µl on nitrocellulose membrane and staining with Ponceau.

The peak fractions of hSUMO1 were pooled and applied to the ÄKTA HiLoad 26/60 S200 preparative gelfiltration column, which was already equilibrated in icecold SUMO enzyme transport buffer. The flow rate of the ÄKTA was set to 1.5 ml/minute and 5 ml fractions were collected. The fractions were analysed via a chromatogram (a protein density profile) and via SDS-PAGE where 5 µl of each peak fraction were analysed on a 12.5% gel. (Fractions 19-46 were tested on a coomassie gel and fractions 33 to 37 were collected and pooled.)

HA-SUMO1 elution fractions were applied to the Äkta S200 10/300 GL preparative gelfiltration column which was equilibrated in transport buffer complemented with 1mM DTT, 1µg/ml aprotinin and 1µg/ml LP). The flow rate was set to 0.5 ml/min and 0.5 ml fractions were collected and analysed via a chromatogram and the most promising fractions via SDS-PAGE. The best fractions of HA-SUMO1 were collected and pooled.
Aliquots of 10 µl, 20 µl, 50 µl, 100 µl, 200 µl, 500 µl and 1ml of SUMO1 and HA-SUMO1 were prepared, frozen in liquid nitrogen and stored at -80°C. The protein concentration was estimated by comparing serial dilutions of the protein to serial dilutions of a reference protein of known concentration via SDS PAGE with subsequent staining with Coomassie Brilliant Blue. The concentration of SUMO1 was estimated as 2 µg/µl = 173 µM and of HA-SUMO1 as 1.5 µg/µl = 100 µM.

6.8.3. EXPRESSION AND PURIFICATION OF HUMAN UBC9

On the first day one aliquot of BL21 gold competent bacteria was transformed with hUbc9 in pET23a. 5 ml of LB complemented with ampicillin was added to the bacteria and the culture was grown during the day at 37°C 200 rpm. In the afternoon the culture was transferred to 80 ml LB with ampicillin and grown overnight. The next day the culture was diluted in 12 litres LB/ampicillin and grown to an OD_{600} of 0.5 to 0.6. Then 1 ml aliquot of the culture was taken, centrifuged at 4000 rpm at room temperature, the LB was removed and the bacterial pellet was resuspended in 50 µl of 2x SDS-Laemmli buffer and boiled at 95°C for 5 minutes.
Expression of the human Ubc9 protein was induced by addition of IPTG at a final concentration of 1 mM (from a 1000x stock solution). The culture was incubated at 37°C 200 rpm for 4 hours. Samples of the induced culture were taken every hour.
After the 4 hours bacteria were harvested by centrifugation at 4°C, at 4000 rpm and for 20 minutes in a Sorvall RC-5 Superspeed refrigerated centrifuge with a Sorvall GS-3 rotor. The

pellets were resuspended in 3x30 ml (15ml/litre culture) of icecold Ubc9 lysis buffer (25 mM Na-phosphate pH 6.5, 50 mM NaCl, 0.1 mM PMSF, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP). The pellets were frozen in liquid nitrogen and stored at -80°C.

On day 3 the pellet was thawed and 0.1 mM PMSF 1 mM DTT and 1 µl/ml of aprotinin and LP, respectively, were added. The suspension was divided into ultrazentrifuge tubes for a Beckman rotor 50.2Ti and ultracentrifuged for one hour at 4°C and 100 000g in the ultracentrifuge Beckman (Optima) XL. In the meantime 24 ml SP Sepharose™ high performance beads from GE Healthcare (=2 ml suspension per litre of culture) were filled into a suitable column and equilibrated with icecold Ubc9 lysis buffer by washing 2-3 times with at least one column volume. After the ultracentrifugation the supernatant was quickly transferred to the column containing the equilibrated SP sepharose beads and passed the column by gravity flow. The beads were washed in the column for 2 to 3 times with 6x column volume of Ubc9 lysis buffer.

Ubc9 was eluted from the beads with 35 ml icecold high salt elution buffer (300 mM NaCl). First 10 ml of elution buffer were applied at once and the flow through was discarded. The next 25 ml of elution buffer were added in 1 ml steps and were collected separately. Each fraction was tested for protein by spotting 1 µl on nitrocellulose membrane and staining with Ponceau.

The peak fractions were pooled and applied to the ÄKTA HiLoad 26/60 S200 preparative gelfiltration column that was already equilibrated in icecold SUMO enzyme transport buffer. The flow rate of the ÄKTA was set to 2.5 ml/minute and 5 ml fractions were collected. The fractions were analysed via a chromatogram (a protein density profile) and via SDS-PAGE where 10 µl of each peak fraction were analysed on a 12.5% SDS-PAGE gel. Fractions containing high amounts of Ubc9 (fractions 51 to 53) were pooled and aliquots of 10 µl, 20 µl, 100 µl and 1 ml were prepared, frozen in liquid nitrogen and stored at -80°C.

6.8.4. EXPRESSION AND PURIFCATION OF RANGAP1

On the first day one aliquot of BL21 gold competent bacteria was transformed with RanGAP1 in pET11d. 5 ml of LB complemented with ampicillin was added to the bacteria and the culture was grown during the day at 37°C 200 rpm. In the afternoon the culture was transferred to 40 ml LB with ampicillin and grown overnight. The next day the culture was diluted in 2 litres LB/ampicillin and grown to an OD_{600} of 0.5 to 0.6. Then 1 ml aliquot of the culture was taken, centrifuged at 4000 rpm at room temperature, the LB was removed and the bacterial pellet was resuspended in 50 µl of 2x SDS-Laemmli buffer and boiled at 95°C for 5 minutes.

Expression of the RanGAP protein was induced by addition of IPTG at a final concentration of 1 mM from a 1000x stock solution. The culture was incubated at 37°C 200 rpm for 4 hours. Aliquots of the induced culture were taken every hour.

After the 4 hours the bacteria were harvested by centrifugation at 4°C, at 4000 rpm and for 20 minutes in a Sorvall RC-5 Superspeed refrigerated centrifuge with a Sorvall GS-3 rotor. The pellets were resuspended in 30 ml (15ml/litre culture) of icecold lysis buffer (50 mM Tris.HCl pH 8, 100 mM NaCl, 0.1 mM PMSF, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP), frozen in liquid nitrogen and stored at -80°C.

On day 3 the pellet was thawed, split into ultrazentrifuge tubes for Beckman rotor 50.2Ti 1 mg/ml lysozyme was added to the suspension and incubated for one hour on ice. After ultracentrifugation for 30 min in the ultracentrifuge Beckman (Optima) XL at 4°C and 75000g the pellet was washed two times with RanGAP1 wash buffer 1 (50 mM Tris pH 8, 1% Triton-X100) and one time with RanGAP1 wash buffer 2 (2 M Urea, 50 mM Tris pH 7.4), the pellet was resuspended with a douncer and subsequently ultracentrifuged in the Beckman rotor 50.2Ti at 75000g for 30 min. The remaining pellet was solubilized in RanGAP1 lysis buffer 1 (8 M Urea, 50 mM Tris pH 7.4). The solubilized pellet was dialyzed over night against 2 l RanGAP1 lysis buffer 2 50 mM Tris pH7.4, 150 mM NaCl, 0.1 mM PMSF, 1 mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin/pepstatin. The buffer was changed two times.

