

USPL1, a novel SUMO isopeptidase

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Affidavit

I hereby declare that this doctoral thesis has been written only by the undersigned and without any assistance from third parties.

Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Göttingen, 30th January 2009

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ABSTRACT

Small Ubiquitin-like **M**odifiers (SUMO) are 10 kDa proteins that are covalently attached to hundreds of intracellular proteins to regulate their function. In mammals, three members of the SUMO family are known to be conjugated (SUMO-1,-2,-3). Desumoylating enzymes (isopeptidases) play an essential role by ensuring reversibility of this posttranslational modification. At present, only a small number of these enzymes, members of the Ulp/SENp family, are known. They share a conserved catalytic cysteine protease domain, C48, while remaining quite different in other regions. Mammals express only 6 distinct SENp proteases. This number appears extremely small, if one considers the plethora of SUMO targets that are individually regulated by reversible modification. For comparison, more than 80 different Ubiquitin proteases are currently known. This let us suspect that as yet undiscovered SUMO-specific isopeptidases exist.

The goal of this work was to identify and perform initial characterization of a novel SUMO specific isopeptidase. Here I describe the approaches I undertook to find such an enzyme. The first approach used a FRET-based desumoylating assay developed in our laboratory. I adapted this assay to a high-throughput screen, and screened a partial bacterial expression library of human ORFs. While positive controls could easily be identified, this approach did not result in identification of a novel SUMO isopeptidase. This was due, at least in part, to the small size of the available library. The second approach was based on a biochemical purification strategy using HeLa cell lysates and HA-epitope tagged SUMO-Vinylmethylester (SUMO-VME), a SUMO derivate that specifically and irreversibly reacts with desumoylating enzymes. In addition to enriching already known isopeptidases, this resulted in the identification of USPL1 (**U**biquitin **S**pecific **P**rotease **L**ike 1) as a protein that reacts with SUMO-VME. USPL1 is present in all vertebrates and lower invertebrates but absent in, e.g., fungi, *C. elegans* and plants. It is necessary for zebra fish development. Interestingly, USPL1 is not related to SENPs, but belongs to the **U**biquitin **S**pecific **P**rotease (USP) family. This family has a C19 cysteine protease domain. Upon expression and purification of the catalytic domain of USPL1 I could demonstrate that it indeed is a SUMO specific isopeptidase that exhibits a high specificity for SUMO-2/3, works less efficiently on SUMO1, but does not cleave Ubiquitin. Initial experiments suggest that USPL1 is a nuclear protein and database search revealed a possibility that it may be upregulated upon heat shock.

Abbreviations

AMC - 7-amino-4-methylcoumarin

APS - ammonium persulfate

ATP - Adenosine-5'-triphosphate

Atg12 - autophagy 12 protein

AVP1 - adenoviral protease-1

BSA - bovine serum albumine

CFP - cyan fluorescent protein

DNA - deoxyribonucleic acid

dNTP - Deoxyribonucleotide triphosphate

DEN-1 - Deneddylase-1,

DMEM - Dulbecco's Modified Eagle's Medium

DTT - dithiothreitol

EDTA - ethylenediaminetetraacetic acid

FboxWD40.5 – F-box and WD repeat domain containing 5

FRET - Fluorescence resonance energy transfer

HA - hemagglutinin

HBM - HCF-1 binding motif

HCF-1 Host cell factor 1

HECT - Homologous to E6- Associated Protein (E6AP) C-Terminus

HEK - Human embryonic kidney

GMP-1 - Gap Modifying Protein 1

GST - Glutathione S-transferase

IPTG - isopropyl β -D-1-thiogalactopyranoside

ISG-15 - Interferon-stimulated gene-15

kDa - kiloDalton

kB - Kilobasepair

LB - Luria Bertani

MESNa - Sodium methanethiolate

mRNA - Messenger ribonucleic acid

Nedd8 - Neural precursor cell-expressed developmentally downregulated 8
NPC – nuclear pore complex
OGT-1 O-linked GlcNAc transferase
ORF - open reading frame
OV - ovalbumine
PAA - polyacrylamide
Pc2 - Polycomb protein 2
PBS - Phosphate buffered saline
PCNA - proliferating cell nuclear antigen
PCR - polymerase chain reaction
PIAS - protein inhibitor of activated STAT
PML - Promyelocytic leukaemia
PMSF - phenylmethanesulphonylfluoride
RanBP2 - Ran binding protein 2
RanGAP1 - Ran GTPase activating protein 1
RIPA - Radioimmunoprecipitation assay
RING - Really Interesting New Gene
RNF4 - RING finger protein 4
rRNA - ribosomal ribonucleic acid
SAB - sumoylation assay buffer
SBM - SUMO binding motif
SDS - sodium dodecyl sulfate
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
SENP - SUMO/Sentrin- specific protease
SIM - SUMO interaction motif
Smt3 - suppressor of mif two 3
STUbl - SUMO-Targeted Ubiquitin Ligase
SUMO - Small Ubiquitin-like protein modifier
TEMED - tetramethylethylenediamine
TEV - Tobacco Etch Virus
TDG - Thymine DNA glycosylase

TOPORS - topoisomerase I binding, arginine/serine-rich

Ub - Ubiquitin

UCH – Ubiquitin C-terminal hydrolase

Ulp1 - Ubl specific protease 1

Ufm - Ubiquitin-fold modifier 1,

USP – Ubiquitin Specific protease

USPL1 – Ubiquitin specific protease like protein 1

VME - Vinylmethylester

VS - vinylsufon

YFP – yellow fluorescent protein

WB – Western Blot

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1. INTRODUCTION

1.1 Protein modification with Ubl

Posttranslational protein modifications allow the expansion of the cell's proteome without increasing its genome size. In some cases, this can ensure a faster cellular response to changing conditions or stimuli without the need for new/additional protein synthesis. It is well documented that target proteins can be modified by the addition of phosphate groups, sugars, fatty acids or even entire polypeptides to confer some activity that is distinct from the unmodified native protein. Polypeptides as posttranslational modifications were first described when Ubiquitin was shown to be covalently attached to histones (Goldknopf & Bush, 1975). At that time, neither the function nor the mechanism of this attachment were known. Work pioneered by Avram Hershko and Aaron Ciechanover elucidated the role of ubiquitin in ATP-dependent protein degradation (Ciechanover *et al.*, 1980, Hershko *et al.*, 1980, Wilkinson *et al.*, 1980). For their discovery they were awarded the Nobel prize. Till date, many proteins functioning in a manner similar to Ubiquitin have been discovered and are collectively known as Ubiquitin like-proteins or Ubls (Welchman *et al.*, 2005, Kerscher *et al.*, 2006). Among these, Ubiquitin is best characterized for its role in protein degradation, SUMO for the regulation of a variety of cellular processes, and Atg12 and Atg8 for a role in autophagy. A list of the currently known Ubls that are expressed in human cells and their relation to ubiquitin are summarized in Table 1. Although the sequence homology between the Ubls is not high, they share a conserved 3 dimensional fold, termed the Ubiquitin fold or β -grasp (Figure 1) (Welchman *et al.*, 2005, Kerscher *et al.*, 2006). Another common feature of Ubls is a glycine residue at the C-terminus and often two glycins are present (Gly-Gy motif). The carboxy group of the C-terminal glycine forms an isopeptide bond with the epsilon amino group of a lysine residue of a substrate. A general outline of protein modification with Ubl is shown in Figure 2. Most Ubls

are expressed as inactive precursors in which the glycine used for conjugation is followed by a short peptide, which renders it unavailable for conjugation with

<i>Ubl</i>	<i>% identity with Ubiquitin</i>
<i>Ubiquitin</i>	100
<i>Nedd8</i>	52
<i>FAT10</i>	30 / 42
<i>ISG15</i>	32 / 37
<i>SUMO-1</i>	20
<i>SUMO-2</i>	16
<i>SUMO-3</i>	16
<i>SUMO-4</i>	15

Table 1. Some of the Ubls expressed in human and their relationship to Ubiquitin. In case of FAT10 and ISG15, which have two beta-grasp domains, sequence identity is shown for each domain.

substrate. C-terminal hydrolases cleave this peptide bond, exposing the carboxy group of the glycine for conjugation to protein substrate (Amerik and Hochstrasser 2004, Love *et al.*, 2007). Ubl processed in such way can be conjugated to target proteins via an enzymatic cascade involving at least 3 steps (reviewed in Hershko & Ciechanover 1998). First, the C-terminus of Ubl is activated in an ATP dependent manner by E1 activating enzyme (E1), and a thioester bond is formed between Ubl and the catalytic cysteine of E1. The second step involves transfer of Ubl to the active cysteine of E2 conjugating enzyme (E2). The third step is transfer of Ubl to a lysine residue in substrate protein either directly from E2 or with the help of E3 ligases. For Ubiquitin two distinct classes of E3 ligases have been identified: HECT- and RING-Type. HECT E3 ligases have a catalytic cysteine to which thioester charged Ubl is transferred from E2 and subsequently put on a target. RING-type E3 ligases do not have such a catalytic activity, instead they bring the E2 enzyme and a substrate together. (Hershko & Ciechanover 1998, Welchman *et al.*, 2005, Kerscher *et al.*, 2006).

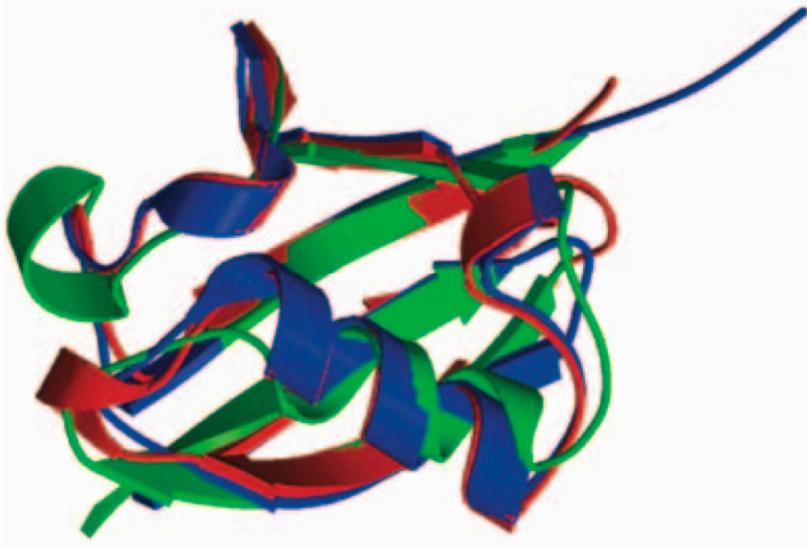


Figure 1. Overlay of Ubl structures. Ubiquitin is shown in blue, SUMO in green and Nedd8 in red. (Taken from Welchman et al 2005)

Protein modification with Ubl is a fully reversible process due to the action of isopeptidases. These enzymes cleave an isopeptide bond between Ubl and a target. Both the target and the Ubl can be used for a next round of conjugation (Amerik and Hochstrasser 2004, Nijman *et al.*, 2005).

There is only one known class of E1 and E2 enzymes (Welchman *et al.*, 2005, Kerscher *et al.*, 2006) and these are related for the different Ubls. In contrast, Ubl proteases for the specific Ubls can be very different from each other. For example SUMO proteases belong to the SENP/Ulp family, Ufm-1 is processed by completely unrelated UfSP proteases and Ubiquitin proteases fall into five

different classes, none of them related to SENP/Ulp or UfSPs (Amerik and Hochstrasser 2005, Routenberg *et al.*, 2007).

Even though Ubl enzymes are specific for one Ubl, some of them can work on more than one Ubl (Kerscher *et al.*, 2006). For example, E1-L2 is an activating

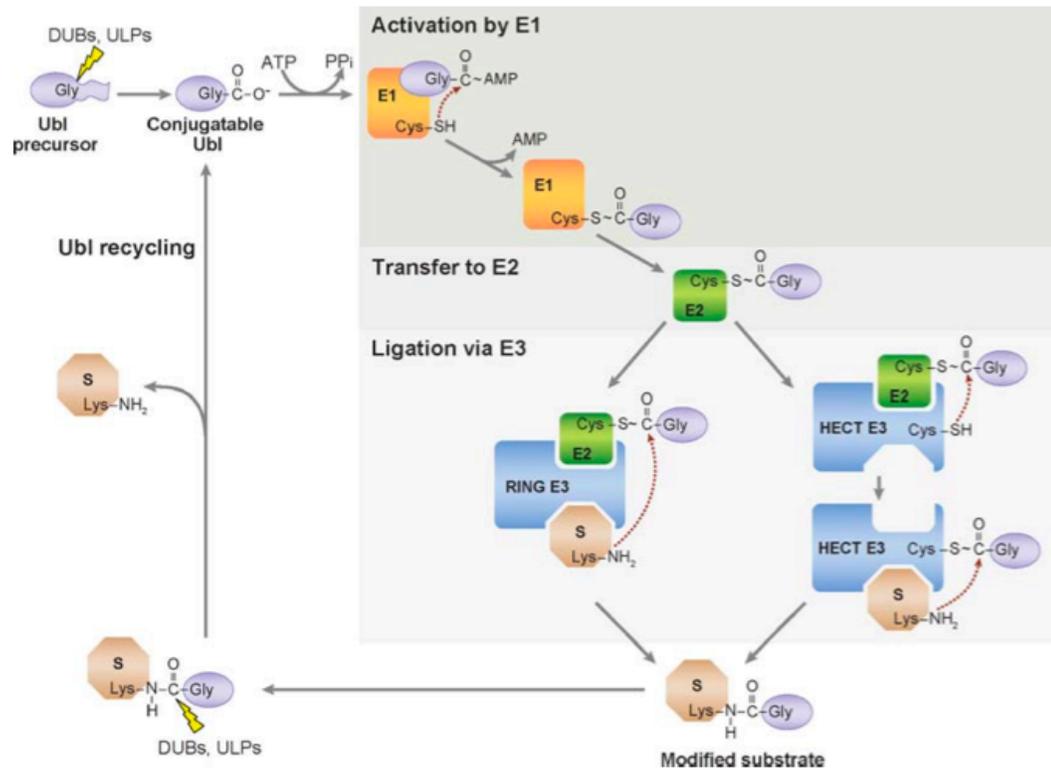
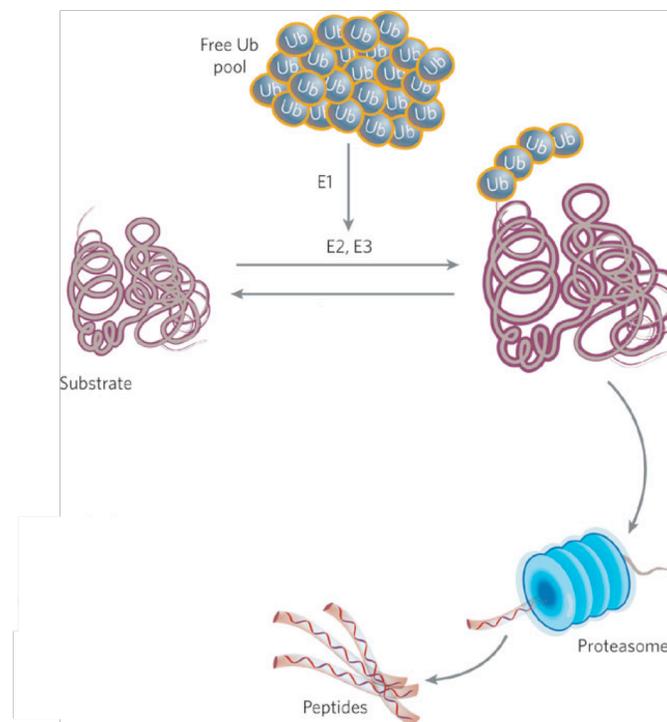


Figure 2. A General outline of the protein modification with Ubl mechanism. Newly synthesized Ubl is processed by a protease. The processed form can be activated in ATP dependent manner by an E1 activating enzyme, which transfers it to an E2 conjugating enzyme. The final step of modification involves formation of an isopeptide bond between the carboxy group of C-terminal glycine in Ubl and the amino group of a lysine residue in the substrate. Modification is reversed by action of isopeptidases. After deconjugation both Ubl and target can undergo the next round of modification (taken from Kerscher *et al.* 2006).

(E1) enzyme for Ubiquitin and FAT10 (Chiu *et al.*, 2007), while UCH-L1 and USP21 are proteases with dual specificity for Ubiquitin and Nedd-8 (Wada *et al.*, 1998, Gong *et al.*, 2000).

1.1.1 Ubiquitin-mediated protein degradation

The best known and characterized function for Ubis is ubiquitin-mediated protein degradation. Figure 3 outlines this process. Proteins destined for degradation are modified with Ubiquitin chains, in which the ubiquitin that is placed directly on a target is itself an attachment site for a second ubiquitin molecule, which becomes an attachment site for a third one and so on. The growing chain is being recognized by ubiquitin binding proteins, which direct such polyubiquitinated



proteins for degradation by the proteasome, a large, multisubunit protease (Hochstrasser 1996, Hershko and Ciechanover 1998, Bochtler *et al.*, 1999).

Figure 3. A general outline of Ubiquitin mediated protein degradation. (taken from Rubinsztein 2006).

1.2 SUMO

SUMO (**S**mall **u**biquitin like **m**odifier), also known as Sentrin or GMP-1, is a subfamily of UbIs (Melchior 2000, Johnson 2004). Different organisms vary in the number of SUMO proteins they express. *S. cerevisiae*, *D. melanogaster* and *C. elegans* have only one SUMO protein. Lower vertebrates like *D. rerio* or *X. leavis* have two and mammals have 3 SUMO proteins (SUMO-1,2,3). Primates have a fourth SUMO gene encoding protein SUMO-4, however it is unclear whether it is conjugated to protein substrates or not, owing to the presence of a proline residue, believed to inhibit its processing by SENPs (Owerbach *et al.*, 2005). The SUMO pathway is essential in many organisms including *S. cerevisiae*, *C. elegans* or *A. thaliana* (Johnson *et al.*, 1997, Fraser *et al.*, 2000, Saracco *et al.*, 2007). Human SUMO-1 was discovered as a protein modifying RanGAP and targeting it to nuclear pore complexes (Mahajan *et al.*, 1997, 1998; Matunis *et al.*, 1996, 1998). Soon after, SUMO-2 and 3 were discovered, and it became clear that sumoylation is a commonly used process. SUMO-2 and SUMO-3 are 97% identical, differing mostly within the C-terminal peptide after the Gly-Gly motif, and they both are 50% identical to SUMO-1. So far there is no evidence for functional differences between SUMO-2 and SUMO-3 and they are often referred to as SUMO-2/3. Whereas Ubiquitin is highly conserved in evolution (97% identity between Ubiquitin from human and yeast) SUMO proteins are conserved to a much lower extent between species. All three human SUMOs share only 50% identity with the *S. cerevisiae* SUMO, Smt3p (Lapenta *et al.* 1997, Kamitani *et al.*, 1998). A unique feature of the SUMO family is the N-terminal tail preceding the β -grasp domain. Many targets can be modified with either SUMO-1 or SUMO-2/3, however a number of targets are specifically modified with one isoform only even *in vitro* (Johnson 2004, Meulmeester *et al.*, 2008, Zhu *et al.*, 2008). SUMO-1 is present in the cell predominantly in form of conjugates. SUMO-2/3 under non-stress conditions are present mainly in the unconjugated form. When stress conditions are applied, SUMO-2/3 are rapidly conjugated to their targets (Saitoh *et al.*, 2000). Another feature differing between SUMO-1 and

SUMO-2/3 is chain formation *in vivo*. The N-terminal tail of SUMO-2/3 has a Sumoylation consensus site that is absent from SUMO-1 (Tatham *et al.*, 2001) and this may be the reason that only SUMO-2/3 can form chains *in vivo*. It is not currently known whether modification of a given protein with SUMO-1 has the same or different outcome than modification with SUMO-2, and vice versa.