The next day the solubilized and dialyzed pellet was ultracentrifuged for 30 min at 75000g at 4°C. In the meantime 15 ml Q Sepharose beads were equilibrated with (50 mM tris pH 7.4, 150 mM NaCl and 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP). For the equilibration step the beads were washed for 2-3 times for at least one column volume. After ultracentrifugation the supernatant was applied to the equilibrated beads. The beads were washed with 50 mM tris pH 7.4, 300 mM NaCl and 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP. To elute the bound RanGAP 15 x 1 ml fractions of RanGAP1 elution buffer were applied to the beads. Each fraction was collected and tested for protein by spotting 1 µl on nitrocellulose membrane and staining with Ponceau. The peak fractions were pooled, concentrated and ultracentrifuged for 10 min.

Then the sample was applied to the ÄKTA S200 column which was already equilibrated in icecold degased transportbuffer complemented 1mM DTT, 1µg/ml aprotinin and 1µg/ml LP. The flow rate was set on 0.5 ml/min and fractions of 0.5 ml were collected. The fractions were analysed via a chromatogram (a protein density profile) and via SDS-PAGE where 10 µl of each peak fraction were analysed on a 7 % gel. Fractions (21 to 30) that contained a protein with 65 kDa were united. Aliquots of 10 µl, 20 µl, 50 µl, 100 µl, 200 µl and 500 µl were prepared, frozen in liquid nitrogen and stored at -80°C. The protein concentration was estimated as 0.7 µg/µl= 11 µM with a reference protein on SDS PAGE.

6.8.5. EXPRESSION AND PURIFICATION OF GST-DAXX

On the first day one aliquot of BL21 gold competent bacteria was transformed with pGEX4T-DAXX. 5 ml of LB complemented with ampicillin was added to the bacteria and the culture was grown during the day at 37°C 200 rpm. In the afternoon the culture was transferred to 40 ml LB with ampicillin and grown overnight. The next day the culture was diluted in 2 litres Lb/ampicilliln and grown to an OD_{600} of 0.5 to 0.6. 1 ml aliquot of the culture was taken, centrifuged at 4000 rpm at room temperature, the LB was removed and the bacterial pellet was resuspended in 50 µl of 2x SDS-Laemmli buffer and boiled at 95°C for 5 minutes.
Expression of GST-DAXX was induced with IPTG (final concentration 1 mM). The culture was incubated at 16°C 200 rpm for 5 hours. Aliquots of the induced culture were taken every hour. After the 5 hours bacteria were harvested at 4°C, at 4000 rpm and for 20 minutes in a Sorvall RC-5 Superspeed refrigerated centrifuge with a Sorvall GS-3 rotor. The pellet was resuspended in 30 ml (15ml/litre culture) of icecold lysis buffer (50 mM Tris.HCl pH 8, 100 mM NaCl, 0.1 mM PMSF, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP). The pellet was frozen in liquid nitrogen and stored at -80°C.

The next day the pellet was thawed and split into ultrazentrifuge tubes for Beckmann rotor 50.2Ti. 1 mg/ml lysozyme was added to the suspension and incubated for one hour on ice. Then they were ultracentrifuged for one hour in the ultracentrifuge Beckman (Optima) XL at 4°C at 100 000g. In the meantime 3 ml Glutathione Sepharose 4B (GST-beads) from GE Healthcare (1-2 ml per litre culture) were equilibrated with lysis buffer (50 mM Tris.HCl pH 8, 100 mM NaCl, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP) by washing 2 to 3 times with at least 15 ml buffer. The supernatant was quickly transferred to the equilibrated GST beads and incubated for 1.5 hours at 4°C on rotation. The beads were harvested by centrifugation for 5 minutes at 4°C and 1000 rpm and washed for 2 to 3 times with 10 ml icecold lysis buffer for 5 minutes at 4°C and 1000 rpm. The beads were transferred to a column. Then the bound GST-Daxx protein was eluted with 5 ml icecold GST elution buffer. 10 x 0.5 ml fractions were collected and each fraction was tested for protein by spotting 1 µl on nitrocellulose membrane and staining with Ponceau.

The peak fractions were pooled and concentrated in a vivaspin2 column at 4000 rpm at 4°C. The concentrated solution was ultracentrifuged at 100000g at 4°C for 10 min. The supernatant was applied to the ÄKTA S200 column which was already equilibrated in icecold degased transportbuffer complemented with 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP. The flow rate

was set on 0.5 ml/min and fractions of 0.5 ml were collected. The fractions were analysed via a chromatogram (a protein density profile) and via SDS-PAGE by analyzing 10 µl of each peak fraction on a 5-20% SDS-PAGE. Protein-containing fractions (13 to 15) were collected and pooled. Aliquots of 10 µl, 20 µl, 50 µl, 100 µl, 200 µl and 500 µl were prepared, frozen in liquid nitrogen and stored at -80°C. The protein concentration was estimated as 2 µg/µl= 40 µM with a reference protein on SDS-PAGE.

6.8.6. EXPRESSION AND PURIFICATION OF HIS-TDG

On the first day one aliquot of Rosetta C41 competent bacteria was transformed with pET His Myc TDG. 5 ml of LB complemented with kanamycin was added to the bacteria and the culture was grown during the day at 37°C 200 rpm. In the afternoon the culture was transferred to 40 ml LB with kanamycin and grown overnight. The next day the culture was diluted in 2 litres LB/kanamycin and grown to an OD_{600} of 0.5 to 0.6. Then 1 ml aliquot of the culture was taken, centrifuged at 4000 rpm at room temperature, the LB was removed and the bacterial pellet was resuspended in 50 µl of 2x SDS-Laemmli buffer and boiled at 95°C for 5 minutes.

Expression of His-TDG was induced by addition of IPTG at a final concentration of 1 mM. The culture was incubated at 16°C 200 rpm for 5 hours. Aliquots of the induced culture were taken every hour. After the 5 hours bacteria were harvested via centrifugation at 4°C, at 4000 rpm and for 20 minutes in a Sorvall RC-5 Superspeed refrigerated centrifuge with a Sorvall GS-3 rotor. The pellet was resuspended in 30 ml (15ml/litre culture) of icecold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, 0.1 mM PMSF, 1 mM beta-mercaptoethanol, 1 µg/ml aprotinin and 1 µg/ml LP). The pellet was frozen in liquid nitrogen and stored at -80°C.

The next day the pellet was thawed and divided into ultrazentrifuge tubes for a Beckman rotor 50.2Ti. 1 mg/ml lysozyme was added to the suspension and incubated for one hour on ice followed by ultracentrifugation for one hour in the ultracentrifuge Beckman (Optima) XL at 4°C at 100 000g. In the meantime 5 ml ProBond™ resin (Ni-Beads) from Invitrogen (2-3 ml per litre culture) were equilibrated with lysis buffer (50 mM tris.HCl pH 8, 100 mM NaCl, 1 mM beta-mercaptoethanol, 1 µg/ml aprotinin and 1 µg/ml LP) by washing 2 to 3 times with at least 15 ml buffer. The supernatant was quickly transferred to the equilibrated Ni beads and incubated for 1 hour at 4°C on rotation. The beads were harvested by centrifugation for 5 minutes at 4°C and 1000 rpm and were washed for 2 to 3 times with 10 ml icecold wash buffer (50 mM tris.HCl pH 8, 100 mM NaCl, 1 mM beta-mercaptoethanol, 1 µg/ml aprotinin and 1 µg/ml LP) for 5 minutes at 4°C and 1000 rpm. The beads were transferred to a Sigma column. The bound His-TDG protein was eluted with 7 ml icecold His elution buffer.

14 x 0.5 ml fractions were collected and each fraction was tested for protein by spotting 1 µl on a nitrocellulose membrane and staining with Ponceau. The peak fractions were pooled and concentrated in a vivaspin2 column at 4000 rpm at 4°C. The concentrated protein solution was ultracentrifuged at 100 000g at 4°C for 10 min.

The supernatant was applied to the ÄKTA S200 column which was already equilibrated in icecold transportbuffer complemented with (1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP). The flow rate was set on 0.5 ml/min and fractions of 0.5 ml were collected. The fractions were analysed via a chromatogram (a protein density profile) and via SDS-PAGE where 10 µl of each peak fraction were analysed on a 5-20% Gradient gel. Protein-containing fractions (7 to 9) were pooled and aliquots of 10 µl, 20 µl, 50 µl, 100 µl, 200 µl and 500 µl were prepared, frozen in liquid nitrogen and stored at -80°C. The protein concentration was estimated as 2 µg/µl= 28 µM with a reference protein on SDS-PAGE.

6.8.7. CLONING OF MEF2A AND HSF1

Myc epitope tagged human Hsf1

Full-length hHSF1, cloned into the mammalian expression plasmid pcDNA3.1A (Invitrogen), was kindly provided by Lea Sistonen[123].

Hsf1 was cloned into pET23a by PCR-amplification of the Hsf1 ORF using using PCR with primers that incorporated the EcoRI and HindIII restriction sites.

After digestion with EcoRI and HindIII restriction enzymes Hsf1 was ligated into pET23a in frame a His tag.

GST-tagged Mef2A

Full-length Mef2A, cloned pcDNA3.1 Myc His (A) (Invitrogen), was kindly provided by Lea Sistonen [59].

Mef2A was cloned into pGEX 6p.1 by performing a PCR using PCR primers with EcoRI and XhoI restriction sites. After PCR the amplicon was digested with the EcoRI and XhoI restriction enzymes and ligated into the likewise digested vector pGEX 6p.1

6.8.8. EXPRESSION AND PURIFICATION OF HIS-HSF1

One aliquot of BL21 gold competent bacteria was transformed with the plasmid pET23a His Hsf1. 5 ml of LB complemented with kanamycin was added to the bacteria and the culture was grown for several hours at 37°C 200 rpm. In the afternoon the culture was transferred to 40 ml LB with kanamycin and grown overnight. The next day the culture was diluted in 1 litre LB/kanamycin and grown to an OD_{600} of 0.5 to 0.6. Then 1 ml aliquot of the culture was taken, centrifuged at

4000 rpm at room temperature, the LB was removed and the bacterial pellet was resuspended in 50 µl of 2x SDS-Laemmli buffer and boiled at 95°C for 5 minutes.

Expression of His-Hsf1 was induced with IPTG at a final concentration of 1 mM. The culture was incubated at 16°C 200 rpm for 6 hours. Induced aliquots were taken every hour. After the 6 hours bacteria were harvested by centrifugation at 4°C, at 4000 rpm and for 20 minutes in a Sorvall RC-5 Superspeed refrigerated centrifuge with a Sorvall GS-3 rotor. The pellet was resuspended in 15 ml (15ml/litre culture) of icecold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, 0.1 mM PMSF, 1 mM beta-mercaptoethanol, 1 µg/ml aprotinin and 1 µg/ml LP). The pellet was frozen in liquid nitrogen and stored at -80°C.

The next day the pellet was thawed and split into ultrazentrifuge tubes for Beckman rotor 50.2Ti. 1 mg/ml lysozyme was added to the suspension and incubated for one hour on ice. Then it was ultracentrifuged for one hour in the ultracentrifuge Beckman (Optima) XL at 4°C at 100 000g. In the meantime 3 ml ProBond^{TM} resin (Ni-Beads) from Invitrogen (2-3 ml per litre culture) were equilibrated with lysis buffer (50 mM tris.HCl pH 8, 100 mM NaCl, 1 mM beta-mercaptoethanol, 1 µg/ml aprotinin and 1 µg/ml LP) by washing 2 to 3 times with at least 15 ml buffer.

The supernatant was quickly transferred to the equilibrated Ni beads and incubated for 1 hour at 4°C while rotating. The beads were harvested by centrifugation for 5 minutes at 4°C and 1000 rpm and washed for 2 to 3 times with 10 ml icecold wash buffer (50 mM tris.HCl pH 8, 100 mM NaCl, 1 mM beta-mercaptoethanol, 1 µg/ml aprotinin and 1 µg/ml LP). Then the beads were transferred to a column and the bound His-Hsf1 protein was eluted with 5 ml (10x 0.5 ml) icecold elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 1 mM beta-mercaptoethanol, 1 µg/ml aprotinin and 1 µg/ml LP). Each of the collected fractions was tested for protein by spotting 1 µl on nitrocellulose membrane and staining with Ponceau.

The peak fractions were pooled and applied to the ÄKTA S200 column, which was already equilibrated in icecold transportbuffer complemented with 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP. The flow rate was set to 0.5 ml/min and fractions of 0.5 ml were collected. The fractions were analysed via a chromatogram (a protein density profile) and via SDS-PAGE where 10 µl of each peak fraction were analysed on a 5-20 % gradient gel. Protein-containing fractions (12 to 16) were pooled and aliquots of 10 µl, 20 µl, 50 µl, 100 µl, 200 µl and 500 µl were prepared, frozen in liquid nitrogen and stored at -80°C. The protein concentration was estimated as 2 µg/µl= 25 µM with a reference protein on SDS-PAGE.

6.8.9. EXPRESSION AND PURIFICATION OF GST-MEF2A

On the first day one aliquot of BL21 gold competent bacteria was transformed with the plasmid pGEX6p.1-Mef2A. 5 ml of LB complemented with ampicillin was added to the bacteria and the culture was grown during the day at 37°C 200 rpm. In the afternoon the culture was transferred to 40 ml LB with ampicillin and grown overnight. The next day the culture was diluted in 1 litre LB/ampicillin and grown to an OD_{600} of 0.5 to 0.6. Then 1 ml aliquot of the culture was taken, centrifuged at 4000 rpm at room temperature, the LB was removed and the bacterial pellet was resuspended in 50 µl of 2x SDS-Laemmli buffer and boiled at 95°C for 5 minutes.
Expression of GST-Mef2A was induced with 1 mM IPTG. The culture was incubated at 16°C 200 rpm for 6 hours. Induced aliquots were taken every hour. After the 6 hours bacteria were harvested via centrifugation at 4°C, at 4000 rpm and for 20 minutes in a Sorvall RC-5 Superspeed refrigerated centrifuge with a Sorvall GS-3 rotor. The pellet was resuspended in 15 ml (15ml/litre culture) of icecold lysis buffer (50 mM tris.HCl pH 8, 100 mM NaCl, 0.1 mM PMSF, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP). The pellet was frozen in liquid nitrogen and stored at -80°C.

The next day the pellet was thawed and split into ultrazentrifuge tubes for Beckmann rotor 50.2Ti. 1 mg/ml lysozyme was added to the suspension and incubated for one hour on ice followed by ultracentrifugation for one hour in the ultracentrifuge Beckman (Optima) XL at 4°C at 100 000g. In the meantime 2 ml Glutathione Sepharose 4B (GST-beads) from GE Healthcare (1-2 ml per litre culture) were equilibrated with lysis buffer (50 mM tris.HCl pH 8, 100 mM NaCl, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP) by washing 2 to 3 times with at least 15 ml buffer. The supernatant was quickly transferred to the equilibrated GST beads and incubated for 1.5 hours at 4°C on rotation. The beads were harvested by centrifugation for 5 minutes at 4°C and 1000 rpm. The beads were washed for 2 to 3 times with 10 ml icecold lysis buffer for 5 minutes at 4°C and 1000 rpm and subsequently transferred to a column. The bound GST-Mef2A protein was eluted with 5 ml icecold elution buffer (50 mM Tris, 20 mM glutathione (reduced) pH 8, 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP). 10 x 0.5 ml fractions were collected and each fraction was tested for protein by spotting 1 µl on nitrocellulose membrane and staining with Ponceau.