1.2.1 SUMO modifying enzymes

In contrast to other Ubl activating enzymes, which are single polypeptide chains, the SUMO E1 is composed of two subunits, Aos1 and Uba2. Aos1 corresponds to the N-terminal part of the ubiquitin E1 and Uba2 to its C-terminal part. Both subunits associate together to form a stable enzymatically active complex (Johnson *et al* 1997). After ATP dependent activation, SUMO is transferred to the E2 conjugating enzyme, Ubc9 (Desterro *et al.*, 1997). In contrast to ubiquitination, sumoylation can occur efficiently without E3 and can take place at a specific lysine residue within the SUMO consensus motif. This motif was identified by aligning SUMO-1 modification sites in different targets, and consists of ΨKxE , where Ψ is a bulky hydrophobic residue and x is any residues. Ubc9 interacts directly with this motif, which explains both lysine residue specificity and conjugation without E3 (Bernier-Villamor *et al.* 2002). However, the affinity of Ubc9 for the consensus site is very low and therefore in most cases additional factors are necessary for efficient sumoylation. The first discovered SUMO E3 ligases were Siz1 and Siz2 in *S. cerevisiae*. Siz1 is required for SUMO attachment to the *S. cerevisiae* septins *in vivo* and strongly stimulates septin sumoylation *in vitro* (Johnson and Gupta 2001). Its homologues in animals belong to the PIAS (protein inhibitor of activated **STAT**) family. PIAS/Siz proteins have an SP-RING domain, which is similar to the RING domain of Ubiquitin E3 ligases and believed to have an analogous function (Hochstrasser 2001). PIAS proteins are expressed in all eukaryotes and are implicated in a number of processes including cytokine signaling, hormone signaling and DNA repair (Palvimo 2007). A special type of E3 ligase so far unique to the SUMO pathway

is RanBP2, a nucleoporin localized to the cytoplasmic side of nuclear pore complexes (Pichler *et al.* 2002). It does not belong to either the HECT or RING class of E3 ligases, however it enhances sumoylation by placing SUMO-charged Ubc9 in an optimal position for SUMO transfer (Pichler *et al.*, 2004, Reverter and Lima 2005). RanBP2 *in vitro* enhances sumoylation of many targets, however *in vivo* only topoisomerase II and borealin have been identified as targets (Dawlaty *et al.*, 2008, Klein *et al.*, 2008). Additional proteins e.g. polycomb protein 2 and TOPORS have also been reported to have a SUMO E3 ligase activity, but these findings still need to be confirmed (Kagey *et al.*, 2003, Weger *et al.*, 2005).

1.2.2 Non covalent SUMO interaction

In contrast to the Ubiquitin pathway, for which more than 20 Ubiquitin-binding domains have been identified (reviewed in Hicke *et al.*, 2005), a single SUMO binding motif called SBM (**S**UMO **B**inding **M**otif) or SIM (**S**UMO interaction **m**otif) is known (Song *et al.*, 2004 and 2005, Hecker *et al.*, 2006). This motif consists of hydrophobic residues flanked N- or C-terminally by acidic residues or serins and interacts with beta2-strand of SUMO (Song *et al.*, 2004 and 2005, Hecker *et al.*, 2006). It has been identified in a number of SUMO enzymes and targets (Song *et al.*, 2004, Sehn *et al.*, 2006, Knipscheer *et al.*, 2007, Meulmeester *et al.*, 2008)

1.2.3 Outcomes of SUMO modification

Similar to other Ubls, conjugation of SUMO changes the interaction partners or the conformational state of the substrate protein, therefore the outcomes can be different for different targets and hard to predict. SUMO attachment to a target can provide a new surface for protein-protein interaction, thereby allowing for new binding partners. Alternatively, SUMO can mask existing interaction sites, therefore excluding interaction with proteins binding to the unmodified target. A third mode in which sumoylation influences the protein function is by inducing conformational changes in target. This is mediated via non-covalent interaction

between SUMO and target's SIM (Geiss-Friedlander and Melchior 2007) (Figure 4).

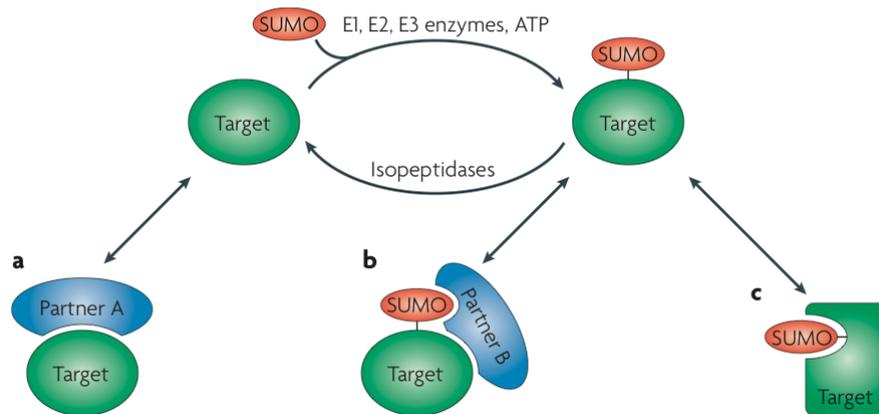


Figure 4. Molecular outcomes of sumoylation. a) Sumoylation interferes with protein-protein interaction by masking of the binding surface in the target. b) Sumoylation induces new interaction by providing additional surface for binding partners. c) Sumoylation induces a conformational change in the target (taken from Geiss-Friedlander and Melchior 2007).

Selected examples for physiological outcomes of sumoylation are given below:

1. Localization

Sumoylation targets vertebrate RanGAP1 to nuclear pore complexes (NPC) by allowing interaction with RanBP2 – a nucleoporin localized at the cytoplasmic side of NPC (Mahajan *et al.*, 1997, 1998; Matunis *et al.*, 1996, 1998). Another example of SUMO regulated localization is targeting of sumoylated proteins to PML-nuclear bodies via SIM mediated interactions between SUMO and PML protein (Shen *et al.*, 2006).

2. Transcriptional repression

Many transcription factors have been identified as targets for sumoylation. In most cases the modification represses the transcription. According to a current model, sumoylation of a transcription factor results in recruitment of transcriptional repressors or chromatin modifying enzymes that inhibit transcription by histone modifications (Geiss-Friedlander and Melchior., 2007). A recent siRNA screen in *D. melanogaster* identified MEP-1, Mi-2, and Sfmbt as SUMO-dependent repressors of Sp3 transcription (Stielow *et al.*, 2008).

3. DNA metabolism and repair

PCNA (proliferating cell nuclear antigen) functions as a sliding clamp during DNA replication. Sumoylation of PCNA prevents unwanted homologous recombination during DNA replication by recruiting antirecombinogenic helicase Srs2 (Pfander *et al.*, 2005). Thymine DNA glycosylase (TDG) is an enzyme involved in DNA mismatch repair and undergoes sumoylation as part of its catalytic cycle. A current model suggests that sumoylation induces a conformational change in enzyme bound to DNA, which results in dissociation from DNA (Hardeland *et al.*, 2002, Baba *et al.*, 2005).

4. Signaling

SUMOylation has also been reported for a number of proteins involved in signaling. For example, a recent study demonstrates that TGF- β receptor is modified by SUMO upon TGF- β stimulation. This enhances signaling by facilitating the recruitment and phosphorylation of Smad3 (Kang *et al.*, 2008).

5. Protein stabilization/degradation

It has been reported for I kappa B that sumoylation and ubiquitination are mutually exclusive since the same lysine residue is target for both SUMO and Ubiquitin modification (Desterro *et al.*, 1998). This observation led to the suggestion that SUMO can act as a repressor of protein degradation. Interestingly, recent findings have revealed that SUMO can also stimulate protein degradation by recruitment of the RNF4/STUbl ubiquitin E3 ligases (Prudden *et al.*, 2007, Sun *et al.*, 2007, Uzunova *et al.*, 2007, Xie *et al.*, 2007, Lallemand-Breitenbach V *et al.*, 2008, Tatham *et al.*, 2008).

1.3 SUMO proteases

SUMO proteases catalyze hydrolysis of a peptide (peptidase/C-terminal hydrolase activity) or isopeptide bond (isopeptidase activity). In spite of their biochemical similarity, the outcomes and functions of these two reactions are very different. Like most Ubls SUMO proteins are synthesized as inactive precursors in which the C-terminal Gly-Gly motif is followed by a short peptide. Hydrolysis of the peptide bond between the last glycine and the following residue exposes the carboxy group of the glycine and makes SUMO available for conjugation. The isopeptidase activity of SUMO proteases results in their ability to remove SUMO from targets, thereby reversing modification (Melchior *et al.*, 2003, Hay 2007, Yeh 2008). So far all known eukaryotic SUMO proteases belong to the Ulp/SEN1 family (Dasso 2007, Yeh 2008). The first SUMO protease was identified in yeast, employing a biochemical screen (Li and Hochstrasser 1999). Pools of bacterial transformants expressing yeast proteins were assayed for their ability to cleave a model substrate – His-Ubiquitin-Smt3c-HA. This screen led to the identification of a previously uncharacterized protein that cleaved SUMO but not ubiquitin. It had no strong similarity to ubiquitin or any other Ubl protease and was named **Ubl specific protease 1 (Ulp1)**. Database searches revealed a number of proteins in different organisms that shared similarity within a single

domain of around 200 amino acids. Inhibitor studies, mutational analysis and sequence alignments resulted in characterization of this domain as the catalytic core of Ulp1. Biochemically, Ulp1 is a cysteine protease with a conserved catalytic triad composed of cysteine, aspartate and histidine in a domain referred to as the “C48 protease domain” This will be henceforth referred to as “catalytic domain” for clarity. Database searches for proteins that have the same conserved catalytic triad led to discovery of a weak similarity between the catalytic domain of Ulp1 and processing proteases of several adenoviruses. Interestingly these proteases cleave a consensus motif similar to glycine-glycine-X of SMT3 and SUMO (Lopez-Otin *et al.*, 1999). Human proteins that have C48 domains were named SENP – for **Sen**trin/SUMO specific protease (Yeh *et al.*, 2000).

1.3.1 SENP/Ulp family

Members of the Ulp/SENP family are present in all eukaryotes. *S. cerevisiae* has two proteins (Ulp1 and Ulp2) while humans have 7 proteins with a C48 domain – SENP1,2,3,5,6,7,8. The family has three main branches: Ulp1-like, Ulp2-like and DEN-1 like (Figure 5). The DEN-1 like branch diverged from the other two branches quite early in evolution (Dasso 2007) and these enzymes are not SUMO specific proteases in spite of having C48 domains. Rather, they act on another Ubl, Nedd8 (Gan-Erdene *et al.*, 2003, Wu. *et al.*, 2003). Members of Ulp1-like and Ulp2-like branches are specific for SUMO. The generic member of the Ulp/SENP family has a C-terminal catalytic domain and a N-terminal domain that contains sequences responsible for the localization. The catalytic domain of the Ulp2-like branch has conserved insertions within the catalytic domain that are absent from Ulp1-like enzymes (Dasso 2007, Reverter and Lima 2008).

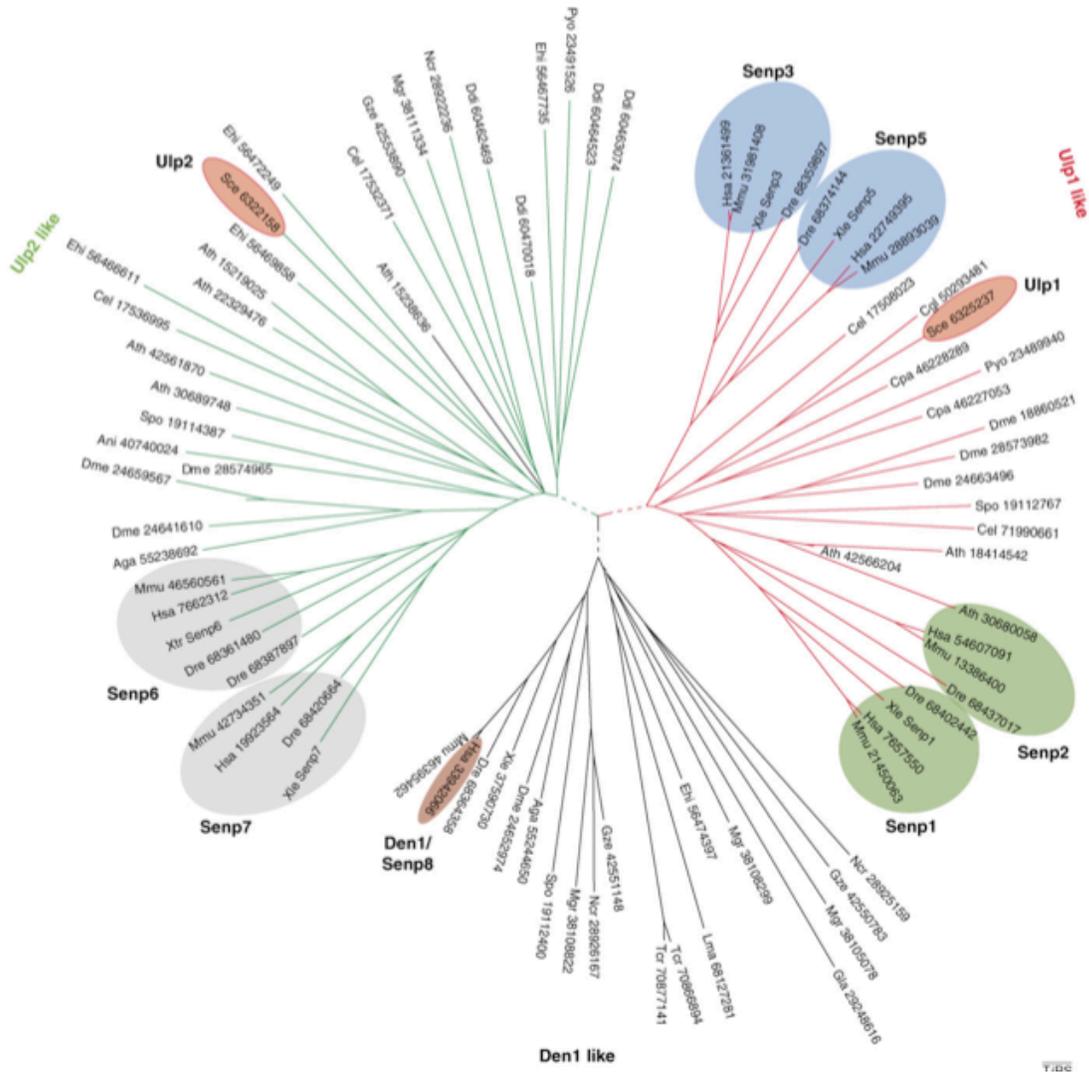


Figure 5. Evolutionary relationship of the Ulp/SENP family members. The Ulp1 like branch including human SENP1,2,3 and 5 is shown in red, the Ulp2 like branch including human SENP6 and 7 is shown in green. The Den1 like branch of enzymes specific for Nedd8, which includes human SENP8 is shown in black (taken from Dasso 2007).

1.3.2 Structure and catalytic mechanism of SUMO proteases

The crystal structure of the C48 domain has been solved for several members of the Ulp1-like branch and for one member of the Ulp-2 like branch, SENP7. The structures provide insights into the mode of SUMO recognition by the catalytic domain and suggest a model for catalysis.

The C48 domain can be divided into two parts: an N-terminal subdomain rich in alpha helices that contains the catalytic cysteine and a C-terminal part composed of five anti-parallel beta strands surrounded by 2 alpha helices, that contains the remaining two residues of the triad (Reverter and Lima 2004). The catalytic domain of SENP shows the highest degree of structural similarity with adenoviral protease-1 fragment (AVP1), representing a prototype of this family of cysteine proteases. AVP1 was reported to exhibit a deubiquitinating activity, however it appears that its physiological function is restricted to processing of viral proteins (Balakirev *et al.*, 2002, Mangel *et al.*, 1993).

In case of ubiquitin specific proteases, residues forming the active site are often misaligned in the structure prior to ubiquitin binding. Ubiquitin binding induces structural rearrangements resulting in formation of the active site. In contrast, the C48 active site is already preformed in the absence of SUMO. The active site is localized to a cavity on the enzyme's surface (Reverter and Lima 2004). Structures of catalytic mutants of SENP1 and SENP2 catalytic domains in complex with sumoylated RanGAP show no specific interaction between RanGAP and the C48 domain, indicating lack of specificity towards specific targets (Reverter and Lima 2006, Shen *et al.*, 2006). Specificity observed *in vivo* must therefore come either from specific interaction of targets with the N-terminal part of the protease or from limiting the localization of the protease to distinct cellular compartments (Reverter and Lima 2006, Dasso 2007).