The peak fractions were pooled and applied to the ÄKTA S200 column which was already equilibrated in icecold degased transportbuffer complemented with 1 mM DTT, 1 µg/ml aprotinin and 1 µg/ml LP. The flow rate was set on 0.5 ml/min and fractions of 0.5 ml were collected. The fractions were analysed via a chromatogram (a protein density profile) and via

SDS-PAGE where 10 µl of each peak fraction were analysed on a 5-20% gradient gel. Protein-containing fractions (16 to 21) were pooled and aliquots of 10 µl, 20 µl, 50 µl, 100 µl, 200 µl and 500 µl were prepared, frozen in liquid nitrogen and stored at -80°C. The protein concentration was estimated as 0.125 µg/µl= 1.6 µM with a reference protein on SDS-PAGE.

6.9. SUMO IN VITRO ASSAYS

6.9.1. SUMOYLATION ACTIVITY COMPARISON OF TWO SEPARATELY PURIFIED UBC9 PREPARATIONS

Table 8 Conditions of the sumoylation activity comparison of two seperatly purified Ubc9 purifications

Enzymatic activity of Ubc9	**Stock conc.**	Final conc. (**Ubc9**)	Final conc. (**S*Ubc9**)	**Volume**
SUMO1	173 µM	4.4 µg (19 µM)	4.4 µg (19 µM)	2.2 µl
E1	4.5 µM	150 ng (70 nM)	150 ng (70 nM)	0.3 µl
"old" Ubc9 No.1	111 µM	**7 µM - 60 nM**	0	2 µl or
"new" Ubc9 No.2	111 µM	0	**7 µM - 60 nM**	2 µl
E2-25K	60 µM	3.5 µg (6.6 µM)	3.5 µg (6.6 µM)	2.2 µl
ATP	100 mM	5 mM	5 mM	1 µl
Sumo Assay Buffer (SAB)	1 x	x µl	x µl	12.3 µl
		20 µl	20 µl	20 µl
Dilution steps of Ubc9 7 µM/1.4 µM/280 nM/60 nM/0				
Incubation at 30 °C for 60 minutes				

6.9.2. SUMOYLATION OF E2-25K

Table 9 Conditions of the SUMOylation assay of E2-25K

Sumo assay	**Stock conc.**	Final conc. (**Ubc9**)	Final conc. (**S*Ubc9**)	**Volume**
E2-25K	170 µM	2.2 µM	2.2 µM	0.134 µl
HA-SUMO1	100 µM	4 µM	4 µM	0.8 µl
E1	4.5 µM	100 nM	100 nM	0.44 µl
Ubc9	111 µM	**500 - 20 nM**	0	2 µl or
S*Ubc9	27 µM	0	**500 - 20 nM**	2 µl
ATP	100 mM	5 mM	5 mM	1 µl
$MgCl_2$	2 M	100 mM	100 mM	1 µl
Sumo Assay Buffer (SAB)	1 x	x µl	x µl	14.63 µl
		20 µl	20 µl	20 µl
Dilution steps of Ubc9/S*Ubc9 500 nM/250 nM/100 nM/20 nM/0				
Incubation at 30 °C for 60 minutes				

6.9.3. SUMOYLATION OF RANGAP1

Table 10 Conditions of the SUMO assay of RanGAP1

Sumo assay	**Stock conc.**	Final conc. (**Ubc9**)	Final conc. (**S*Ubc9**)	**Volume**
RanGAP1	11 µM	2.2 µM	2.2 µM	3.96 µl
HA-SUMO1	100 µM	4 µM	4 µM	0.8 µl
E1	4.5 µM	100 nM	100 nM	0.44 µl
Ubc9	111 µM	**50 - 0.4 nM**	0	2 µl **or**

S*Ubc9	27 µM	0	**50 - 0.4 nM**	2 µl
ATP	100 mM	5 mM	5 mM	1 µl
$MgCl_2$	2 M	100 mM	100 mM	1 µl
Sumo Assay Buffer (SAB)	1 x	x µl	x µl	10.8 µl
		20 µl	20 µl	20 µl
Dilution steps of Ubc9/S*Ubc9 50 nM/10 nM/2 nM/0.4 nM/0				
Incubation at 30 °C for 30 minutes				

6.9.4. SUMOYLATION OF TDG

Table 11 Conditions of the SUMO assay of His-TDG

Sumo assay	**Stock conc.**	Final conc. (**Ubc9**)	Final conc. (**S*Ubc9**)	**Volume**
His-TDG	28 µM	500 ng (357 nM)	500 ng (357 nM)	0.25 µl
HA-SUMO1	100 µM	4 µM	4 µM	0.8 µl
E1	4.5 µM	100 nM	100 nM	0.44 µl
Ubc9	111 µM	**500 - 20 nM**	0	2 µl or
S*Ubc9	27 µM	0	**500 - 20 nM**	2 µl
ATP	100 mM	5 mM	5 mM	1 µl
$MgCl_2$	2 M	100 mM	100 mM	1 µl
Sumo Assay Buffer (SAB)	1 x	x µl	x µl	14.51 µl
		20 µl	20 µl	20 µl
Dilution steps of Ubc9/S*Ubc9 500 nM/250 nM/100 nM/20 nM/0				
Incubation at 30 °C for 60 minutes				

6.9.5. SUMOYLATION OF DAXX

Table 12 Conditions of the SUMO assay of GST-DAXX

Sumo assay	**Stock conc.**	Final conc. (**Ubc9**)	Final conc. (**S*Ubc9**)	**Volume**
GST-DAXX	40 µM	500 ng (625 nM)	500 ng (625 nM)	0.25 µl
HA-SUMO1	100 µM	4 µM	4 µM	0.8 µl
E1	4.5 µM	100 nM	100 nM	0.44 µl
Ubc9	111 µM	**1 µM - 100 nM**	0	2 µl **or**
S*Ubc9	27 µM	0	**1 µM - 100 nM**	2 µl
ATP	100 mM	5 mM	5 mM	1 µl
$MgCl_2$	2 M	100 mM	100 mM	1 µl
Sumo Assay Buffer (SAB)	1 x	x µl	x µl	14.51 µl
		20 µl	20 µl	20 µl
Dilution steps of Ubc9/S*Ubc9 1 µM/500 nM/250 nM/100 nM/0				
Incubation at 30 °C for 60 minutes				

6.9.6. SUMOYLATION OF HSF1

Table 13 Conditions of the SUMO assay of the substrate His-Hsf1

Sumo assay	**Stock conc.**	Final conc. (**Ubc9**)	Final conc. (**S*Ubc9**)	**Volume**
His-Hsf1	25 µM	500 ng (333 nM)	500 ng (333 nM)	1 µl
HA-SUMO1	100 µM	4 µM	4 µM	0.8 µl
E1	4.5 µM	100 nM	100 nM	0.44 µl
Ubc9	111 µM	**1 µM - 100 nM**	0	2 µl or
S*Ubc9	27 µM	0	**1 µM - 100 nM**	2 µl
ATP	100 mM	5 mM	5 mM	1 µl
$MgCl_2$	2 M	100 mM	100 mM	1 µl
Sumo Assay Buffer (SAB)	1 x	x µl	x µl	13.76 µl
		20 µl	20 µl	20 µl
Dilution steps of Ubc9/S*Ubc9 1 µM/500 nM/250 nM/100 nM/0				
Incubation at 30 °C for 60 minutes				