The structure of a covalent thiohemiacetal transition-state complex of SENP2's catalytic domain with SUMO-1 showed that SUMO is recognized at two different sites, the surface of the globular core and the C-terminal tail (Figure 6). Surface recognition depends on salt bridges between side chains of arginin in SUMO

(R63) and a conserved aspartic acid (D413) in the catalytic domain as well as a conserved phenylalanine residue (F441) of the catalytic domain, which is inserted into a hydrophobic pocket on the SUMO surface. Of note, the mutation of the corresponding residues in Ulp1 results in conditional lethality in yeast, indicating the importance of this interaction. The second site of recognition is localized to the C-terminus of SUMO, which adopts the structure of an elongated beta strand and inserts into a hydrophobic tunnel leading to the active site, in which the Gly-Gly motif is capped by conserved tryptophan residues (W410 and W479) (Reverter and Lima 2004). Biochemical analysis revealed that SENPs exhibit specificity towards the type of reaction (deconjugation vs. processing) and SUMO paralogues. Structures of catalytic mutants of either SENP1 or SENP2 catalytic domain in complex with unprocessed SUMO or sumoylated RanGAP led to a model for the mechanism of catalysis and provided a structural basis for SENP specificity (Reverter and Lima 2006, Shen *et al.*, 2006). SENP binds SUMO non-covalently and binding induces cis-trans isomerization of the scissile bond. As a result the amide bond is in a favorable position for the attack by the catalytic cysteine. SENP specificity is determined by two factors: binding affinity to the SUMO paralogue, and SENP's ability to isomerize the scissile bond (Reverter and Lima 2006, Shen *et al.*, 2006). The latter depends on how well the enzyme accommodates the C-terminal side of the scissile bond and how rigid are the residues that are found there. SENP1 binds SUMO-1 and SUMO-2 equally well and deconjugates it from targets with similar efficiencies, however it processes efficiently only full-length SUMO-1. This discrepancy is due to the fact that in the conjugated form SUMO-1 and SUMO-2 have the same C-terminal side of the isopeptide bond, the lysine side chain, but the full length forms differ significantly. In SUMO-2 a rigid proline residue in position P2 to the scissile bond interferes with isomerization, whereas in SUMO-1 a histidine residue in position P1 stabilizes the *cis* conformation of the scissile peptide bond (Shen *et al.*, 2006). SENP2 binds SUMO-2 better than SUMO-1, which explains its preference for SUMO-2-modified substrates. It also seems to be less efficient at isomerizing

scissile bonds in full length SUMO, which explains why it is less active in processing (Shen *et al.*, 2006).

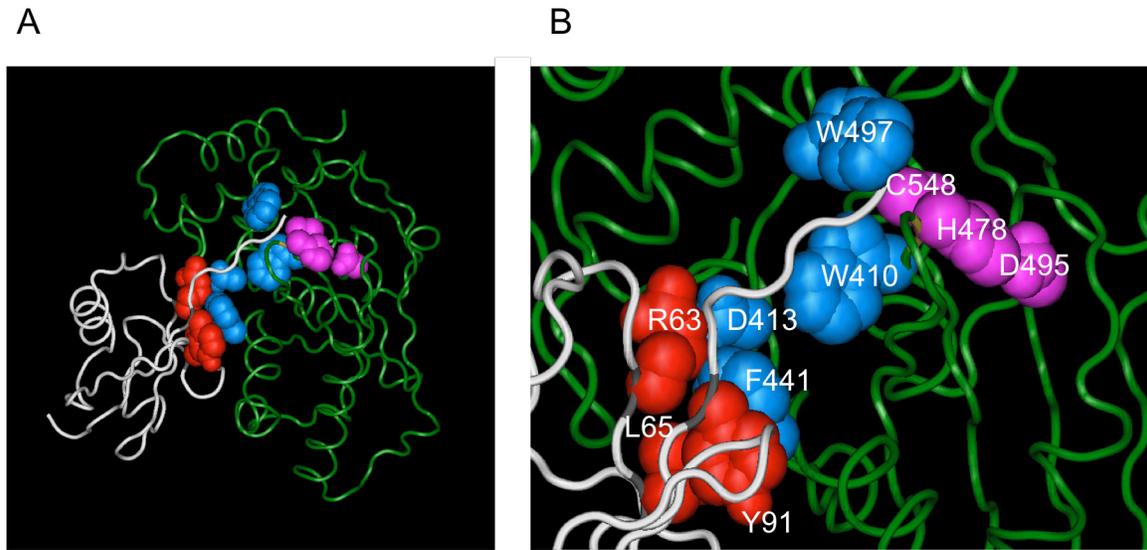


Figure 6. The structure of the SENP2/SUMO1 complex. A) General arrangement of the SENP2/SUMO-1 complex. SENP2 and SUMO-1 backbones are shown in green and white respectively. SENP2 residues participating in SUMO-1 binding are shown in blue and the residues of the catalytic triad in magenta. SUMO-1 residues mediating binding to SENP2 are shown in red. B) SUMO-1 and SENP2 residues participating in binding and catalysis. Color scheme same as in panel A. (Prepared using Cn3D application and PDB file 2HD5)

A recently solved structure of SENP7, which exhibits specificity for SUMO-2 chains, revealed that Ulp2-like proteases differ from Ulp1 not only in sequence but also in structure (Reverter and Lima 2008). Although the structures are similar, they do not align well. SENP7 lacks some elements present in Ulp1 and contains several additional loops. These loops correspond to the insertions observed in the sequence of Ulp2 like proteases. So far there is no structure of SENP7 with SUMO-2 chains and therefore the exact elements determining the preference of Ulp2 like proteases towards SUMO chain cleavage remain to be determined.

1.3.3 Functions of Ulp1 branch proteases

Deletion of Ulp1 in *S. cerevisiae* results in lethality, and the level of Ulp1 activity correlates with its ability to support cell growth. Ulp1 depleted cells experience problems with vegetative growth and arrest at the G2/M phase of the cell cycle. The Δ Ulp1 strain can be partially rescued by the expression of processed form of Smt3, which indicates an important role of Ulp1 in pre-Smt3 processing (Li and Hochstrasser 1999). Ulp1 is localized to the nuclear periphery, where it associates with nuclear pore complexes via interaction with Psp1 and the nuclear import receptors importin α/β (Panse *et al.*, 2003). This localization may regulate its substrate specificity. An expression of the deletion mutant that contains only the catalytic domain of Ulp1 is lethal for the cells, suggesting that Ulp1 anchoring to the NPC may be a way to regulate its activity. Mutants that are not localized to the NPC show much more activity than wild-type (Li and Hochstrasser 2003). In addition to cell division, Ulp1 plays a role in RNA processing and ribosome biogenesis (Dasso 2007).

Humans have four members of the Ulp1-like branch, which fall into two subgroups. The first one consists of SENP1 and SENP2. SENP1 is active both in processing and in deconjugation. For processing it favors SUMO-1 as a substrate. It can also process SUMO-2, but shows a very limited activity towards SUMO-3. SENP1 can deconjugate SUMO-1 and SUMO-2 from substrates with equal efficiencies (Shen *et al.*, 2006). SENP2 on the other hand prefers SUMO-2 to SUMO-1 for deconjugation and is a poor processing enzyme (Reverter and Lima 2006). SENP1 is localized to the nucleoplasm and nuclear speckles, whereas SENP2 localizes to the nucleoplasmic side of the NPC (Bailey and O'Hare 2004, Hang and Dasso 2002). Localization to the NPC is similar to that of Ulp1, however the mechanism may be different. SENP2 interacts with Nup153, but it is not known whether this interaction is direct or if it requires importin α/β (Hang and Dasso 2002, Zhang *et al.*, 2002). The role of the NPC localization has not been directly addressed in human cells. Analogous to Ulp1 NPC

localization, it may be a way to restrict enzymatic activity only to a certain localization. Overexpression of a SENP2 mutant, which can no longer localize to the NPC, results in a stronger reduction of SUMO conjugates than overexpression of a wild-type protein (Hang and Dasso 2002). SENP2 has also been implicated in transcriptional regulation (Best *et al* 2002). Considering the localization of other SUMO enzymes to the NPC (Ubc9,RanBP2) it is possible that SENP2 has a role in nuclear transport. SENP1 is essential for embryonic development (Cheng *et al.*, 2007). It has been implicated in regulation of transcription, keratinocyte differentiation and prostate cancer development (Cheng *et al.*, 2006, Deyrieux *et al.*, 2007).

The second subgroup of the Ulp1 branch consists of SENP3 and SENP5. Both of these enzymes localize to the nucleolus, and are specific for SUMO2/3 in deconjugation reaction, having almost no processing activity (Gong and Yeh 2006, Di Bacco *et al.*, 2006). SUMO-2/3 conjugates are absent from the nucleolus. Localization of SENP3/5 to the nucleolus may be a way to exclude SUMO2/3 conjugates from this compartment. Upon depletion of SENP5 strong enrichment of SUMO2/3 conjugates in nucleoli can be observed (Di Bacco *et al.*, 2006). Multiple functions have been associated with SENP3/5, including cell cycle control, rRNA processing and regulation of mitochondrial morphology (Di Bacco *et al.*, 2006, Zunino *et al.*, 2007, Kuo *et al.*, 2008, Haindl *et al.*, 2008, Klein *et al.*, 2008).

1.3.4 Functions of Ulp2-like SUMO proteases

Ulp-2 like proteins appear to be specialized for SUMO chain cleavage. They are more proficient in chain disassembly than in deconjugation, but have no activity in processing (Dasso 2007, Reverter and Lima 2008).

Contrary to Ulp1, Ulp2 is not essential for vegetative growth of *S. cerevisiae*. Deletion of Ulp2 results in increased sensitivity to hydroxyurea and DNA damaging agents like UV or gamma radiation. Ulp2 is also required for normal

recovery from spindle check point arrest. The delta Ulp2 strain exhibits higher rates of chromosome loss and cells exhibit problems with rDNA condensation and shows premature loss of chromosomal cohesion during mitosis. Although not essential for normal growth, Ulp2 is needed for sporulation. In early meiosis the mRNA levels of ULP2 increase 10 fold. The deletion mutant arrests in meiotic prophase (Li and Hochstrasser 2000). Ulp2 was implicated in remodeling of synaptonemal complexes - protein complexes involved in chromosomal pairing and crossing over (Cheng *et al.*, 2006).

Ulp2 has a different substrate specificity than Ulp1, as judged from the accumulation of SUMO conjugates in delta strains. Ulp2 depletion leads to accumulation of high molecular weight sumoylated species. The defects can be rescued by overexpression of a SUMO mutant that does not form chains, indicating that Ulp2 is responsible for chain disassembly (Bylebyl *et al.*, 2003).

Humans have two members of the Ulp2 like branch – SENP6 and SENP7. Recent characterization revealed that similar to Ulp2 SENP6 and SENP7 are very efficient in disassembling SUMO chains (Mukhopadhyay *et al.*, 2006, Reverter and Lima 2008) . In deconjugation reactions both enzymes prefer SUMO-2/3 over SUMO1, but have no processing activities. Both enzymes are nuclear proteins. Depletion of SENP6 leads to the accumulation of SUMO-2/3 species within PML nuclear bodies (Mukhopadhyay *et al.*, 2006).

1.4 Are there more SUMO specific isopeptidases?

The small number of SUMO proteases is in clear contrast to the ubiquitin pathway, in which the number of proteases is close to one hundred. Ubiquitin proteases fall into five different families, none of which are related to SUMO proteases. The biggest group is the Ubiquitin Specific Proteases family (USP), a family of cysteine proteases with more than fifty members in humans (Nijman *et al* 2005). Three out of the remaining four families are also cysteine proteases, whereas enzymes belonging to the last family are metallo proteases (Amerik and Hochstrasse 2004, Nijman *et al.*, 2005). The plethora of ubiquitin proteases

allows us to suspect that yet undiscovered SUMO proteases exist. In agreement with this, unpublished data from our laboratory as well as from studies conducted by Suzuki *et al.* in 1999 suggest the presence of a SUMO isopeptidase activity that correlates with a protein of ~30kDa. The smallest known member of the SUMO proteases (SENP5) is 65 kDa in size therefore it is reasonable to suspect that the activity observed by us is distinct. This supports the hypothesis that a novel yet unidentified protease exists.

1.5 Specific Aim

A specific aim of my work was to identify a new SUMO specific isopeptidase and to perform its initial characterization. Two approaches were used: an *in vitro* FRET based desumoylation assay was applied to screen a bacterial expression library of human ORFs, and chemically modified SUMO was used to purify desumoylating enzymes from HeLa cell lysates. As a result Ubiquitin specific protease like protein 1 (USPL1) was identified as a protease specific for SUMO but not Ubiquitin.

2. Materials and methods

2.1 Materials

2.2.1 Equipment

Automated robotic station BioMek2000 (Beckman Coulter)
Bacterial incubator (Kelvitron t Heraeus, Hanau)
Cell culture incubator (Hera cell Heraeus, Hanau)
Centrifuge J6MI (Beckman Coulter, München)
Centrifuge Avanti J30I (Beckman Coulter, München)
Centrifuge Allegra X-15R (Beckman Coulter, München)
Chromatography system Äkta Purifier (GE Healthcare, München)
Chromatography system Äkta Prime (GE Healthcare, München)
Documentation system Gel Jet Imager Intas, Göttingen
Documentation system LAS 3000 Fujifilm, Tokyo (Japan)
Elektrophoresis and blotting chambers (Workshop MPI for Biochemistry, Martinsried)
Film developing machine Curix 60 (Agfa, Köln)
high pressure homogenizer EmulsiFlex-C3 (Avestin)
Microplate reader, Fluoroskan Ascent (Labsystems)
Photometer SmartSpec (Plus Bio-Rad, München)
Power supply Variomag Biomodul 40B H+P (Labortechnik, München)
Rotors JS 4.2, JA 30.50Ti, SX4750, TLA 100.3 (Beckman Coulter, München)
Rotors Type45, Type60Ti, Type70.1Ti (Beckman Coulter, München)
Scanner 4990 (Photo Epson, Meerbusch)
Shaking incubator Innova 4230 (New Brunswick Scientific, Edison, NJ,USA)
Sterile cell culture hood Hera safe (Heraeus, Hanau)
Table centrifuge 5415C, 5424 (Eppendorf, Hamburg)
Thermomixer compact (Eppendorf, Hamburg)
Ultracentrifuge OptimaMax (Beckman Coulter, München)
Ultracentrifuge Optima L-80 XP (Beckman Coulter, München)
Vacuum pump LABOPORT N480.3FTP (KNF Neuberger, Freiburg)

Water purification system Ultra Clear (SG, Barsbüttel)

2.1.2 Commonly used reagents

Unless indicated otherwise Millipore water was used for preparation of all buffers and stock solutions.

2.1.2.1 Stock solutions:

Ampicillin 100 mg/ml

Aprotinin, 1000x 1 mg/ml

ATP 20 mM HEPES pH 7.4, 100 mM ATP, 100 mM magnesium acetate

Chloramphenicol 30 mg/ml

Dithiothreitol (DTT) 1 M

Kanamycin 50 mg/ml

Leupeptin/Pepstatin, 1000x 1 mg/ml each, in DMSO

PMSF 100 mM in 2-propanol

Puromycin 1 mg/ml in PBS

2.1.2.2 Commonly used buffers

Phosphate buffered saline (PBS) PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , pH 7.5)

PBST PBS supplemented with 0.2 % (v/v) Tween 20

Transport buffer 20 mM HEPES, 110 mM KOAc, 2 mM $\text{Mg}(\text{OAc})_2$, 1 mM EGTA pH 7.3)

Sumoylation assay buffer (SAB) transport buffer supplemented with 0.2 mg/ml ovalbumine, 0.05 % (v/v) Tween 20, 1 mM DTT, 1 μM PMSF and 1 $\mu\text{g/ml}$ each of aprotinin, leupeptin, pepstatin

DNA loading dye (stock 6x) 10 mM Tris-HCl, 50 mM EDTA, 1 % (w/v) SDS, 30 % (w/v) glycerol, 0.1 % (w/v) bromophenol blue, 0.1 % (w/v) xylencyanol, pH 8

TE buffer (10mM Tris, 1mM EDTA, pH 7.5)

2.1.2.3 Bacterial strains and culture media

LB (Luria-Bertani)

1% (w/v) bacto-tryptone, 0.5 % (w/v) yeast extract, 1 % (w/v) sodium chloride, pH 7) sterilized by autoclaving

LB plates

LB medium with addition of 1.5% (w/v) bacto-agar

In case when antibiotics were used LB medium and plates was supplemented with either 100 µg/ml ampicillin or 50 µg/ml of kanamycin or 30 µg/ml chloramphenicol.

Bacterial strains

E.coli DH5α F⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d/lacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K⁻ m_K⁺), λ-

E.coli BL21 (DE3) F⁻ ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])

E.coli (DE3) pLysS F⁻ ompT, hsdS(r_ñ, m_ñ), dcm, gal, λ(DE3), pLysS, Cmr

E.coli pLysE F⁻ ompT, hsdS(r_ñ, m_ñ), dcm, gal, λ(DE3), pLysE, Cmr

E.coli Rosetta F⁻ ompT hsdS(r⁻ m⁻) gal dcm (DE3) pRARE (Cm)

E.coli Rosetts2 F⁻ ompT hsdS(r⁻ m⁻) gal dcm (DE3) pRARE2 (Cm)

2.1.2.4 Cell lines and culture medium

DMEM (Gibco) supplemented with 10 % (v/v) FBS,

Cell lines

HEK293T - HEK293 human embryonic kidney cells immortalized by transformation with adenovirus 5 DNA (Graham *et al.*, 1977), and containing SV40 large antigen allowing episomal propagation of SV40 promoter containing plasmid (Lebkowski *et al.*, 1985)

HeLa - human cervix carcinoma cell line

2.1.2.5 Primers

All primers were custom orders from Operon

#1209 GGAAAGGAAGGATCCATGATGGATTCTCCGAAGAT
#1210 CCAAACCAACTCGAGTCAATAATTCTCAAACAGATA
#1211 GGAAAGGAACCATGGGGATGATGGATTCTCCGAAGATTGGA
#1212 CCAAACCAACTCGAGTCATTCCAGTTTAGATGTACATCCTTC
#1213 GGAAAGGAACCATGGGGATGCCACTGGAGAGCAAATGTACA
#1214 CCAAACCAACTCGAGTCATATTTTTCTTTCCCAAATAACAAT
#1231 GAAAAATGCTTATGCTCTCAGCTGGTTAGACTGTATCCTGTC
#1232 GACAGGATACAGTCTAACCAGCTGAGAGCATAAGCATTTTTC
#1234 CCAAACCAACTCGAGTCAAGCAGGAAGCTTCAAATTTCTT
#1251 CCAAACCAACTCGAGTCAAAGTGGAAGGCAGGCAGCTTC
#1331 CCAAACCAACTCGAGTTCCAGTTTAGATGTACATCCTTC
#1332 GGAAAGGAAGTCAAGGATGATGGATTCTCCGAAGATT
#1355 CCAAACCAAGGATCCCCATAATTCTCAAACAGATA

2.1.2.6 Antibodies

epitope	type	dilution for WB	source	Concentration [mg/ml]
HA peptide	mouse monoclonal	1:1000	Covance	2.5-3.5
HA peptide	mouse monoclonal	1:1000	Niman et al. 1983	0.4
GFP	rabbit polyclonal	1:1000	Santa Cruz	0.2
GST	rabbit polyclonal (serum)	1:10000	kindly provided by Prof. Ludger Hengst	

Table 2. Primary antibodies

Horseradish peroxidase-conjugated secondary antibodies anti-mouse and anti-rabbit for western blot analysis were obtained from Dianova and were used at a dilution of 1:10.000

2.2 Methods

2.2.1 Cloning

Bacterial strain

E. coli DH5 α

Media

LB (Lysogeny broth)

LB plates

For DNA isolation bacteria were cultured in LB medium supplied with appropriate antibiotic(s) for overnight (12-18 hours) at 37°C with vigorous shaking (120-180 rpm).

After DNA transformation bacteria were plated on an LB plate with appropriate antibiotic(s) and incubated overnight (12-18 hours) at 37°C.

2.2.1.1 DNA isolation

DNA isolation was based on alkaline lysis method (Birnboim and Doly 1979). SDS breaks the cell membrane, while sodium hydroxide denatures proteins and genomic DNA. Plasmid DNA stays in solution and can be recovered.

Small scale DNA isolation (mini prep)

Materials

Solutions of:

P1 (50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml RNase A, pH 8)

P2 (200 mM NaOH, 1% (v/v) SDS)

P3 (3 M KOH pH 5.5)

2-propanol

70% ethanol

TE buffer

LB medium supplied with appropriate antibiotic

A single bacterial colony was used to inoculate 3 ml of LB medium with appropriate antibiotic. After overnight growth (16-20 hours) the cells were pelleted by centrifugation, suspended in 300 µl of P1 solution, lysed by addition of 300 µl of P2 solution and incubated for 5 min at room temperature. Adding 300 µl of P3 solution precipitated proteins and genomic DNA. After 30 minutes on ice soluble fraction was obtained by centrifugation, from which DNA was precipitated by adding 0.8 volumes of 2-propanol and centrifugation. Precipitated DNA was

washed with 70% ethanol and dried. Obtained DNA pellet was solubilized in 50 μ l of TE buffer.

Medium scale plasmid DNA isolation (midi prep)

Materials

LB medium supplemented with appropriate antibiotic

Macherey&Nagel Midi Prep Kit

TE

A single bacterial colony was used to inoculate 50 ml of LB medium with appropriate antibiotic. After overnight growth (16-20 hours) the cells were pelleted by centrifugation. DNA was purified using a Macherey & Nagel kit following the manufacturers' instructions. Concentration and purity were determined by measuring absorbance at 280 and 260 nm.

2.2.1.2 Restriction digestion

Materials

Restriction enzymes supplied with reaction buffer (Fermentas)

For control restriction 10 μ l (approximately 0.5-3 μ g) of mini prep DNA was digested using 2 U of an enzyme for 1 hour at appropriate temperature (generally 37°C) in total reaction volume of 50 μ l.

For preparative digestion 2 μ g of DNA was digested using 10 U of an enzyme for 2 hours at appropriate temperature (generally 37°C).

2.2.1.3 DNA separation and extraction

Materials

TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 7.7)

TE buffer

DNA was resolved by electrophoresis on agarose gel (1-2% (w/v) agarose in TAE buffer at 80 V. DNA was visualized by staining in an ethidium bromide bath and exposure to UV light (365nm). Gel slices containing desired DNA were excised and DNA was extracted using a Macherey & Nagel kit following the manufacturers' instructions. DNA was eluted in 30 µl of TE buffer.

2.2.1.4 Ligation

The ligation procedure is based on T4 phage DNA ligase's ability to catalyze formation of phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA (Weiss *et al.*, 1968)

Materials

ATP stock solution

T4 DNA ligase supplied with reaction buffer (Fermentas)

Ligations were performed using 50-100 ng of vector with triple molar excess of insert. Reactions were performed in presence of 5 nM ATP and 2 Weiss Units of T4 DNA ligase in a total volume of 20 µl for one 1 hour at room temperature. To inactivate the ligase, reactions were incubated at 65°C for 15 minutes. Usually, 10 µl of reaction was used for bacterial transformation.

2.2.1.5 PCR reactions

Polymerase chain reaction (PCR) is based on a logarithmic amplification of desired DNA fragment using primers flanking it on 5' and 3' (Kleppe and Khorana 1971). The sequential cycles and the logarithmic amplification are possible due to application of thermostable polymerase that is not deactivated during denaturing process (Mullis *et al.*, 1986)

Materials

Phusion polymerase supplied with reaction buffer (Finnzymes)

Mix of four dNTPs (Fermentas)

Primers (custom order, Operon)

To amplify DNA fragment 100ng of template DNA was mixed with forward and reverse primers (final concentration of 500 nM each), each of four dNTPs (final concentration of each 250 μ M), 1 U of polymerase in a final volume of 50 μ l. Reaction was subjected to 30 sec of initial denaturation at 98°C, followed by 30 cycles of: 10 seconds of denaturation at 98°C, 30 seconds of annealing at appropriate temperature, 25 seconds/1kB of amplified fragment of extension at 72°C. The final step involved 10 minutes of extension at 72°C.

Annealing temperature was determined using an online tool provided by manufacturer (www.finnzymes.fi/tm_determination.htm). The exact PCR conditions are summarized in Table 3.

primers	T annealing [°C]	time of extension [sec]
#1209/#1210	55	75
#1213/#1251	71	20
#1213/#1234	71	20
#1213/#1214	71	20
#1231/1232	72	180
#1211/#1212	67	20
#1211/#1331	67	20
#1332/1355	69	75

Table 3. PCR conditions.

2.2.1.6 Site-directed mutagenesis

The approach described here is adopted from stratagen site-directed mutagenesis kit. Using of primers complementary to each other and partially complementary to template DNA allows for introduction of desired mutation. Contrary to PCR the newly synthesized DNA is not a template for the further

synthesis. Restriction digestion with *DpnI* removes template (if methylated) DNA but not newly synthesized.

Materials

Same as for PCR reaction

DpnI (Fermentas)

Primers (custom order, Operon)

The reaction was set up as for the PCR using increasing amount of DNA, from 5 to 50 ng of template DNA. Extension time was modified to 30 seconds/1kB of the template. Primers were designed using the online the onlinetool, PrimerX. (www.bioinformatics.org/primerx/). Annealing temperature was calculated using an online tool (www.finnzymes.fi/tm_determination.htm). 25 μ l of the reaction product was digested with 1 U of *DpnI* enzyme for 1 hour at 37°C and transformed into *E. coli*. Conditions for site directed mutagenesis of USPL1 are given in Table 3.

#	construct	primers/source of the insert	template	restrictio site	purpose	remarks
1	pcDNA3.1-HA-USPL1	#1209/#1210	HeLa cDNA (kindly provided by Dr. Ruth Geiss-Friedlander)	BamHI/Xho1	mammalian expression	N-term HA tag
2	pcDNA3.1-FLAG-USPL1	construct 1		BamHI/Xho1	mammalian expression	N-term FLAG-tag
3	pETM30-USPL1cat(212-514)	#1213/#1251	construct 1	NcoI/XhoI	bacterial expression	N-term HIS-GST-TEV
4	pETM30-USPL1cat(212-498)	#1213/#1234	construct 1	NcoI/XhoI	bacterial expression	N-term HIS-GST-TEV
5	pETM30-USPL1cat(212-502)	#1213/#1214	construct 1	NcoI/XhoI	bacterial expression	N-term HIS-GST-TEV
6	pcDNA3.1-HA-USPL1 C236S	#1231/1232	construct 1	BamHI/Xho1	mammalian expression	N-term HA tag catalytic mutant
7	pETM30-USPL1cat(212-514)C236S	#1231/1232	construct 3	BamHI/Xho1	bacterial expression	N-term HIS-GST-TEV catalytic mutant
8	pETM30-USPL1(1-212)	#1211/#1212	construct 1	NcoI/XhoI	bacterial expression	N-term HIS-GST-TEV
9	pET28b-USPL1	construct 1		BamHI/Xho1	bacterial expression	N-term His
10	pET28b-USPL1-1-212	#1211/#1331	construct 1	NcoI/XhoI	bacterial expression	C-term His
11	pEYFP-USPL1	#1332/1355	construct 1	XhoI/BamHI	mammalian expression	N-term YFP
12	pECFP-USPL1	#1332/1355	construct 1	XhoI/BamHI	mammalian expression	N-term CFP

Table 4. Constructs prepared and used during this work

2.2.1.7 Transformation of bacteria

Materials:

Aliquot of competent *E.coli* (available as a general lab stock)

LB medium (without antibiotics)

LB plate supplemented with required antibiotic

Bacteria were transformed using the heat shock method (Hanahan 1983): an aliquot of competent bacteria was thawed and incubated on ice with DNA for 15 minutes. Cells were subjected to a heat shock (42°C 45 sec) and incubated on ice for 2 minutes. 900 µl of LB medium without antibiotics was added and cells were grown for 1 hour at 37°C before plating them on LB-agar containing appropriate antibiotic.

2.2.2 Cell culture methods

2.2.2.1 Cultivation of mammalian cell lines

Materials

DMEM (PAA)

Trypsine (Gibco)

EDTA (PAA)

Human embryonic kidney (HEK293T) cell line

penicillin and streptomycin

glutamine (cell culture grade) Gibco

PBS

HEK293T were grown in DMEM medium supplemented with 10% fetal bovine serum, glutamine and antibiotics (penicillin and streptomycin) at 37°C with 5% CO₂. After reaching confluence they were split in 1 to 10 ration. To do so, cells were washed twice with sterile PBS (warmed up to 37°C) and incubated with trypsin/EDTA. After they detached fresh medium was added to stop the trypsinization reaction and to dilute the cells to desired density before placing

them on a fresh dish.

2.2.2.2 Transfection of HEK293T cells by calcium phosphate method.

Materials

1/10 TE buffer (1mM Tris, 0.1 mM EDTA pH 7.5)

500 mM CaCl₂

2 x HBS (50mM HEPES, 280 mM NaCl, 15mM NaPO₄ pH 7.1)

10⁶ HEK 293T cells were plated on the 10 cm dish 24 hours before transfection. 2 µg of plasmid DNA was mixed with 1/10 TE so that the final volume was 250 µl and 250 µl of 500 mM CaCl₂ was added. To the DNA/ CaCl₂ solution 500 µl of 2 x HBS was added in a dropwise manner with vigorous shaking. The mixture was incubated until visible precipitate was formed (usually 10-20 minutes) and added to the cells. The medium was exchanged 6 hours after transfection.

2.2.3 Biochemical methods

2.2.3.1 Preparation of HeLa cell lysate

Materials

Frozen HeLa cell pellet (RELIATech, Wolfenbüttel)

Transport buffer (TB) supplemented with 1 mM DTT, 1 µM PMSF and 1 µg/ml each of aprotinin, leupeptin, pepstatin

HeLa cell pellet was thawed in 2 volumes of TB, centrifuged at 1500g for 10 minutes to remove cell nuclei and debris. Supernatant was centrifuged first at 15.000 g for 25 minutes, and then at 100.000 g for 60 minutes. The supernatant from the last step is referred to as a HeLa cell lysate. It was aliquoted, flash frozen in liquid nitrogen and stored at -80°C.

2.2.3.2 Preparation of detergent extracts of HEK293T

Materials

PBS

Lysis buffer (100 mM Tris, 150 mM NaCl, 5% Glycerol, 1% Triton X-100, pH 7.5) supplemented with 1 mM DTT, 1 μ M PMSF and 1 μ g/ml each of aprotinin, leupeptin, pepstatin)

All steps were performed at 4°C. 1 ml of lysis buffer was used for one 10 cm dish of HEK293T cells at 60-70% confluence ($\sim 7 \times 10^6$ cells). The medium was aspirated, cells were washed carefully twice with ice cold PBS and lysed in 1 ml of lysis buffer for 30 minutes on the rocking platform. Lysate was collected and centrifuged for 10 minutes at 14.000 rpm in a table-top centrifuge. The supernatant containing extracted proteins was transferred to a new tube and, if not used immediately, it was flash-frozen in liquid nitrogen.

2.2.3.3 Immunopurification of HA-tagged USPL1 from detergent extracts

Materials

Anti-HA-agarose (Sigma)

HA peptide (Biomol)

All steps were performed at 4°C and samples were centrifuged in a tabletop centrifuge at 2000 rpm for 20 seconds unless indicated otherwise. The first step was omitted in case of a freshly prepared extract. 1 ml of detergent extract was centrifuged in a tabletop centrifuge at 14.000 rpm for 10 minutes. 950 μ l were transferred to a fresh tube and incubated with 15 μ l of anti-HA agarose for 2 hours. After centrifugation, beads were extensively washed with a lysis buffer and transferred to a fresh tube. Bound proteins were eluted twice with 30 μ l of an HA-peptide (0.2 mg/ml in lysis buffer) at 30°C for 15 minutes. Eluates were combined.

2.2.3.4 Labeling of HeLa cell lysate proteins with SUMO-Vme and their enrichment by immunopurification.

Materials

SUMO –Vinylmethyl ester, SUMO-VME (see later part of this chapter)

HeLa cell lysate

Protein-A-agarose (Roche)

Anti-HA-agarose (Sigma)

HA peptide (Biomol)

1 ml of Transport buffer + 1% Triton X-100

TNE buffer (Tris 100 mM, NaCl 150 mM pH=8.0)

RIPA buffer (50 mM Tris-HCl 150 mM NaCl, 1 % (v/v) nonidet P-40, 0.5 % (w/v) Na-desoxycholate, 0.1 % (w/v) SDS, , pH 8)

All buffers supplemented 1 mM DTT, 1 μ M PMSF and 1 μ g/ml each of aprotinin, leupeptin, pepstatin

The amounts of used reagents are optimal to label and enrich proteins using 1.5 ml of HeLa cell lysate at ~10mg/ml protein concentration.

The HeLa cell lysate was centrifuged at 100.000 g for 20 minutes at 4°C, and incubated for 30 minutes at 37°C with 600 ng of SUMO-1-VME or 450 ng of SUMO-2-VME. From now on all steps were carried out at 4°C. To remove aggregated or precipitated proteins, lysates were centrifuged for 20 minutes at 100.000 g. Supernatants were incubated with 20 μ l of Protein-A agarose for 1 hour for preclearing. After pelleting Protein-A agarose (2000 rpm, 20 seconds) supernatant was incubated with 20 μ l of Anti-HA-agarose for 2 hours. Beads were collected by centrifugation and subjected to the following washes:

- 1) 1 ml of TNE buffer
- 2) 1 ml of the TNE buffer with NaCl concentration adjusted to 500 mM
- 3) 1 ml of RIPA buffer

4) 1 ml of TB + 1% Triton X-100

Subsequently beads were transferred to a fresh tube, washed with 1 ml 1 ml of TB +1% Triton X-100, and the bound proteins were eluted twice with 30 μ l of an HA-peptide (0.2 mg/ml in TB + 1% Triton X-100) for 15 minutes at 30°C. Eluates were combined and flash frozen.

2.2.3.5 Enrichment of the paralogue specific proteases.

The general scheme of the purification is shown in Figure 11.

The first step involved labeling of proteins of HeLa cell lysates by treatment with SUMO-1-VME. The procedure was carried out as described above, however the amount of used SUMO-1-Vme was 1000 ng per 1.5 ml of lysate. The elution amount of used protein-A-agarose or anti-HA-agarose and elution volume remained unchanged. The supernatant after immunoprecipitation was kept.

The second step involved the labeling of proteins with SUMO-2-Vme using HeLa lysate depleted of SUMO-1-Vme reactive proteins (supernatant from the first step). For labeling, 600 ng SUMO-2-VME was used and the immunopurification was carried out as in the first step omitting incubation with protein-A-agarose.

2.2.3.6 Identification of labeled proteins by Mass Spectrometry

For Mass Spectrometry analysis the enrichment of paralogue specific proteases was carried out using 25 ml of HeLa cell extract, 10 μ g of SUMO-1-VME and 10 μ g of SUMO-2-VME. The amount of used Protein-A-agarose and anti-HA-agarose was 120 μ l. The Elution volume was 250 μ l.

Mass Spec identification of immunoprecipitated proteins was carried out in collaboration with Dr. Henning Urlaub (Department of Mass Spectrometry, Max-Planck Institute for Biophysical Chemistry, Göttingen). Sample preparation and MS analysis was performed by Monika Raabe. I carried out peptide identification using MASCOT software.

2.2.3.7 SDS-PAGE electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to the system described by Laemmli (Laemmli, 1970)

Materials

Acrylamide solution, 30 %, 37.5:1 AA:bisAA (Applichem)

ammonium persulfate (APS) 10% (w/v) stock solution

TEMED

Resolving gel solution (0.4 M Tris-HCl pH 8.8, 0.1 % (w/v) SDS, acrylamide at desired concentration)

Stacking gel solution (50 mM Tris-HCl pH 6.8, 0.1 % (w/v) SDS, 4 % (w/v) acrylamide)

Laemmli buffer (25 mM Tris, 192 mM glycine, 0.05 % (w/v) SDS, pH=8.3)

2x sample buffer (100 mM Tris, 4% (w/v) SDS, 0.2 % (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM DTT, pH=6.8)

4x sample buffer (200 mM Tris, 8% (w/v) SDS, 0.4 % (w/v) bromophenol blue, 40% (v/v) glycerol, 400 mM DTT, pH=6.8)

Unstained 10 kDa protein marker (Fermentas)

Gel casting chamber

Polyacrylamide gel preparation

Gels were prepared in batch by putting polyacrylamide solutions between glass plates in a casting chamber that accommodated eight gels.

~70 ml of desired PAA concentration resolving gel solution was prepared. Polymerization was initiated by addition of APS (0.06% (w/v) final concentration) and TEMED (0.06% (v/v) final concentration), the solution was poured into the chamber and overlaid with 2-propanol to prevent oxygen access. After the gel polymerized, 2-propanol was removed and stacking gel solution in which polymerization was initiated by addition of APS (0.1 % (w/v) final concentration) and TEMED (0.1 % (v/v) final concentration) was put on the top of resolving gel.

If not used immediately after polymerization gels were stored at 4°C up to one week.

5-20% resolving gradient gels were prepared by placing equal volumes of 5% and 20% gel solutions in a cylindrical gradient mixer connected to the casting chamber.

Polypeptides in sample were denatured by adding sample buffer to final concentration of 1x and incubation at 95°C for 5 minutes. After loading sample on the gel electrophoresis was performed in Laemlli buffer at 22 mA per gel until the dye run out from the gel.

2.2.3.8 Coomassie staining

Materials

Coomassie blue stain solution (0.025% Coomassie R-250, 10% (v/v) Acetic Acid, 40% (v/v) methanol)

Destaining solution (10% (v/v) Acetic Acid, 40% (v/v) methanol)

After the electrophoresis the gels were incubated in Coomassie blue stain solution until completely stained (usually between 60 minutes to overnight). To destain, gels were incubated for several hours at room temperature on the rocking platform with frequent solution changes.