6.9.7. SUMOYLATION OF MEF2A

Table 14 Conditions of the SUMO assay of the substrate GST-Mef2A

Sumo assay	**Stock conc.**	Final conc. (**Ubc9**)	Final conc. (**S*Ubc9**)	**Volume**
GST-Mef2A	1.6 µM	1 µg (641 nM)	1 µg (641 nM)	8 µl
HA-SUMO1	100 µM	4 µM	4 µM	0.8 µl
E1	4.5 µM	100 nM	100 nM	0.44 µl
Ubc9	111 µM	**1 µM - 100 nM**	0	2 µl or
S*Ubc9	27 µM	0	**1 µM - 100 nM**	2 µl
ATP	100 mM	5 mM	5 mM	1 µl
$MgCl_2$	2 M	100 mM	100 mM	1 µl
Sumo Assay Buffer (SAB)	1 x	x µl	x µl	6.76 µl
		20 µl	20 µl	20 µl
Dilution steps of Ubc9/S*Ubc9 1 µM/500 nM/250 nM/100 nM/0				
Incubation at 30 °C for 60 minutes				

6.10. PROTO ARRAY

Purified enzymes were sent to Invitrogen ProtoArray Service at – 20°C, the α-HA.11 antibody from Covance was sent at 4 °C. The probing and detection of the ProtoArray was performed by the ProtoArray Service.

E1 was used as a 4.5 µM solution and diluted to a final concentration of 100 nM in all assays. Ubc9 and S*Ubc9 were diluted from a 111 µM and 27 µM stock solution to either 100 nM or 500 nM working concentration (Table 16). HA-SUMO was diluted from a 100 µM solution to a final concentration of 4 µM. Energy regeneration solution (Boston Biochem) was used at a final concentration of 1X.

Table 15 ProtoArray Assay conditions

	Stock	100 nM Ubc9	500 nM Ubc9	100 nM S*Ubc9	500 nM S*Ubc9	Sumo/E1 only	Neg. control
HA-SUMO1 (4 µM)	100 µM	4 µl	4 µl	4 µl	4 µl	4 µl	-
E1 (100 nM)	4.5 µM	2.22 µl	2.22 µl	2.22 µl	2.22 µl	2.22 µl	-
Ubc9	111 µM	0.09 µl	0.45 µl	-	-	-	-
S*Ubc9	27 µM	-	-	0.37 µl	1.85 µl	-	-
ATP (5 mM)	100 nM	5 µl	5 µl	5 µl	5 µl	5 µl	-
Sumo Assay Buffer	1 x	88.69 µl	88.33 µl	88.41 µl	86.93 µl	88.78 µl	100 µl
	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl

The slides were blocked in 4-well QuadriPERM trays (Greiner) with 5 ml blocking buffer (50mM HEPES pH 7.5, 200mM NaCl, 0.08% Triton X-100, 25% glycerol, 20mM reduced glutathione, 1mM DTT and 1% BSA) for 60 minutes at 4°C on a shaker at 50 rpm (circular shaking).

Reactions were prepared in Sumo Assay Buffer and incubated at 30 °C for 10 minutes. Then 120 µl of each reaction mixture was applied to the slides and overlayed with a Lifterslip (Erie Scientific) taking care not to produce bubbles. Slides were incubated at 30°C for 60 minutes, followed by three 5 minute washes with 5 ml assay buffer from Invitrogen (50 mM tris pH 7.5, 50 mM NaCl, 5 mM $MgSO_4$, 0.1 % Tween 20 and 1 % BSA).

Then the arrays were washed with three 5 minute washes using 5 ml 0.5 % SDS, three 5 minute washes with 5 ml assay buffer and one 5 minute wash with 5 ml PBST (1X PBS, 0.1% Tween-20, 1X Synthetic Block). Arrays were then incubated with assay buffer containing 1:1500 dilution of mouse anti-HA antibody (end concentration of 3.3 – 4.6 µg/ml) for 90 minutes at 4 °C and then washed four times with PBST. Arrays were then incubated with 5 ml of the secondary detection

reagent AlexaFluor 647-conjugated rabbit anit-mouse IgG (Invitrogen, end concentration of 1 µg/ml) in assay buffer) at 4 °C for 90 minutes and then washed 5 times with PBST.

All arrays were quickly rinsed in water to remove residual salts. Arrays were dried by centrifugation at 1000 rpm for 2 minutes in a table top centrifuge equipped with a plate rotor. The arrays were then scanned using an Axon GenePix 4000B fluorescent microarray scanner at 668nm. Data was acquired with GenePix 6.0 software and processed in ProtoArray Prospector.

ABBREVATIONS

ADP	Adenosine di-phosphate
Aos	activation of Smt3p
AP	aprotinin
ATP	Adenosine tri-phosphate
Daxx	Death domain-associated protein 6
DTT	Dithiothreitol
FPLC	fast protein liquid chromatography
GST	glutathione-S-transferase
HA	hemagglutinin
HIS	poly-histidine
HSF	Heat Shock Factor
IPTG	Isopropyl-β-D-thiogalactopyranosid
LP	leupeptin / pepstatin
MEF2A	Myocyte Enhancement Factor 2A
NDSM	negative charged dependent SUMO motif
OD600	Optical Density at the wavelength 600 nm
PDSM	phosphorylation dependent SUMO motif
PI	protease inhibitors
PIAS	protein inhibitor of activated STAT
PML	promyelocytic leukemia
RanGAP	Ran GTP activating protein
S*	modified with SUMO
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
*	Conjugated

INDEX OF FIGURES

INDEX OF TABLES

LITERATURE

1. Geiss-Friedlander, R. & Melchior, F. Concepts in sumoylation: a decade on. *Nature reviews. Molecular cell biology* **8**, 947-56 (2007).

2. Boddy, M.N., Howe, K., Etkin, L.D., Solomon, E. & Freemont, P.S. PIC1, a novel ubiquitin-like protein which interacts with the PML component of a multiprotein complex that is disrupted in acute promyelocytic leukaemia. *Oncogene* **13**, 971-982 (1996).

3. Matunis, M., Coutavas, E. & Blobel, G. A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J. Cell Biol.* **135**, 1457-1470 (1996).

4. Mahajan, R., Delphin, C., Guan, T., Gerace, L. & Melchior, F. A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* **88**, 97-107 (1997).

5. Johnson, E.S. Protein modification by SUMO. *Annual review of biochemistry* **73**, 355-82 (2004).

6. Melchior, F. SUMO — NONCLASSICAL UBIQUITIN. *Annu. Rev. Cell Dev. Biol* **16**, 591-626 (2000).

7. Müller, S., Hoege, C., Pyrowolakis, G. & Jentsch, S. SUMO , UBIQUITIN ' S MYSTERIOUS COUSIN. *Nat. Rev. Mol. Cell Biol.* **2**, 202-210 (2001).

8. Hay, R.T. SUMO: a history of modification. *Molecular cell* **18**, 1-12 (2005).

9. Hochstrasser, M. Origin and function of ubiquitin-like proteins. *Nature* **458**, 422-9 (2009).

10. Pichler, A. *et al.* SUMO modification of the ubiquitin-conjugating enzyme E2-25K. *Nature structural & molecular biology* **12**, 264-269 (2005).