2.2.3.9 Immunoblotting

Materials

Nitrocellulose membrane (Schleicher & Schuell)

Primary and secondary antibodies

Transfer buffer (25 mM Tris, 193 mM glycine, 20 % (v/v) methanol, 0.036 % (w/v) SDS)

PBST solution

Blocking solution (5% skim milt in PBST)

Primary and secondary antibodies

X-ray film (GE Healthcare)

ECL kit (Millipore)

PonceauS solution (0.5 % (w/v) PonceauS in 1 % (v/v) acetic acid)

Whatman paper stacks were soaked in transfer buffer. The membrane was soaked first with water and then with transfer buffer. The electrophoresis sandwich was built up as following: from the bottom: 3 stacks of the Whatman paper, membrane, gel, 3 stacks of the Whatman paper. The proteins were transferred at 200 mA for 1 hour at room temperature in a semi-dry western blotting chamber. After transfer, the membrane was stained with PonceauS solution and incubated in a blocking solution for at least 1 hour at room temperature. The primary antibody was applied for 1 hour at room temperature in blocking buffer solution. The secondary antibody was applied for 1 hour at room temperature in blocking solution. After each antibody incubation the membrane was washed 4 times with PBST solution for 15 minutes at room temperature. Proteins were visualized using ECL kit and exposed on a X-ray film. An exposure time of 2 minutes was usually sufficient. Film was developed using automated developer.

2.2.4 Recombinant protein purification

2.2.4.1.USPL1 catalytic domain

Materials

pET30M-USPL1cat(212-514) expression plasmid

E.coli (DE3) Rosetta2 strain

5L baffled Erlenmeyer flask

LB medium supplemented with kanamycine 50 µg/ml and chloramphenicol 34 µg/ml

IPTG

Lysis buffer (50 mM Tris, 100 mM NaCl, pH 8.0) supplemented with 1 μ g/ml each of aprotinin, pepstatin, leupeptin and 1mM PMSF and DTT

High pressure homogenizer (Avestin)

Elution buffer (10 mM reduced glutathione in lysis buffer)

Glutathione Sepharose 4B (GE Healthcare)

Reduced glutathione

Ni-NTA Agarose (QIAGEN)

TEV protease (our laboratory common stock)

HiLoad 26/60 Superdex 75 pg, GE Healthcare

AKTA purifier (GE Healthcare)

Transport buffer supplemented with 1 μ g/ml each of aprotinin, pepstatin, leupeptin and 1mM PMSF and 1 mM DTT

Protein concentrator with 10kDa cut off, VIVASPIN, (Sartorius)

Low protein binding filters – 0.2 μ m (Acrodisc LC25, PALL Life Sciences)

The construct, which expression resulted in soluble catalytic domain contained amino acid residues 212-514 of USPL1 (see results section for further details).

One aliquot of competent bacteria was transformed by heat shock method with pET30M-USPL1cat plasmid. After recovery the whole aliquot was transferred directly into 20 ml of LB medium containing antibiotics, and grown overnight (14-18 hours) at 37°C, 150 rpm shaking. The following day the culture was diluted to OD₆₀₀ between 0.05 and 0.1 in a final volume of 1 liter, and grown until OD₆₀₀ reached 0.6-0.7. Bacteria were induced by adding IPTG to final concentration of 0.5 mM and grown for 16-20 hours at 15°C, 150 rpm shaking. The bacteria were collected by a centrifugation and suspended in 50 ml of ice cold lysis buffer. From now all steps were carried out at 4°C. Bacteria were lysed using high pressure homogenizer, and obtained lysate was cleared by centrifugation (100.000 g, 60 minutes). Supernatant was incubated for 2 hours at a rocking platform with 5 ml of glutathione-agarose equilibrated with lysis buffer. Agarose was collected by passage through a plastic column and washed with 25 ml of lysis buffer. Bound protein was eluted twice with 10 ml of lysis buffer

supplemented with 50mM glutathione. Eluted HIS-GST-TEV-USPL1cat fusion protein was digested for at least 12 hours with TEV protease. 10 µg of HIS-TEV protease was sufficient to digest 1 mg of the fusion protein. To remove uncleaved protein, free HIS-GST and HIS-TEV, 2 ml of NiT-Agarose was added. After 2 hours of incubation the supernatant was collected. At this point DTT was added to 5mM final concentration and supernatant was concentrated to 2 ml using protein concentrator. Aggregated proteins were removed by centrifugation and subsequent passage through a 0.2 µm filter. Sample was applied to a preparative S-75 column equilibrated in Transport buffer, mounted on an Akta Purifier. Protein containing fractions were analyzed by 12% SDS-PAGE followed by coomassie staining. The cleanest fractions containing USPL1cat were pooled, concentrated down to 1ml, aliquoted and flash frozen. Aliquots were stored at -80°C.

2.2.4.2 Purification of YFP-SUMO and CFP-GAP

Purification was performed according to published protocols (Bossis *et al.*, 2005, Stankovic-Valentin *et al.*, 2009)

Materials

E.coli (DE3) Rosetta strain

LB medium supplemented with ampicilline 100 µg/ml

LB agar plate supplemented with ampicilline 100 µg/ml

IPTG

YFP-SUMO Lysis buffer (50 mM Tris, 50 mM NaCl, 1 mM EDTA pH=8.0)

CFP-SUMO Lysis buffer (50 mM Tris, 20 mM NaCl, 1 mM EDTA pH=8.0)

DTT, aprotinin, pepstatin, leupeptin, PMSF, DTT

Buffer A (50 mM Tris pH=8.0) with 1 µg/ml each of aprotinin, pepstatin, leupeptin and 1mM PMSF and 1 mM DTT

Buffer B (50 mM Tris pH=8.0) with 1 µg/ml each of aprotinin, pepstatin, leupeptin and 1mM PMSF and 1 mM DTT

HiLoad 26/60 Superdex 75 pg (preparative S-75 column), GE Healthcare

HiTrap Q FF 5 ml (Q sepharose column), GE Healthcare
AKTA prime (GE Healthcare)
AKTA purifier (GE Healthcare)
Lysozyme stock solution (25mg/ml)
Transport buffer supplemented with 1 μ g/ml each of aprotinin, pepstatin,
leupeptin and 1mM PMSF and 1 mM DTT
Protein concentrator with 10kDa cut off, VIVASPIN, (Sartorius)
Low protein binding filters – 0.2 μ m (Acrodisc LC25, PALL Life Sciences)

The procedure is the same for YFP-SUMO-1 and YFP-SUMO-2.

E. coli Rosetta (DE3) were transformed with appropriate plasmids, plated on LB agar and incubated overnight at 37°C. A single colony was used to inoculate 500 ml of LB medium. The culture was grown overnight at 37°C with 150 rpm shaking. The following day bacteria were collected by centrifugation and resuspended in fresh 2 l of LB medium. Protein expression was induced by adding IPTG to final concentration of 1mM and the culture was grown for 6 hours. Bacteria were collected by centrifugation, the pellet was resuspended in 50 ml of appropriate lysis buffer and subjected to a freeze thaw cycle. At this point protein inhibitors were added were added to final concentration of 1 μ g/ml each, DTT to 1 mM and lysozyme to 1 mg/ml. From now on all procedures were carried out at 4°C. The mixture was incubated for 60 minutes on ice with frequent shaking, and clarified by centrifugation (100.000 g 60 minutes). After passage through 0.2 μ m filter 25 ml of supernatant was loaded on a Q-sepharose column (equilibrated with 95% buffer A, 5% buffer B for YPF-SUMO purification, or 98% buffer A, 2% buffer B for CFP-GAPtail purification) mounted to the AKTA prime. The column was washed with 20 mls of appropriate lysis buffer and proteins were eluted with linear gradient of NaCl up to 500 mM, with 200 ml length of the gradient. Fractions of 5 ml were collected and the ones with yellow color were analyzed by 12% SDS-PAGE followed by coomassie staining. The same procedure was applied to the remaining 25 ml of supernatant. Fractions containing the fusion

protein were concentrated using a protein concentrator, precipitated protein was removed by centrifugation and the supernatant was applied to a S-75 preparative column equilibrated with transport buffer. Yellow fractions were analyzed by 12% SDS-PAGE followed by coomassie staining. The cleanest fractions were concentrated down to 1 mg/ml, aliquoted, flash frozen and stored at -80°C. The yield was 10 mg of YFP-SUMO and 2 mg of CFP_Gtail per 1 liter of culture

2.2.4.3 SUMO-VME synthesis and purification

SUMO-Vinylmethylester (SUMO-VME) is a recombinant SUMO modified at the C-terminus with vinylmethylester, which resembles peptide bond, and can covalently bind SUMO proteases. The synthesis of SUMO-VME is based on an intein-based chemical ligation method (Chong *et al.*, 1997, Cotton and Muir 1999). (See results section for further details).

The procedure can be divided into two steps: 1) preparation Strep-TEV-HA-SUMO-MESNa 2) Synthesis and purification of Strep-TEV-HA-SUMO-VMe. The procedure is identical for SUMO-1 and SUMO-2

Materials

E. coli (DE3) BL21 strain

LB medium supplemented with ampicilline 100 µg/ml

LB agar plate supplemented with ampicilline 100 µg/ml

pTXB3-Strep-TEV-HA-SUMO-1 and pTXB3-Strep-TEV-HA-SUMO-2 expression plasmids (Provided by Dr. E. Meulmeester)

MESNa (sodium 2-sulfanylethanesulfonate)

glycinevinylmethylester-tosyl (stock solution 250 µM) (Provided by Dr. Huib Ova, NKI, Amsterdam)

N-hydroxysuccinimide (stock solution 1 M)

NaOH (0.5 M)

HCl (0.5 M)

IPTG

Lysis buffer (20 mM Hepes, 50 mM NaCl, pH=6.5) supplemented with 1 μ g/ml each of aprotinin, pepstatin, leupeptin and 1mM PMSF

Elution buffer (20 mM Hepes, 50 mM NaCl, 50 mM MESNa, pH=6.5) supplemented with 1 mg/ml each of aprotinin, pepstatin, leupeptin and 1mM PMSF

Buffer A (20 mM Hepes, pH=6.5) supplemented with 1 mM DTT, 1 mg/ml each of aprotinin, pepstatin, leupeptin and 1mM PMSF

Buffer B (20 mM Hepes, 1 M NaCl pH=6.5) supplemented with 1 mM DTT, 1 mg/ml each of aprotinin, pepstatin, leupeptin and 1mM PMSF

chitin beads (NEB)

MonoQ HR 5/5 (GE Healthcare)

HiLoad 26/60 Superdex 75 pg (preparative S-75 column), GE Healthcare

AKTA purifier (GE Healthcare)

Protein concentrator with 10kDa cut off, VIVASPIN, (Saturious)

Low protein binding filters – 0.2 mm (Acrodisc LC25, PALL Life Sciences)

High pressure homogenizer (Avestin)

PD-10 desalting column (GE Healthcare)

2.2.4.2.1 Preparation of Strep-TEV-HA-SUMO-MESNa

An aliquot of the competent *E.coli* (DE3) BL21 bacteria was transformed either with pTXB3-Strep-TEV-HA-SUMO-1 or pTXB3-Strep-TEV-HA-SUMO-2 by the heat shock method and plated on LB agar with ampicillin. The following day a single colony was used to inoculate 20 ml of LB, which was grown overnight at 37°C with 150 rpm shaking. The following day the bacteria were collected by centrifugation and the pellet was suspended in 2 liters of LB medium with ampicillin, grown until an O.D₆₀₀ between 0.6-0.7 was reached and induced with 1 mM IPTG. After 4 hours the bacteria were pelleted by centrifugation, suspended in 60 ml of ice cold lysis buffer and lysed using high pressure homogenizer. The lysate was clarified by centrifugation (100.000 g for 60

minutes at 4°C) and the supernatant was loaded on 5 ml of chitin beads packed in a plastic column, previously equilibrated with the lysis buffer at room temperature. After loading, beads material was washed with 50 ml of lysis buffer and 15 ml of elution buffer. At this point the flow of the column was stopped and the beads material was incubated overnight at room temperature in 15 ml of elution buffer to cleave column bound fusion protein. The eluate was collected and the column was washed with additional 15 ml of elution buffer to elute the rest of the protein. Both eluates were concentrated down to 1 ml using a protein concentrator and loaded onto a preparative S-75 column equilibrated with 20 mM Hepes pH=7.5, Fractions were analyzed by SDS-PAGE. Fractions containing clean Strep-TEV-HA-SUMO-MESNa were concentrated down to 2 mg/ml protein concentration.

2.2.4.2.2.Synthesis and purification of Strep-TEV-HA-SUMO-Vme

To obtain Strep-TEV-HA-SUMO-Vme, to 1 ml of Strep-TEV-HA-SUMO-MESNa 250µl of glycinevinylmethylether-tosyl stock solution and 150 µl of N-hydroxysuccinimide stock solution were added, pH was adjusted to 8.0 with NaOH and the mixture was incubated for 2 hours at 37°C with gentle shaking. The reaction was stopped by addition of 50 µl of HCl solution. Using a PD-10 column buffer was exchanged to buffer A. The mixture was loaded on a MONO-Q column mounted on an AKTA purifier and washed with 5 ml of buffer A. Strep-TEV-HA-SUMO-Vme was separated from Strep-TEV-HA-SUMO-MESNa by applying a linear gradient of NaCl up to 400 mM in total volume of 20 ml using buffer A and buffer B and collecting 0.5 ml fractions. Protein containing fractions were analyzed by 15% SDS-PAGE followed by commasie staining. Strep-TEV-HA-SUMO-Vme was eluted at 290 mM NaCl. The fractions were aliquoted, flash frozen and stored at -80°C.

2.2.5 Enzymatic reactions and assays

All reactions were pipetted on ice

2.2.5.1 Preparation of the isopeptidase conjugate

Materials

CFP-GAP

YFP-SUMO

E1 SUMO enzyme (our laboratory's common stock, purification described in Bossis *et al.*, 2005, Werner *et al.*, 2009)

E2 SUMO enzyme (our laboratory's common stock purification described in Bossis *et al.*, 2005, Werner *et al.*, 2009)

Sumoylation assay (SAB)

Apyrase (sigma)

To prepare 50 ml of 1.25 μ M CFP-GAPtail*YFP-SUMO conjugate (100 μ g/ml) 2.5 mg of CFP-GAPtail, 2.5 mg of YFP-SUMO, 60 μ g E1 and 60 μ g E2 were pipetted together. 500 μ l of 100 mM ATP solution was added and final volume of 50 ml was achieved by adding SAB buffer. The mixture was incubated at 37°C for 40 minutes. ATP was depleted by addition of 100 U of apyrase. The conjugate was divided into aliquots, which after flash freezing in liquid nitrogen were stored at -80°C.

2.2.5.2 Preparation of RanGAP-SUMO-2 conjugate

Materials

RanGAP and SUMO-2 (provided by Dr. Andreas Werner)

E1 SUMO enzyme (our laboratory's common stock purification described in Bossis *et al.*, 2005, Werner *et al.*, 2009)

E2 SUMO enzyme (our laboratory's common stock purification described in Bossis *et al.*, 2005, Werner *et al.*, 2009)

Sumoylation assay (SAB)

To prepare 500 μ g of RanGAP-SUMO-2 conjugate 450 μ g of RanGAP and 200 μ g of SUMO-2 was incubated with 1 μ g of E1, 2 μ g of E2 in the presence of 1 mM ATP. The final volume of 1 ml was achieved by addition of SAB buffer. The

mixture was incubated for 60 minutes at 37°C, and loaded on an S-75 analytical column to remove unconjugated SUMO and remaining ATP. Fractions were analyzed by SDS-PAGE, and concentrated down to 0.5 ml. The final concentration was 1 mg/ml (14 μ M).

2.2.5.3 FRET-based desumoylation assay

Materials

CFP-GAPtail-YFP-SUMO conjugate

Black micro titer 386-well plates (Greiner)

Microplate reader, Fluoroskan Ascent (Labsystems)

The conjugated CFP-Gaptail-YFP-SUMO exerts FRET (Fluorescence Resonance Energy Transfer) signal, due to a radiation free excitation of the YFP component. Cleavage of the isopeptide bond results in loss of FRET. This is a basis for the enzymatic assay developed in our laboratory (Bossis *et al.*, 2005, Stankovic-Valentin *et al.*, 2009)

To follow desumoylation 20 μ l of the 1.25 μ M conjugate are mixed with 5 μ l of analyzed sample. To follow the reaction two values were measured: fluorescence emission at 485 nm after excitation at 430 nm, and fluorescence emission at 527 nm after exciting at 430 nm. The ratio of emission at 527 to emission at 485 is proportional to the amount of conjugated components and decreases during deconjugation reaction. Plotting this ratio values as a function of time allows to monitor kinetics of desumoylation reaction.

All measurements were taken in a microplate reader. For enzymatic assays measurements were taken every 1 minute for 30-60 minutes with the integration time of 100 milliseconds.

2.2.5.4 Screen of bacterial expression library

Materials

Lysates of bacterial expression library of human ORFs, in 384 well plates 10 plates, 3840 clones (provided by Dr. Erich Wanker, Max-Delbrück Center, Berlin), (Grelle *et al.*, 2006)

CFP-GAPtail-YFP-SUMO-1 conjugate

Automated robotic station – BioMek2000 (Transcriptome analysis laboratory, University of Göttingen)

SAB buffer

Black micro titer 386-well plates (Greiner)

Microplate reader, Fluoroskan Ascent (Labsystems)

The screen was performed in collaboration with Dr. Reinert Hitt (Transcriptome analysis laboratory, University of Göttingen), who programmed and operated the robotic station. In the first step the following plates were prepared:

- 1) for dilution of the bacterial library 95 μ l of SAB buffer was pipetted into each well of an empty plate.
- 2) For deconjugation assay 20 μ l of 1.25 μ M substrate was pipetted into each well

In a second step the library was diluted. 5 μ l of each lysate was transferred from the library plate into the corresponding well of the SAB buffer filled plate.

In a third step 5 μ l of each diluted extract was transferred into the corresponding well of the substrate filled plate. After 30 minutes of incubation at 37°C fluorescence values for 430/485 excitation/emission and 430/527 excitation/emission were measured 3 times for each well. The average values were used to determine the 527/485 fluorescence ratio.

2.2.5.5 Labeling of recombinant proteins with SUMO-VME

Materials

SEN1 catalytic domain (N- terminal GST-tag) (our laboratory's common stock)

USPL1cat domain

USPL1cat domain C236S

HA-USPL1 (immuprecipitated from mammalian cells)

SUMO-1-Vme

SUMO-2-Vme

1-2 μg of recombinant catalytic domains were incubated in the presence of 1 μg of SUMO-Vme for 30 minutes at 37°C. TB buffer was used as a negative control. Reactions were stopped by adding sample buffer to a final concentration of 1x. Samples were analyzed by 12 or 14% SDS-PAGE followed by Coomassie staining.

For HA-USPL1 overexpressed in HEK293T cells, the concentration of immunopurified USPL1 was not known. Transfection and immunopurification are described in sections 2.2.2.2 and 2.2.3.3. Total volume of eluate was 60 μl and was divided into 3 parts, and incubated with either 100 ng of SUMO-1-Vme, 100 ng of SUMO-2-Vme or elution buffer. The eluate of the immunoprecipitation from cells transfected with an empty vector was used as a control. Samples were analyzed by 5% SDS-PAGE followed by anti-HA western blotting.

2.2.5.6. SUMO cleavage

Materials

RanGAP modified with SUMO-1 (provided by Dr. Andreas Werner)

RanGAP modified with SUMO-2

USPL1cat

TB buffer supplemented with 5 mM DTT and 1 $\mu\text{g}/\text{ml}$ each of aprotinin, leupeptin, pepstatin

Sumoylated RanGAP at a concentration of 2 μM was incubated with 4 μM USPL1cat for 0-120 minutes. The reaction was stopped by addition of the sample buffer to the final concentration of 1x and analyzed by 8% SDS-PAGE followed by Coomassie staining.

2.2.5.7 Chain cleavage

Materials

SUMO-2 chains (provided by Sarah Schulz, PhD student in our laboratory)

USPL1cat

SENP1cat (common stock of our laboratory)

USP5 (provided by Sarah Schulz, PhD student in our laboratory)

TB buffer supplemented with 5 mM DTT and 1 $\mu\text{g/ml}$ each of aprotinin, leupeptin, pepstatin

5 μl of SUMO-2 chains and were incubated with 5 μM USPL1cat or 150 nM USP5 for 0-60 minutes at 37°C. The reaction was terminated at different time point and samples were analyzed by 5-20% gradient SDS-PAGE followed by Coomassie staining.

2.2.5.8 Binding assay

Material

SUMO-1-Sepharose, SUMO-2-Sepharose, Ovalbumin-Sepharose, Ubiquitin-Sepharose, protein concentration 1 mg/ml (provided by Sarah Schulz, PhD student in our laboratory)

SAB buffer

Washing buffer (TB buffer supplemented 0.05 % (v/v) Tween-20)

To 20 μg of USPL1cat 20 μl of either SUMO-1-Sepharose, SUMO-2-Sepharose, Ubiquitin-Sepharose or Ovalbumin-Sepharose, and SAB was added to the final volume of 200 μl . Samples were incubated for 2 hours on a rocking platform at 4°C. Beads material was collected by centrifugation (tabletop centrifuge 2000 rpm, 20 sec) and washed 3 times with the washing buffer. Beads bound protein was eluted by adding 40 μl of 1x Sample buffer and incubating at 95°C for 5 minutes. Eluates were analyzed by 15% SDS-PAGE followed by coomassie staining.

2.2.5.9 Ubiquitin cleavage assay

Materials

Ubiquitin AMC (Biomol)

USP1cat

USP5 (Sarah Shulz)

TB buffer

Black micro titer 386-well plates (Greiner)

Microplate reader, Fluoroskan Ascent (Labsystems)

The reaction was pipetted into a black microtiter plate. 5 μ M Ubiquitin-AMC was used as a substrate, 50 nM USPL1cat and 5 nM USP5 were used in a total volume of 25 μ l. AMC fluorescence was measured at 360 nm excitation 465 nm emission filter pair every 6 seconds for 25 minutes with integration time of 100 ms. AMC fluorescence values were plotted against time.

3. RESULTS

3.1 Search for SUMO specific isopeptidases

The aim of this work was to identify a novel SUMO specific isopeptidase. To do this we used two different approaches:

- a) A screen of a bacterial expression library of human ORFs using a FRET-based desumoylation assay.
- b) Biochemical purification of SUMO specific proteases from HeLa cells lysates using SUMO-Vinylmethylester (VME).

3.3.1 A high-throughput screen for a SUMO isopeptidases

To identify novel SUMO specific proteases we screened a bacterial expression library of human ORFs for desumoylating activity. This library was developed by Dr. Erich Wanker (Max-Delbrück Center, Berlin) (Grelle *et al.*, 2006), who provided us with lysates in 384-well format (see materials and methods chapter for a detailed description of the library). The library consists of *E. coli* clones, transformed with human ORFs in a prokaryotic expression vector. Each clone is transformed with a different ORF. Bacterial clones are grown, induced and lysed in 384-well plate format. Lysates can be screened in high-throughput manner for activity of interest if the appropriate assay is available.

To screen the library for desumoylating activity I used a FRET-based desumoylation assay developed in our laboratory (Bossis *et al.* 2005, Stankovic-Valentin *et al.*, 2009) (see materials and methods section for a detailed description of the assay). When CFP-GapTail and YFP-SUMO are unconjugated, excitation at 435nm (excitement maximum for CFP) results in a strong emission by CFP-GapTail at 485 nm, but only in minimal YFP-SUMO excitement and emission at 527 nm. After excitation of CFP-GapTail conjugated with YFP-SUMO, part of the energy of excited CFP-GapTail is transferred to YFP-SUMO due to Fluorescence resonance energy transfer (FRET) between these components. As

a result the emission of CFP-GapTail decreases and the emission of YFP increases. Direct read out of the assay are fluorescence values for excitation at 435 nm with emission at 485 nm (CFP-GapTail excitation and emission) and excitation at 435 nm with emission at 527 nm (CFP-GapTail excitation, YFP-SUMO emission). Changes in the ratio between fluorescence emission of CFP and YFP (485/527 nm ratio) are proportional to changes in the amount of the conjugated components and can be used to monitor the progress of sumoylation/desumoylation reaction. Fluorescence measurements can be performed in multi well plates, which allows adaptation of the assay to a high-throughput screen.

The buffer in which measurements are done may influence the fluorescence. The buffer used for the preparation of the bacterial lysates differs significantly from the sumoylation assay buffer in which the assay was originally developed. Therefore I tested whether this lysis buffer is compatible with the assay. When undiluted lysate is added into reaction in a standard amount (5 μ l of lysates to 20 μ l of 1 μ M substrate) the signal to noise ratio becomes too low to interpret accurately obtained results (data not shown). To overcome this problem the lysates were diluted with sumoylation assay buffer. 20 fold dilution of the lysates allowed for undisturbed measurement.

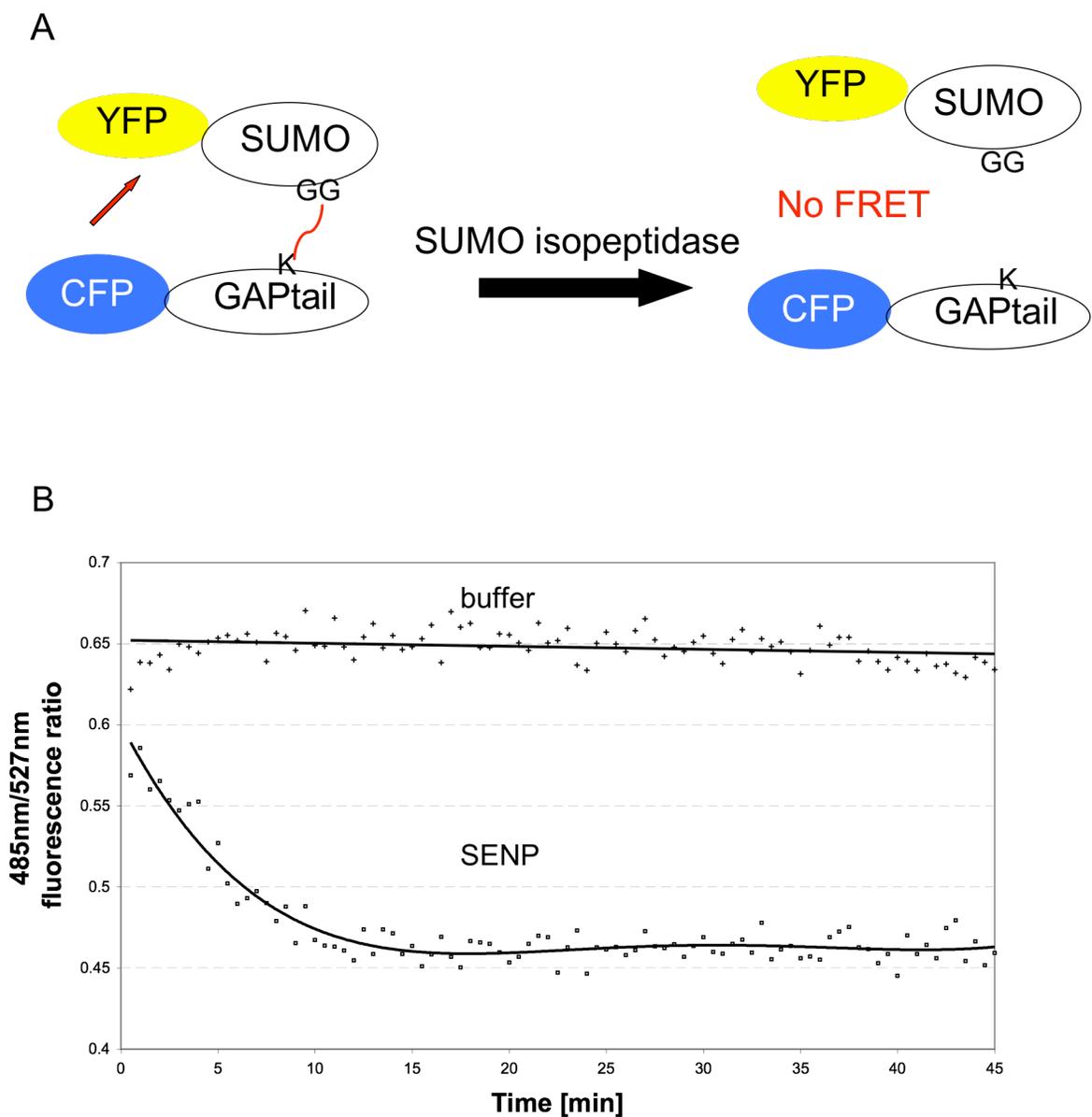
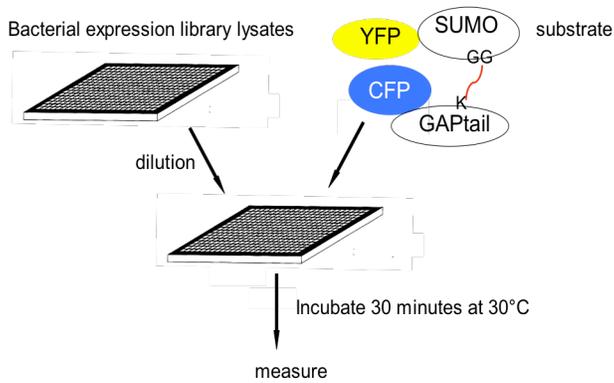


Figure 7. FRET-based assay as a tool to study isopeptidases. **A.** The principle of the assay. Desumoylation leads to the loss of FRET between CFP and YFP. **B.** CFP-GAP conjugated with YFP-SUMO-1 ($1\mu\text{M}$) was incubated with either GST-SEN1cat ($1\mu\text{M}$) or SAB buffer at 30°C , in a 384-well microtiter plate in total volume of $25\mu\text{l}$. The kinetics of reaction was followed by exciting CFP and measuring YFP and CFP fluorescence emission.

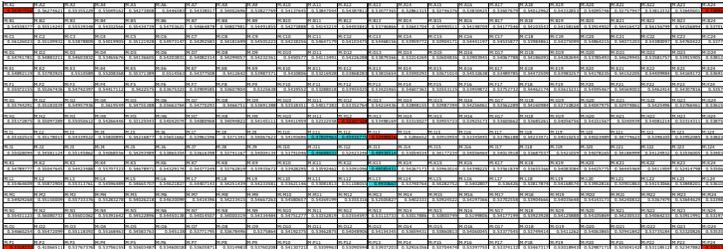
Next I tested whether the assay can be applied to a high-throughput screen. For this, our collaborators included 6 colonies of *E.coli* the expressing catalytic domain of SENP1 (SENP1cat) in 6 unspecified wells of a 384 well plate. None of the clones present in the rest of the wells contained known SUMO isopeptidase. My goal was to identify the wells containing SENP1cat. Together with Dr. Reiner Hitt (Transcriptome analysis laboratory, the University of Göttingen) we screened the plate using an automated robotic station as shown in Figure 8A. The lysates were diluted with SAB into a fresh plate and subsequently pipetted into a plate containing CFP-GapTail conjugated with YFP-SUMO-1. After incubation, I measured the fluorescence in each well. The results are summarized in Figure 8B and C. Six wells (shown in red) had a 485/527nm emission ratio corresponding to complete deconjugation, indicating presence of SENP1cat. Six wells, adjacent to SENP1cat containing wells, had the fluorescence ratio significantly lower than fully conjugated substrate (shown in blue), however the deconjugation was not complete. Our collaborators confirmed that SENP1cat was indeed present in the wells with ratios corresponding to fully deconjugated substrate. The wells with significant signal loss did not contain proteins of which it was known to possess SUMO specific isopeptidase activity or any other protease activity. However it turns out that these wells were contaminated with SENP1cat from adjacent wells: lysates of these clones grown separately showed no desumoylating activity. This confirmed that the activity observed during the screen was a result of a spill over. The fact that all six wells containing SENP1cat were easily identified and that a spill over could be detected indicated that the FRET assay is sensitive enough to use for high-throughput screening.

I screened the whole library containing 3860 different clones (Figure 8D). As a positive control I used a plate containing SENP1cat. Unfortunately no desumoylating activity was detected in any of the wells except for SENP1cat. Later I discuss the possible reasons for not detecting desumoylating activity and propose alterations in the screen.

A



B



C

well	E527/E485 ratio	content
A1	0.34	SENP
P1	0.33	
A24	0.33	
P24	0.33	
H12	0.32	
I13	0.33	
I11	0.48	directly adjacent to SENP - spill over
J11	0.45	
I12	0.46	
J13	0.49	
K13	0.48	
L13	0.49	no SENP
H15	0.54	
D16	0.54	
L20	0.54	
I3	0.53	
P11	0.54	

D

plates	# of clones	fluorescence ratio > 0.5	fluorescence ratio < 0.5
10	3840	3840	0

Figure 8. The FRET-based assay can be used to screen bacterial expression libraries.

A. Schematic representation of the screen. Bacterial lysates are diluted with SAB, incubated with substrate and the fluorescence is measured. **B.** Testplate with positive control. Bacterial clones expressing SENP1cat were placed on the plate in 6 unspecified wells. The plate was screened as shown in panel A. Wells in which FRET was completely lost are shown in red. Wells with significant loss of FRET are shown in blue. **C.** Testplate screen summary. **D.** Summary of the whole library screen.

3.3.2 Biochemical purification of SUMO-isopeptidases using SUMO-VME

As our screen did not result in identification of a novel SUMO protease we decided to take an alternative approach based on biochemical purification. To do this we used SUMO-VME, which is a recombinant mature SUMO modified chemically on the C-terminus with vinylmethylester. Vinylmethylester resembles peptide bond and once attacked by a protease it forms a stable covalent bond with the catalytic residue of an enzyme.

Chemical probes used to study the Ubiquitin system were developed several years ago (Borodovsky *et al.*, 2001 and 2002). They were successfully used to identify new Ubiquitin and other Ubl like proteases. They can also be used to study enzymatic activity of proteases. For ubiquitin proteases, the chemical with the broadest spectrum of specificity is vinylmethylester. The protocol for synthesis of Ubiquitin-VME was developed by Dr. Huib Ovaa (NKI, Amsterdam) and adopted to SUMO technology by Erik Meulmeester, a postdoc in our laboratory. He generated a construct for bacterial expression of HA-SUMO-intein-chitin binding domain protein. The N-terminal HA epitope allows for immunopurification of SUMO-VME-modified proteins using anti-HA antibodies. Recombinant SUMO fusion protein and the outline of the procedure generating the reactive SUMO-VME species are shown in Figure 9.

Expression, purification and synthesis of SUMO electrophilic traps

SUMO-VME were obtained by chemical ligation method (Chong *et al.*, 1997, Cotton and Muir 1999). SUMO-1 or SUMO-2 lacking the last glycine residue were expressed recombinantly in *E.coli* as a fusion with intein and chitin binding domains. To obtain reactive thioester (Strp-HA-TEV-SUMO-MESNa), proteins were bound to chitin-beads followed by subsequent transthioesterification with MESNa. Strep-HA-TEV-SUMO-MESNa was purified using size-exclusion chromatography, and used for chemical ligation with

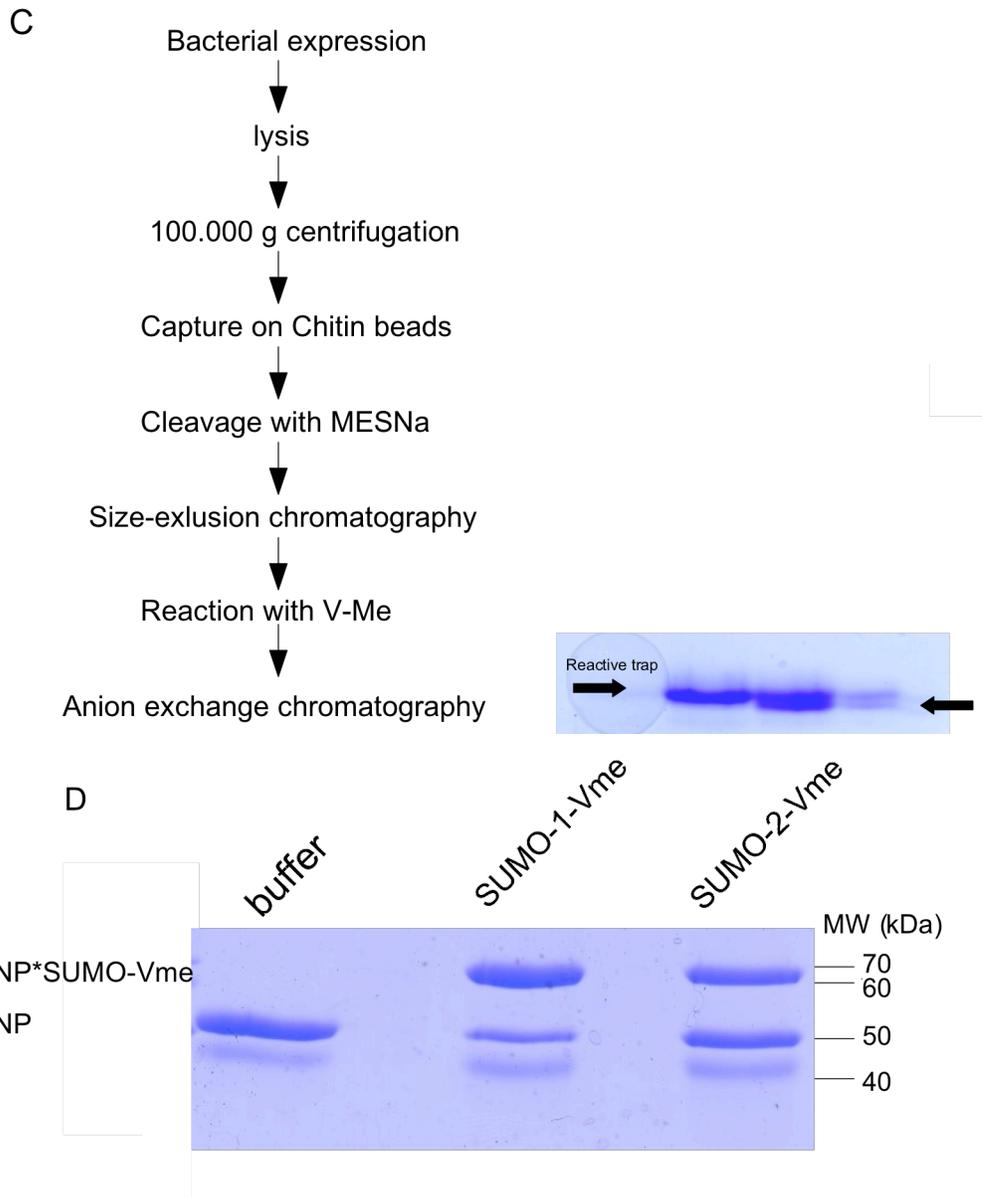


Fig 9. Generation, purification and testing of SUMO-VMEs.