11. Shen, T., Lin, H., Scaglioni, P., Yung, T. & Pandolfi, P. The mechanisms of PML-nuclear body formation. *Mol Cell* **24**, 331-339 (2006).

12. Maderböck, K. & Pichler, A. SUMO control. *Conjugation and Deconjugation of Ubiquitin Family Modifiers Subcellular Biochemistry* 158-169 (2010).

13. Bayer, P. *et al.* Structure determination of the small ubiquitin-related modifier SUMO1. *J. Mol. Biol.* **280**, 275-286 (1998).

14. Lois, L., Lima, C. & Chua, N. Small ubiquitin-like modifier modulates abscisic acid signaling in Arabidopsis. *Plant Cell* **15**, 1347-59 (2003).

15. Kurepa, J. *et al.* The small ubiquitin-like modifier (SUMO) protein modification system in Arabidopsis. Accumulation of SUMO1 and -2 conjugates is increased by stress. *J Biol Chem* **278**, 6862-72 (2003).

16. Guo, D., Li, M. & Zhang, Y. A functional variant of SUMO4, a new I kappa B alpha modifier, is associated with type 1 diabetes. *Nat Genet* **36**, 837-41 (2004).

17. Bohren, K., Nadkarni, V. & Song, J. A M55V polymorphism in a novel SUMO gene (SUMO-4) differentially activates heat shock transcription factors and is associated with susceptibility to type 1 diabetes mellitus. *J Biol Chem* **279**, 27233-8 (2004).

18. Bylebyl, G., Belichenko, I. & Johnson, E. The SUMO isopeptidase Ulp2 prevents accumulation of SUMO chains in yeast. *J Biol Chem* **278**, 44113-20 (2003).

19. Evdokimov, E., Sharma, P. & Lockett, S. Loss of SUMO1 in mice affects RanGAP1 localization and formation of PML nuclear bodies, but is not lethal as it can be compensated by SUMO2 and SUMO3. *J Cell Sci* **121**, 4106-13 (2008).

20. Saitoh, H. & Hinchey, J. Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J Biol Chem.* **275**, 6252-8 (2000).

21. Matunis, M., Coutavas, E. & Blobel, G. A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol.* **135**, 1457-70 (1996).

22. Sramko, M., Markus, J., Kabát, J., Wolff, L. & Bies, J. Stress-induced inactivation of the c-Myb transcription factor through conjugation of SUMO-2/3 proteins. *J Biol Chem* **281**, 40065-75 (2006).

23. Tatham, M.H. *et al.* Polymeric Chains of SUMO-2 and SUMO-3 Are Conjugated to Protein Substrates by SAE1/SAE2 and Ubc9. *J Biol Chem.* **276**, 35368-74 (2001).

24. Desterro, J., Rodriguez, M., Kemp, G. & Hay, R. Identification of the enzyme required for activation of the small ubiquitin-like protein SUMO-1. *J Biol Chem.* **274**, 10618-24 (1999).

25. Desterro, J., Thomson, J. & Hay, R. Ubch9 conjugates SUMO but not ubiquitin. *FEBS Lett.* **417**, 297-300 (1997).

26. Johnson, E. & Blobel, G. Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. *J Biol Chem.* **272**, 26799-802 (1997).

27. Hochstrasser, M. SP-RING for SUMO: new functions bloom for a ubiquitin-like protein. *Cell* **107**, 5-8 (2001).

28. Jackson, P. A new RING for SUMO: wrestling transcriptional responses into nuclear bodies with PIAS family E3 SUMO ligases. *Genes Dev.* **15**, 3053-8 (2001).

29. Pichler, A., Gast, A., Seeler, J.S., Dejean, A. & Melchior, F. The nucleoporin RanBP2 has SUMO1 E3 ligase activity. *Cell* **108**, 109-20 (2002).

30. Johnson, E., Schwienhorst, I., Dohmen, R. & Blobel, G. The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. *EMBO J* **16**, 5509-19 (1997).

31. Walden, H., Podgorski, M. & Schulman, B. Insights into the ubiquitin transfer cascade from the structure of the activating enzyme for NEDD8. *2003* **422**, 330-4

32. Epps, J. & Tanda, S. The Drosophila semushi mutation blocks nuclear import of bicoid during embryogenesis. *Curr Biol* **8**, 1277-80 (1998).

33. Jones, D., Crowe, E., Stevens, T. & Candido, E. Functional and phylogenetic analysis of the ubiquitylation system in Caenorhabditis elegans: ubiquitin-conjugating enzymes, ubiquitin-activating enzymes, and ubiquitin-like proteins. *Genome Biol.* **3**, RESEARCH0002 (2002).

34. Seufert, W., Futcher, B. & Jentsch, S. Role of a ubiquitin-conjugating enzyme in degradation of S- and M-phase cyclins. *Nature* **373**, 78-81 (1995).

35. Tanaka, K. *et al.* Characterization of a fission yeast SUMO-1 homologue, pmt3p, required for multiple nuclear events, including the control of telomere length and chromosome segregation. *Mol Cell Biol* **19**, 8660-72 (1999).

36. Al-Khodairy, F., Enoch, T., Hagan, I. & Carr, A. The Schizosaccharomyces pombe hus5 gene encodes a ubiquitin conjugating enzyme required for normal mitosis. *J Cell Sci* **108**, 475-86 (1995).

37. van Wijk, S. & Timmers, H. The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. *FASEB J* **4**, 981-93 (2010).

38. Tong, H., Hateboer, G., Perrakis, A., Bernards, R. & Sixma, T. Crystal structure of murine/human Ubc9 provides insight into the variability of the ubiquitin-conjugating system. *J Biol Chem.* **272**, 21381-7 (1997).

39. Bernier-Villamor, V., Sampson, D. a, Matunis, M.J. & Lima, C.D. Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. *Cell* **108**, 345-56 (2002).

40. Reverter, D. & Lima, C.D. Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup 358 complex. *Nature* **435**, 687-692 (2005).

41. Yunus, A.A. & Lima, C.D. Lysine activation and functional analysis of E2-mediated conjugation in the SUMO pathway. *Nature Structural & Molecular Biology* **13**, 491-499 (2006).

42. Knipscheer, P. *et al.* Ubc9 sumoylation regulates SUMO target discrimination. *Molecular cell* **31**, 371-82 (2008).

43. Johnson, E. & Gupta, A. An E3-like factor that promotes SUMO conjugation to the yeast septins. *Cell* **106**, 735-44 (2001).

44. Kagey, M., Melhuish, T. & Wotton, D. The polycomb protein Pc2 is a SUMO E3. *Cell* **113**, 127-37 (2003).

45. Weger, S., Hammer, E. & Heilbronn, R. Topors acts as a SUMO-1 E3 ligase for p53 in vitro and in vivo. *FEBS Lett.* **579**, 5007-12 (2005).

46. Qu, J. *et al.* Nitric oxide destabilizes Pias3 and regulates sumoylation. *PLoS One* **2**, 1085 (2007).

47. Roscic, A., Moller, A. & Calzado, M. Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2. *Mol Cell* **24**, 77-89 (2006).

48. Yang, X. *et al.* Plk1-mediated Phosphorylation of Topors Regulates p53 Stability. *J Biol Chem.* **284**, 18588–18592 (2009).

49. Cheng, C. *et al.* SUMO modifications control assembly of synaptonemal complex and polycomplex in meiosis of Saccharomyces cerevisiae. *Genes Dev.* **20**, 2067-81 (2006).