A. Schematic representation of SUMO electrophilic traps – HA tag, Strep tag and TEV cleavage site are indicated. **B.** The scheme of electrophilic trap synthesis. **C.** Left – The flowchart of SUMO-VME generation (synthesis and purification), right - fractions of MONO-Q containing reactive SUMO-Vme **D** . SUMO electrophilic traps react with desumoylating enzymes. 2 μ g of recombinant GST-SEN1cat was incubated for 30 minutes at 37°C in the presence of 1 μ g SUMO-1-VME, 1 μ g SUMO-2-VME or buffer. Samples were analyzed by SDS-PAGE followed by coomassie staining.

SUMO-VME can modify endogenous proteins present in HeLa cell lysate.

To identify putative novel SUMO protease I tested whether SUMO-VME can modify endogenous human proteins. The HeLa cell lysate was incubated with increasing amounts of either SUMO-1-VME or SUMO-2-VME, centrifuged to remove precipitated or aggregated proteins, precleared by incubation with protein A beads and subjected to an anti-HA immunoprecipitation. Proteins bound specifically to an anti-HA antibody via an HA epitope were eluted by competition with HA-peptide. Eluates were resolved by SDS-PAGE and analyzed by immunoblotting with an α -HA antibody (Figure 10 A and B) . Multiple bands were observed both in case of SUMO-1-VME and SUMO-2-VME samples, whereas the control, in which no SUMO-VME was present in the extract remained empty, suggesting that each of detected bands was a protein that specifically reacted with a trap. Overall patterns of proteins immunoprecipitated with SUMO-1 and SUMO-2-VME were similar, however a very conspicuous band of approximate 85 kDa in size was present only in samples incubated with SUMO-2 VME. This band could represent an isopeptidase that is specific for SUMO-2. To enrich proteins reacting specifically with SUMO-2-VME HeLa, cell extract was first incubated with SUMO-1 Vme, and subjected to immunoprecipitation with an anti-HA antibodies. Proteins specifically bound to anti-HA antibodies were eluted with HA-peptide (fraction S1). The supernatant that was partially depleted from SUMO-1 reacting proteins was then incubated with SUMO-2-VME. Labeled proteins were immunoprecipitated and eluted from the beads as for SUMO-1-VME (fraction S2). Eluates were resolved by SDS-PAGE and analyzed by immunoblotting with an anti-HA antibody. Also here a strong band was present at 85 kDa only in case of SUMO-2-VME labeled proteins, indicating the presence of a SUMO-2 specific protease (Figure 11).

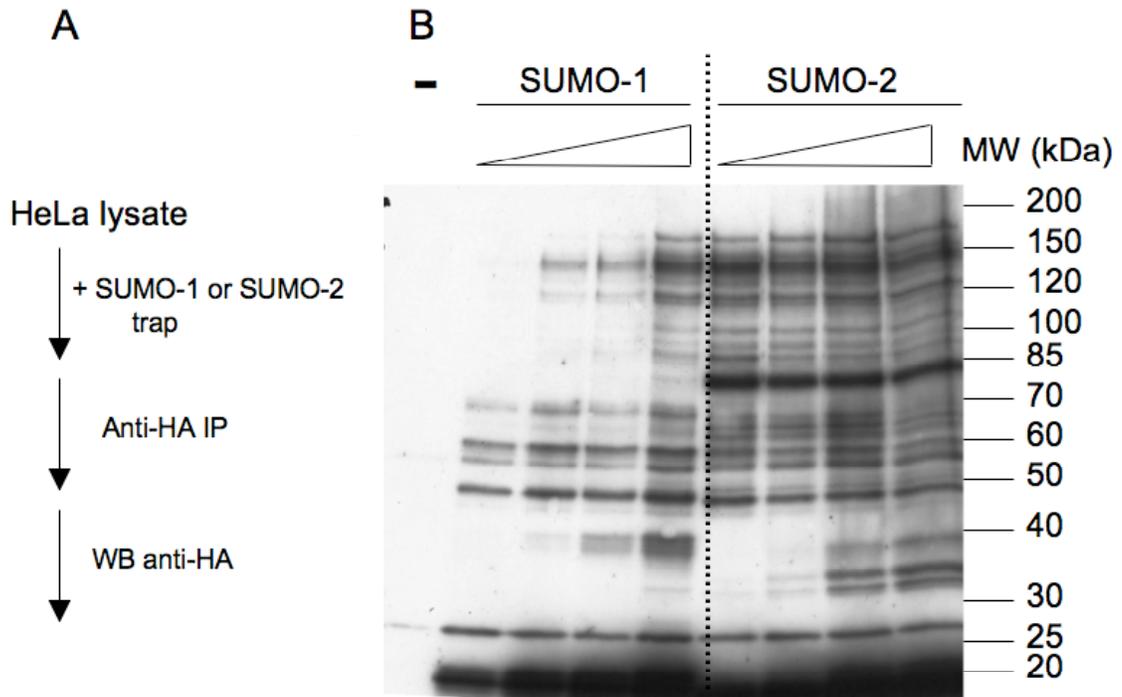


Figure 10. Enrichment of SUMO-1-VME and SUMO-2-VME modified proteins.

A. The scheme of the purification. **B.** 1.5 ml of HeLa cell lysate (10mg/ml) was incubated for 30 minutes at 37°C with increasing amounts of either SUMO-1-VME (75-600 ng), SUMO-2-VME (250ng - 2 µg) or buffer (-). Lysates were centrifuged at 100.000 g and subjected to an anti-HA IP followed by HA-peptide elution. Eluates were analysed by SDS-PAGE followed by anti-HA Western Blotting.

Having established a protocol allowing biochemical purification of SUMO-VME modified proteins I upscaled the procedure to identify these proteins by mass spectrometry.

25 ml HeLa cell lysate was incubated with 10 µg of SUMO-1-VME followed by anti-HA immunoprecipitation and HA-peptide elution. Supernatant was incubated with 10 µg of SUMO-2-VME, and the same procedure as for SUMO-1-VME was followed. Protein identification was performed in collaboration with Dr. Henning Urlaub (Max-Planck Institute for Biophysical Chemistry, Göttingen). Eluates were resolved by SDS-PAGE, gel lanes containing proteins were cut into slices from which protein was extracted.

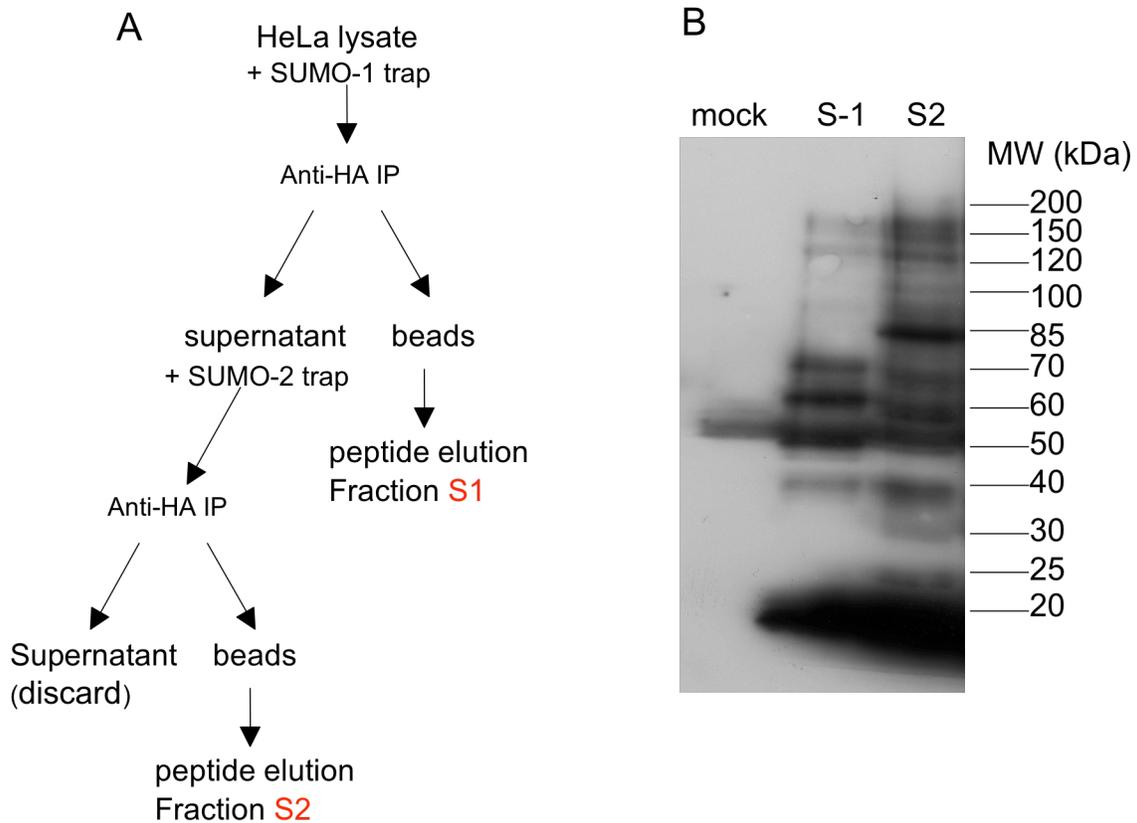


Fig 11. Sequential application of SUMO-VMEs can be used to enrich for paralogue specific isopeptidases.

A. Scheme of purification. **B.** 1.5 ml (10 mg/ml) of HeLa cell lysate was incubated with 1 μ g of SUMO-1-VME for 30 minutes at 37°C. Afterwards lysates were subjected to centrifugation (100.00 g 30 minutes at 4°C) and anti-HA IP followed by HA-peptide elution (eluted fraction – S1). The supernatant was incubated with 1 μ g of SUMO-2-VME, centrifuged and subjected to anti-HA IP followed by peptide elution. Lysate without trap was used as a control. Eluates were analyzed by SDS-PAGE followed by anti-HA Western Blotting.

After trypsin digestion samples were analysed by mass spectrometry (Figure 12). Identified proteins are shown in Tables 2 and 3 for SUMO-1-VME and SUMO-2-VME respectively.

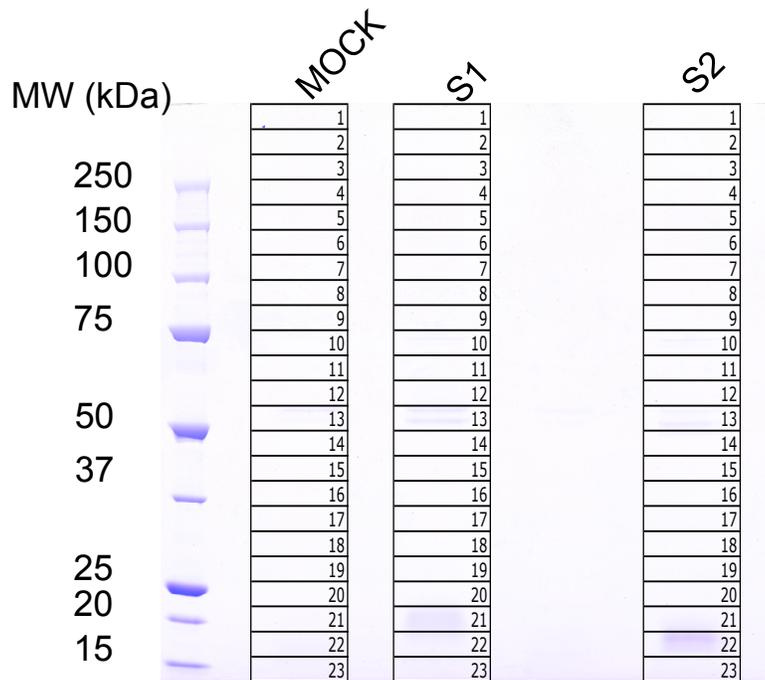


Figure 12. Gel with immunoprecipitated proteins. Gel picture provided by Monika Raabe, (Department of Mass Spectrometry, Max-Planck Institute for biophysical chemistry, Göttingen).

The majority of identified proteins were known SUMO isopeptidases. Four out of six members of the SENP family were identified. One of them, SENP5, was identified only upon SUMO-2-VME labeling. Presence of SENP proteins in many gel slices, including those corresponding to lower than predicted molecular weight of the full length protein suggested presence of splice variants or limited proteolysis products.

Interestingly four proteins of which no link to the SUMO pathway was known were also identified. In case of SUMO-1-VME USPL1 and F-boxWD40.5 were identified. In case of SUMO-2-VME USPL1, F-boxWD40.5, HCF-1 and OGT-1 were found.

Slice	Protein	accession number	# of peptides	score
1	SEN6	gi 7662312	8	256
2	SEN6	gi 7662312	9	206
3	SEN6	gi 7662312	1	45
4	SEN6	gi 7662312	18	464
	USPL1	gi 55639683	3	98
5	SEN6	gi 7662312	24	577
	USPL1	gi 55639683	3	149
6	SEN6	gi 7662312	26	566
	USPL1	gi 55639683	2	99
	USPL1	gi 55639683	3	63
7	SEN6	gi 7662312	13	407
	SEN1	gi 7657550	1	41
8	SEN6	gi 7662312	2	103
9	SEN6	gi 7662312	4	94
10	SEN1	gi 7657550	4	153
11	F-box and WD-40 domain protein 5	gi 24308129	9	373
	SEN1	gi 7657550	5	167
12	SEN1	gi 7657550	3	120
	SEN6	gi 7662312	1	29
14	SEN1	gi 7657550	4	192

Table 5. Proteins identified upon SUMO-1-VME treatment. Known SUMO-isopeptidases are shown in red, other proteins are shown in blue.

HCF-1

Host Cell factor-1 is a conserved and very abundant (100.000 per cell) chromatin associated protein (Wysocka *et al.*, 2001). It is synthesized as a precursor and migrates to the nucleus where it undergoes cleavage near the middle of the protein (Wilson *et al.*, 1993). Generated subunits remain associated non-covalently (Wilson *et al.*, 1995). HCF-1 is involved in the regulation of cell proliferation and functions as a transcriptional regulator (Goto *et al.*, 1997, Wysocka and Herr 2003). It interacts with a number of transcription factors like Sp1 or members of the E2F family and chromatin modifying enzymes like histone deacetylase or methylase (Vogel *et al.* 2000, Tyagi *et al.*, 2007, Wysocka *et al.*, 2003). HCF-1 also interacts with OGT-1 and is a target for O-glycosylation.

slice	Protein	accession number	# of peptides	score
3	HCF-1	gi 558349	2	42
4	HCF-1	gi 98986457	4	115
	SENP6	gi 55626950	2	108
5	SRNP6	gi 7662312	11	286
	HCF-1	gi 98986457	8	235
	USPL1	gi 55639683	3	96
6	HCF-1	gi 98986457	10	410
	SENP6	gi 7662312	11	198
	OGT-1 O-linked GlcNAc transferase isoform 1	gi 32307150	2	93
	USPL1	gi 55639683	1	81
7	O-linked GlcNAc transferase isoform 1	gi 32307150	9	307
	SENP6	gi 7662312	3	100
	HCF-1	gi 55664732	6	99
8	SENP5	gi 14250319	4	202
	SENP6	gi 9963808	2	78
	HCF-1	gi 558349	3	63
	F-box and WD-40 domain protein 5	gi 10438817	2	25
	Hs-CUL-1	gi 1381142	1	24
9	SENP6	gi 7662312	2	38
	SENP5	gi 14250319	1	33
	HCF-1	gi 558349	2	23
	FBXW5 protein [Homo sapiens]	gi 10438817	1	21
10	SENP5	gi 14250319	9	384
11	F-box and WD-40 domain protein 5	gi 10438817	6	217
	SENP5	gi 14250319	3	130
12	SENP1	gi 7657550	2	43
	SENP6	gi 7662312	1	36

Table 6. Proteins identified upon treatment with SUMO-2-VME. Known SUMO-isopeptidases are shown in red, other proteins are shown in blue.

The role of this modification is unclear (Wysocka *et al.*, 2003). Many of the HCF-1 interactors bind to it via a tetrapeptide termed the HCF-1 binding motif (HBM) [D/E]HxY (Wysocka and Herr 2003). Recently it was shown that via the coupling of E2F proteins to chromatin modifying complexes, HCF-1 regulates posttranslational modifications of histones associated with E2F responsive genes. By doing so it affects the transcription of these genes, many of which are in cell proliferation. (Tyagi *et al.* 2007). We identified HCF-1 only upon SUMO-2-

Vme labeling, and hypothesized that either it associates with very stably SUMO-2 or a desumoylating enzyme that was purified specifically with SUMO-2. SENP5 was the only SENP that was purified exclusively with SUMO-2. SENP5 has two HBMs (127-130 DHEY and 459-462 DHPY) and is the only human SENP protein that possesses such motif. HCF-1 therefore most probably copurified with SENP5. Cells lacking functional HCF-1 arrest in the cell cycle, which is reminiscent of the phenotype of cells depleted from SENP5 (Di Bacco *et al.* 2006). Sumoylation of transcription factors is known to inhibit transcription, probably by recruiting transcriptional repressors (Hay 2005, Stielov *et al.* 2008). Sumoylation of histones also represses transcription. An attractive hypothesis that will be followed in the future is that HCF-1 attracts the desumoylating enzyme SENP5 to desumoylate a transcription factor or histone or both.

OGT-1

OGT-1 (O-linked GlcNAc transferase) is an enzyme involved in O-glycosylation of serine residues. It is known to interact with HCF-1, which itself is a target for O-glycosylation.