50. Hay, R. SUMO-specific proteases: a twist in the tail. *Trends Cell Biol.* **17**, 370-6 (2007).

51. Mossessova, E. & Lima, C. Ulp1-SUMO crystal structure and genetic analysis reveal conserved interactions and a regulatory element essential for cell growth in yeast. *Mol Cell.* **5**, 865-76 (2000).

52. Mukhopadhyay, D. *et al.* SUSP1 antagonizes formation of highly SUMO2/3-conjugated species. *J Cell Biol.* **174**, 939-49 (2006).

53. Mahajan, R., Gerace, L. & Melchior, F. Molecular characterization of the SUMO-1 modification of RanGAP1 and its role in nuclear envelope association. *J Cell Biol.* **140**, 259-70 (1998).

54. Duprez, E. *et al.* SUMO-1 modification of the acute promyelo- cytic leukaemia protein PML: implications for nuclear localisation. *J Cell Sci* **112**, 381–393 (1999).

55. Sternsdorf, T., Jensen, K., Reich, B. & Will, H. The nuclear dot protein sp100, characterization of domains necessary for dimerization, subcellular localization,

and modification by small ubiquitin-like modifiers. *J Biol Chem* **274**, 12555-66 (1999).

56. Muller, S. *et al.* c-Jun and p53 activity is modulated by SUMO-1 modification. *J Biol Chem* **275**, 13321-9 (2000).

57. Anckar, J. & Sistonen, L. SUMO: getting it on. *Biochemical Society transactions* **35**, 1409-13 (2007).

58. Hietakangas, V. *et al.* Phosphorylation of Serine 303 Is a Prerequisite for the Stress-Inducible SUMO Modification of Heat Shock Factor 1. *Molecular and Cellular Biology* **23**, 2953-2968 (2003).

59. Hietakangas, V. *et al.* PDSM, a motif for phosphorylation-dependent SUMO modification. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 45-50 (2006).

60. Mohideen, F. *et al.* A molecular basis for phosphorylation-dependent SUMO conjugation by the E2 UBC9. *Nature structural & molecular biology* **16**, 945-52 (2009).

61. Yang, S., Galanis, A., Witty, J. & Sharrocks, A. An extended consensus motif enhances the specificity of substrate modification by SUMO. *EMBO Journal* **25**, 5083-93 (2006).

62. Yang, X. & Grégoire, S. A recurrent phospho-sumoyl switch in transcriptional repression and beyond. *Mol Cell* **23**, 779-86 (2006).

63. Minty, A., Dumont, X., Kaghad, M. & Caput, D. Covalent modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. *J Biol Chem* **275**, 36316-23 (2000).

64. Song, J., Durrin, L., Wilkinson, T., Krontiris, T. & Chen, Y. Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *Proc Natl Acad Sci U S A* **101**, 14373-8 (2004).

65. Hecker, C., Rabiller, M., Haglund, K., Bayer, P. & Dikic, I. Specification of SUMO1- and SUMO2-interacting motifs. *J Biol Chem* **281**, 16117-27 (2006).

66. Minty, A., Dumont, X., Kaghad, M. & Caput, D. Covalent modification of p73α by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1- interacting proteins and a SUMO-1 interaction motif. *J Biol Chem* **275**, 36316–36323 (2000).

67. Gareau, J.R. & Lima, C.D. The SUMO pathway: emerging mecha- nisms that shape specificity, conjugation and recognition. *Nat. Rev. Mol. Cell Biol.* **11**, 861–871 (2010).

68. Song, J., Durrin, L., Wilkinson, T., Krontiris, T. & Chen, Y. Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *Proc Natl Acad Sci U S A* **101**, 14373-14378 (2004).

69. Meulmeester, E., Kunze, M., Hsiao, H.H., Urlaub, H. & Melchior, F. Mechanism and consequences for paralog- specific sumoylation of ubiquitin-specific protease 25. *Mol. Cell* **30**, 610–619 (2008).

70. Steinacher, R. & Schär, P. Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation. *Curr Biol* **15**, 616-23 (2005).

71. Sun, H., Leverson, J. & Hunter, T. Conserved function of RNF4 family proteins in eukaryotes: targeting a ubiquitin ligase to SUMOylated proteins. *EMBO J* **26**, 4102-12 (2007).

72. Jefferson, J., Perry, P., Tainer, J.A. & Boddy, M. A SIM-ultaneous role for SUMO and ubiquitin. *Trends in Biochemical Sciences* **33**, 201-108 (2008).

73. Hannich, J.T. *et al.* Defining the SUMO-modified proteome by multiple approaches in Saccharomyces cerevisiae. *The Journal of biological chemistry* **280**, 4102-10 (2005).

74. Baba, D. *et al.* Crystal structure of thymine DNA glycosylase conjugated to SUMO-1. *Nature* **435**, 979-82 (2005).

75. Kerscher, O. SUMO junction-what's your function? New insights through SUMO-interacting motifs. *EMBO reports* **8**, 550-5 (2007).

76. Borden, K. Pondering the promyelocytic leukemia protein (PML) puzzle: possible functions for PML nuclear bodies. *Mol Cell Biol* **15**, 5259-69 (2002).

77. Stehmeier, P. & Muller, S. Phospho-regulated SUMO interaction modules connect the SUMO system to CK2 signaling. *Molecular cell* **33**, 400-9 (2009).

78. Knipscheer, P., van Dijk, W.J., Olsen, J.V., Mann, M. & Sixma, T.K. Noncovalent interaction between Ubc9 and SUMO promotes SUMO chain formation. *The EMBO journal* **26**, 2797-807 (2007).

79. Papouli, E. *et al.* Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p. *Mol Cell* **19**, 123-33 (2005).

80. Pfander, B., Moldovan, G., Sacher, M., Hoege, C. & Jentsch, S. SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. *Nature* **436**, 428-33 (2005).

81. Ulrich, H. Mutual interactions between the SUMO and ubiquitin systems: a plea of no contest. *Trends Cell Biol* **15**, 525-32 (2005).

82. Hardeland, U., Steinacher, R., Jiricny, J. & Schar, P. Modification of the human thymine-DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover. *EMBO J* **21**, 1456-1464 (2002).

83. Baba, D. *et al.* Crystal structute of SUMO-3-modified thymine-DNA glycosylase. *J Mol Biol* **359**, 137-147 (2006).

84. Lin, J., Ohshima, T. & Shimotohno, K. Association of Ubc9, an E2 ligase for SUMO conjugation, with p53 is regulated by phosphorylation of p53. *2004* **573**, 15-18

85. Desterro, J., Rodriguez, M. & Hay, R. SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. *Mol Cell* **2**, 233-9 (1998).

86. Sternsdorf, T., Jensen, K. & Reich, B. The nuclear dot protein sp100, characterization of domains necessary for dimerization, subcellular localization and modification by small ubiquitin-like modifiers. *J Biol Chem* **274**, 12555-66 (1999).

87. Xu, Z. & Au, S. Mapping residues of SUMO precursors essential in differential maturation by SUMO-specific proteases, SENP1. *Biochem J* **386**, (2005).

88. Shao, R., Zhang, F. & Tian, F. Increase of SUMO-1 expression in response to hypoxia: direct interaction with HIF-1 alpha in adult mouse brain and heart in vivo. *FEBS Letters* **569**, 293-300 (2004).

89. Lallemand-Breitenbach, V. *et al.* Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. *Nat Cell Biol* **10**, 547-55 (2008).

90. Matic, I., Macek, B., Hilger, M., Walther, T. & Mann, M. Phosphorylation of SUMO-1 occurs in vivo and is conserved through evolution. *J Proteome Res* **9**, 4050-7 (2008).

91. Boggio, R., Colombo, R., Hay, R., Draetta, G. & Chiocca, S. A mechanism for inhibiting the SUMO pathway. *Mol Cell* **16**, 549-61 (2004).

92. Bossis, G. & Melchior, F. Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. *Mol Cell* **21**, 349-57 (2006).

93. Cheng, J., Bawa, T., Lee, P., Gong, L. & Yeh, E. Role of desumoylation in the development of prostate cancer. *Neoplasia* **8**, 667-76 (2006).

94. Gong, L., Millas, S., Maul, G. & ET, Y. Differential regulation of sentrinized proteins by a novel sentrin-specific protease. *J Biol Chem* **275**, 3355-9 (2000).

95. Hang, J. & Dasso, M. Association of the human SUMO-1 protease SENP2 with the nuclear pore. *J Biol Chem* **277**, 19961-6 (2002).

96. Zhang, H., Saitoh, H. & Matunis, M. Enzymes of the SUMO modification pathway localize to filaments of the nuclear pore complex. *Mol Cell Biol* **18**, 6498-508 (2002).

97. Yeh, E. SUMOylation and De-SUMOylation: wrestling with life's processes. *J Biol Chem* **284**, 8223-7 (2009).

98. Li, S. & Hochstrasser, M. The Ulp1 SUMO isopeptidase: distinct domains required for viability, nuclear envelope localization, and substrate specificity. *J Cell Biol.* **160**, 1069-81 (2003).

99. Ihara, M., Yamamoto, H. & Kikuchi, A. SUMO-1 modification of PIASy, an E3 ligase, is necessary for PIASy-dependent activation of Tcf-4. *Mol Cell Biol* **25**, 3506-18 (2005).

100. Wu, J., Matunis, M., Kraemer, D., Blobel, G. & Coutavas, E. Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin A homologous domain, and a leucine-rich region. *J Biol Chem* **270**, 14209-13 (1995).

101. Joseph, J., Tan, S., Karpova, T., McNally, J. & Dasso, M. SUMO-1 targets RanGAP1 to kinetochores and mitotic spindles. *J Cell Biol.* **156**, 595-602 (2002).

102. McDoniels-Silvers, A.L., Nimri, C.F., Stoner, G.D., Lubet, R.A. & You, M. Differential gene expression in human lung adenocarcino- mas and squamous cell carcinomas. *Clinical Cancer Research* **8**, 1127 (2002).

103. Ledl, A.., Schmidt, D. & Muller, S. Viral oncoproteins E1A and E7 and cellular LxCxE proteins repress SUMO modification of the retinoblastoma tumor suppressor. *Oncogene* **24**, 3810 (2005).

104. Moschos, S.J. *et al.* SAGE and antibody array analysis of melanoma-infiltrated lymph nodes: identification of Ubc9 as an important molecule in ad- vanced-stage melanomas. *Oncogene* **26**, 4216 (2007).

105. Kim, J.H.. *et al.* SUMOylation of pontin chromatin-remodeling complex reveals a signal integration code in prostate cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 20973 (2007).

106. Kobayashi, S. *et al.* FHL2, UBC9, and PIAS1 are novel es- trogen receptor alpha-interacting proteins. *Endocr. Res* **30**, 617 (2004).

107. Sentis, S., Le Romancer, M., Bianchin, C., Rostan, M.C. & Corbo, L. Sumoylation of the estrogen receptor alpha hinge region regulates its transcriptional activity. *Mol. Endocrinol.* **19**, 2671 (2005).

108. Geletu, M. *et al.* Target proteins of C/EBPalphap30 in AML: C/EBPalphap30 enhances sumoylation of C/EBPalphap42 via up-regulation of Ubc9. *Blood* **110**, 3301-9 (2007).

109. Li, M. *et al.* SUMO wrestling with type 1 diabetes. *Journal of molecular medicine (Berlin, Germany)* **83**, 504-13 (2005).

110. Mo, Y.-Y. & Moschos, S.J. Targeting Ubc9 for cancer therapy. *Expert Opin Ther Targets* **6**, 1203-16 (2005).

111. Bartek, J. & Hodny, Z. SUMO boosts the DNA damage response barrier against cancer. *Cancer cell* **17**, 9-11 (2010).

112. Okura, T. *et al.* Protection against Fas/APO-1- and tumor necrosis factor-mediated cell death by a novel protein, sentrin. *J Immunol* **157:**, 4277–4281 (1996).

113. Lee, Y., Jang, M., Lee, J., Choi, E. & Kim, E. SUMO-1 represses apoptosis signal-regulating kinase 1 activation through physical interaction and not through covalent modification. *EMBO reports* **10**, 949-55 (2005).

114. Bossis, G. & Melchior, F. SUMO: regulating the regulator. *Cell division* **1**, 13 (2006).

115. Lin, D.-Y. *et al.* Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. *Molecular cell* **24**, 341-54 (2006).

116. Takahashi, H., Hatakeyama, S., Saitoh, H. & Nakayama, K.I. Noncovalent SUMO-1 binding activity of thymine DNA glycosylase (TDG) is required for its SUMO-1 modification and colocalization with the promyelocytic leukemia protein. *J. Biol. Chem.* **280**, 5611–5621 (2005).

117. Vermeulen, N. *et al.* Identification of a novel autoantigen in inflammatory bowel disease by protein microarray. *Inflamm Bowel Dis* **17**, 1291-300 (2011).

118. Bauer, M. *et al.* Protein networks involved in vesicle fusion, transport, and storage revealed by array-based proteomics. *Methods Mol Biol* **781**, 47-58 (2011).

119. Levy, D. *et al.* A proteomic approach for the identification of novel lysine methyltransferase substrates. *Epigenetics Chromatin* **24**, 19 (2011).

120. Merbl, Y. & Kirschner, M.W. Large-scale detection of ubiquitination substrates using cell extracts and protein microarrays. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 2543-8 (2009).

121. Lyons, G.E., Micales, B.K., Schwarz, J., Martin, J.F. & Olson, E.N. Expression of Mef2 genes in the mouse central nervous system suggests a role in neuronal maturation. *J. Neurosci.* **15**, 5727–5738 (1995).

122. Castro, I. A SUMOylation motif in Aurora-A: implications for spindle dynamics and oncogenesis. *Frontiers in Oncology* **1**, 1-16 (2011).

123. Holmberg, C.I. *et al.* Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *The EMBO journal* **20**, 3800-10 (2001).

CURRICULUM VITAE

PERSÖNLICHE DATEN

Name	Lisa Kirschner
Geburtsdatum	18.März 1985
Geburtsort	Wien
Nationalität	Österreich
Kontakt	lisa.kirschner@gmx.at

AUSBILDUNG

Seit 2003	Diplomstudium der Molekularen Biologie
08-12/2008	Erasmus Stipendium in Kopenhagen
Juni 2003	Matura mit gutem Erfolg am BG Perchtoldsdorf
1995-2003	BG Perchtoldsdorf, neusprachlicher Zweig
1991-1995	Volkschule Rodaun

PRAKTIKA

09-12/2010	Wahlbeispiel bei Dr. Ralf Steinborn, Veterinär Universität Wien
05/2009- 05/2011	Diplomarbeit bei Dr. Andrea Pichler
04/2009	Praktikum bei AWS
09- 12/2008	Wahlbeispiel bei Dr. Cord Brakebusch
01-06/2008	Mitarbeit bei Dr. Jürgen Knoblich
04/2006-06/2008	Mitarbeit bei Dr. Andrea Pichler