FboxWD40#5

Proteins containing Fbox and WD40 domains are substrate recognition subunits of cullin1-based E3-ubiquitin ligases. These ligases are composed of several subunits: the scaffold subunit is cullin1 that on one terminus binds both Roc1 and Skp1. Roc1 is a RING finger protein that recruits E2 enzymes, and Skp1 binds receptor recognition subunits. The Fbox module of FboxWD40 proteins binds to Skp1, and the WD40 domain binds substrates (Petroski and Deshaies 2005). We hypothesized that FboxWD40 5 is a receptor subunit responsible for SUMO ubiquitination. This idea is now investigated by other in our laboratory.

Surprisingly none of those three proteins had a domain that could be a protease domain. Therefore the most reasonable explanation for their co-immunopurification is that they interact very stably but noncovalently either with SUMO or enzymes of SENP family.

3.2 USPL1 is a SUMO isopeptidase

The last protein identified from the immunopurification with SUMO-VME is USPL1. **U**biqSpecific **P**rotease like protein 1 is a member of the Ubiquitin Specific Peptidases Family, the largest family of the known Ubiquitin peptidases (Nijman *et al* 2005). A generic member of this family has a C19 cysteine protease domain (catalytic domain), in which the catalytic triad consists of cystein, apartate and histidine. USPL1 was suggested to be an inactive ubiquitin protease due to the absence of a non-catalytic histidine residue, conserved among other members of the USP family (swiss-prot/Q5W0Q7)..

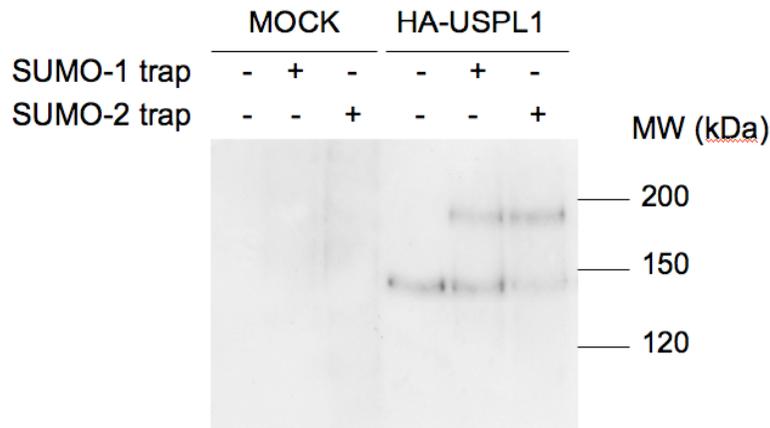


Fig 13. USPL1 reacts with SUMO-VMEs.

HEK 293T cells were transfected with HA-USPL1 using calcium phosphate precipitation method (10µg DNA per 10 cm dish at 80% confluency). 24 H after transfection lysates were prepared and subjected to anti-HA IP, followed by HA-peptide elution (60µl of final eluate). Eluates were divided in 3 parts and incubated with either SUMO-1-VME, SUMO-2-VME or buffer. Samples were analyzed by SDS-PAGE followed by anti-HA Western Blotting.

The alignment of the USPL1 C19 domain with C19 domains of ubiquitin specific proteases revealed the presence of a conserved catalytic triad in USPL1 (Figure 20). Therefore I decided to test whether USPL1 is a SUMO isopeptidase. First, I tested if USPL1 purified from mammalian cells can be labeled with SUMO-VME. In order to do so, HEK293 cells were transfected with HA-USPL1. One day after transfection cells were lysed, HA-USPL1 was immunopurified, eluted from the beads with an HA-peptide and incubated with either SUMO-1-VME, SUMO-2-VME or buffer. Samples were analyzed by immunoblotting with anti-HA antibodies. HA-USPL1 could be labeled both by SUMO-1 and SUMO-2-VME (Figure 13). This fact strongly suggested that USPL1 is a SUMO protease.

To further confirm that USPL1 is a SUMO isopeptidase I cloned and expressed its catalytic domain (USPL1cat). The region chosen for a recombinant expression was picked up based on sequence alignment of USPL1 from different species and its secondary structure prediction. Three different fragments were chosen for expression trials, residues 212-489, 212-502 and 212-514. To determine optimal expression conditions I tested different *E.coli* strains, temperatures, IPTG concentrations and expression times (summarized in Figure 14). A soluble protein was obtained by expressing the residues 212-514 of USPL1 N-terminally tagged with His-GST in *E.coli* Rosetta2 strain at 15°C for 16 hours after induction with 0.5 mM IPTG. The protein was purified by glutathione affinity chromatography, followed by TEV cleavage, removal of the HIS-GST tag as well as uncleaved fusion protein by Nickel affinity chromatography. The final step involved the size-exclusion chromatography. With this protocol I obtained protein of very high purity with a yield of 1mg per 1 liter of bacterial culture (Figure 14).

A

E.coli Rosetta

tag	residues	temperature	IPTG concentration	induction time	solubility	yield
N-term HIS-GST	212-489	37	1 mM	3H	-	n/d
N-term HIS-GST	212-489	25	0.1 mM	6H	-	n/d
N-term HIS-GST	212-489	16	0.1 mM	12-16H	-	n/d
N-term HIS-GST	212-502	37	1 mM	3H	-	n/d
N-term HIS-GST	212-502	25	0.1 mM	6H	-	n/d
N-term HIS-GST	212-502	16	0.1 mM	12-16H	-	n/d
N-term HIS-GST	212-514	37	1 mM	3H	-	n/d
N-term HIS-GST	212-514	25	0.1 mM	6H	-	n/d
N-term HIS-GST	212-514	16	0.1 mM	12-16H	-	n/d

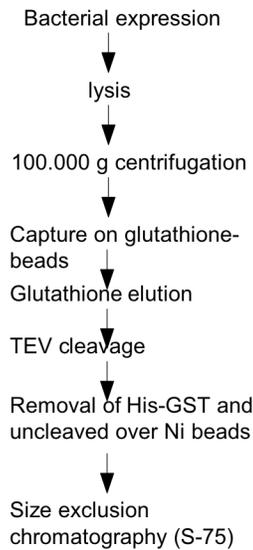
E.coli pLysS

tag	residues	temperature	IPTG concentration	induction time	solubility	yield
N-term HIS-GST	212-489	37	1 mM	3H	-	n/d
N-term HIS-GST	212-489	25	0.1 mM	6H	-	n/d
N-term HIS-GST	212-489	16	0.1 mM	12-16H	-	n/d
N-term HIS-GST	212-502	37	1 mM	3H	-	n/d
N-term HIS-GST	212-502	25	0.1 mM	6H	-	n/d
N-term HIS-GST	212-502	16	0.1 mM	12-16H	-	n/d
N-term HIS-GST	212-514	37	1 mM	3H	+	n/d
N-term HIS-GST	212-514	25	0.1 mM	6H	+	n/d
N-term HIS-GST	212-514	16	0.1 mM	12-16H	+	100ug/10l culture

E.coli Rosetta2

tag	residues	temperature	IPTG concentration	induction time	solubility	yield
N-term HIS-GST	212-514	37	1 mM	3H	+	n/d
N-term HIS-GST	212-514	25	0.5 mM	6H	+	n/d
N-term HIS-GST	212-514	16	0.5 mM	12-16H	+	1 mg/1l culture

B



C

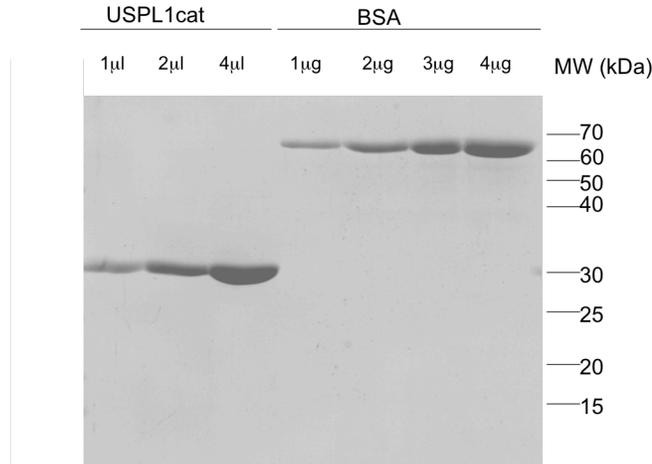


Figure 14. Expression and purification of USPL1 catalytic domain

A. Summary of expression trials. **B** Scheme of purification. **C** SDS-PAGE followed by Coomassie staining was used to determine the purity and the concentration of recombinant USPL1cat .

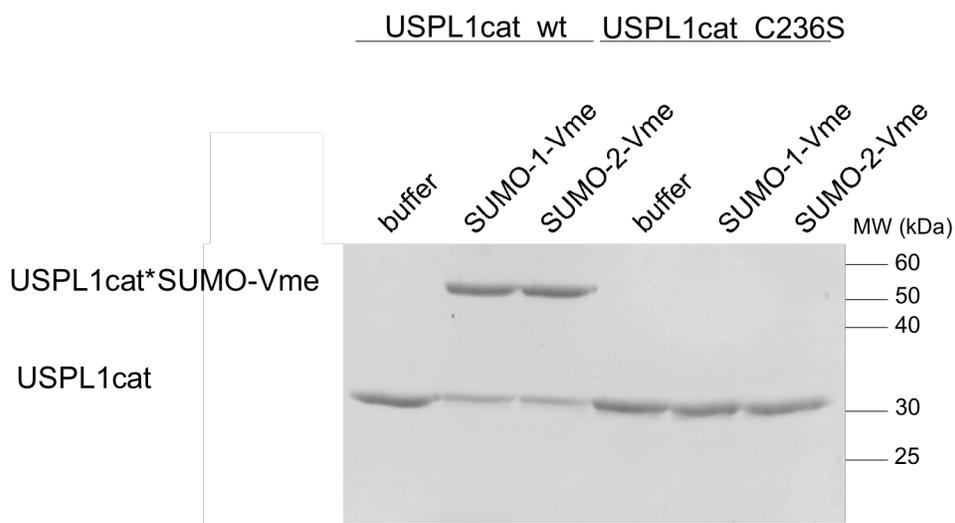


Figure 15. The reaction of USPL1 with SUMO traps depends on the catalytic cysteine C236.

1 μ g of recombinant USPL1cat wild type (wt) or catalytic mutant (C236S) was incubated at 37° for 30 minutes with 1 μ g of either SUMO-1-VME, SUMO-2-VME or buffer (total volume 20 μ l). Samples were analyzed by SDS-PAGE followed by Coomassie staining.

To examine whether USPL1 is modified by SUMO-VME at the active site I generated also a mutant version of USPL1cat, in which the predicted catalytic cysteine was replaced by serine. Both proteins were next subjected to SUMO-VME labeling. Wild type USPL1 catalytic domain was indeed labeled upon treatment with SUMO-1-VME or SUMO-2-VME, while the mutant showed no reactivity (Figure 15). This result indicated that SUMO-VME indeed labels the catalytic cysteine of USPL1, and that this reaction does not require any additional factors, therefore strongly suggesting that USPL1 is a SUMO protease.

To demonstrate actual isopeptidase activity it was necessary to show that USPL1 can remove SUMO from a target. To do this, RanGAP *in vitro* sumoylated with either SUMO-1 or SUMO-2 was incubated with catalytic amount of USPL1cat for different times. Samples were analyzed by SDS-PAGE and Coomassie staining. USPL1 was able to remove both SUMO-1 and SUMO-2 from RanGAP, however the reaction was more efficient for RanGAP modified with SUMO-2 (Figure 16).

This experiment shows that USPL1 is indeed a SUMO isopeptidase that exhibits strong preference towards SUMO-2.

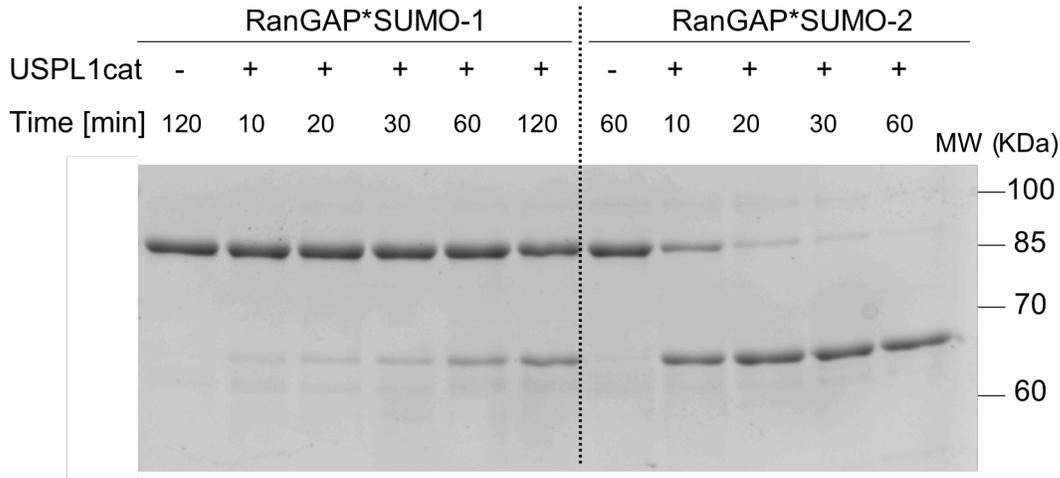


Figure 16. USPL1cat is a SUMO isopeptidase with preference towards SUMO-2.

RanGAP sumoylated *in vitro* with either SUMO-1 or SUMO-2 (2 μ M), was incubated at 37°C with USPL1cat (4nM) or buffer (-) in total volume of 20 μ l for indicated times. The reaction was terminated by adding SDS sample buffer and boiling. Samples were analyzed by SDS-PAGE followed by Coomassie staining. (RanGAP1-SUMO-1 provided kindly by Dr. Andreas Werner).

Similar to Ubiquitin, SUMO-2 can form polymeric chains *in vivo*. Members of the USP family differ in their specificity towards the type of Ubiquitin chain and at least in some cases this specificity is a feature of the catalytic domain. We were interested whether USPL1cat can cleave SUMO-2 chains. To address this matter, *in vitro* prepared SUMO-2 chains were incubated with USPL1cat for different time periods and the samples were analyzed by SDS-PAGE. For positive control the catalytic domain of SENP1 was used, for negative control the Ubiquitin specific isopeptidase of the USP family, USP5, was used. USPL1cat was able to cleave SUMO-2 chains (Figure 17).

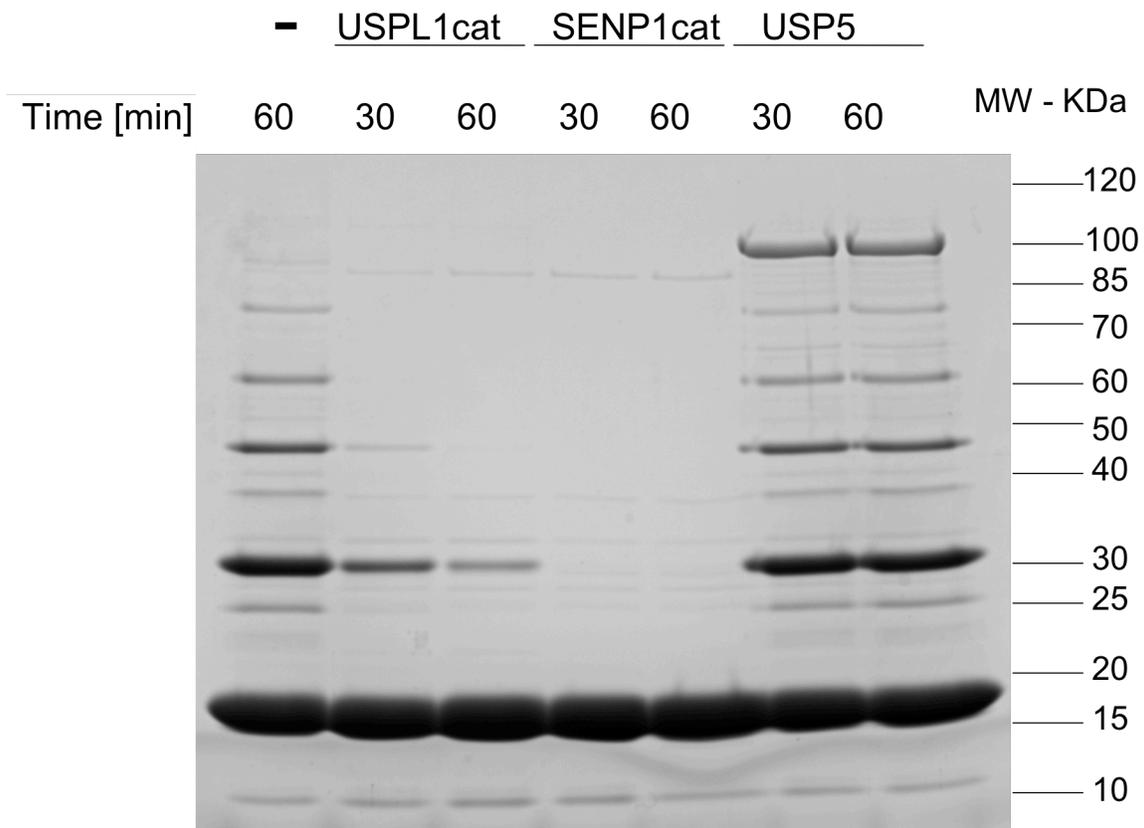


Figure 17. USPL1cat can cleave SUMO-2 chains.

5nM USPL1 or SENP1cat or 150 nM USP5 were incubated with 5 μ l *in vitro* prepared SUMO-2 chains in total a reaction volume of 40 μ l for indicated times at 37°C. Reaction was stopped by adding sample buffer. Samples were analyzed by SDS-PAGE followed by Coomassie staining. (SUMO-2 chains and USP5 kindly provided by Sarah Schulz).

Based on sequence homology, USPL1 is classified as a ubiquitin isopeptidase. Several of the USP family members exhibit specificity toward UbIs, whereas other members can act both on Ubiquitin and other UbIs (see introduction). Therefore it was necessary to test USPL1 specificity towards Ubiquitin. Binding of ubiquitin by the catalytic domain is necessary for the ability of ubiquitin proteases to cleave it, therefore USPL1cat ability to bind SUMO and Ubiquitin was examined. USPL1 catalytic domain was incubated with either SUMO-1, SUMO-2 or Ubiquitin immobilized on sepharose beads. USP5 (a known

interactor of ubiquitin) was used as a positive control for Ubiquitin beads. After extensive washing bound protein was eluted by the addition of SDS sample buffer and boiling. The eluates were analyzed by SDS-PAGE. USPL1 bound both SUMO-1 and SUMO-2 but not Ubiquitin (Figure 18). This suggested that USPL1 can not cleave ubiquitin.

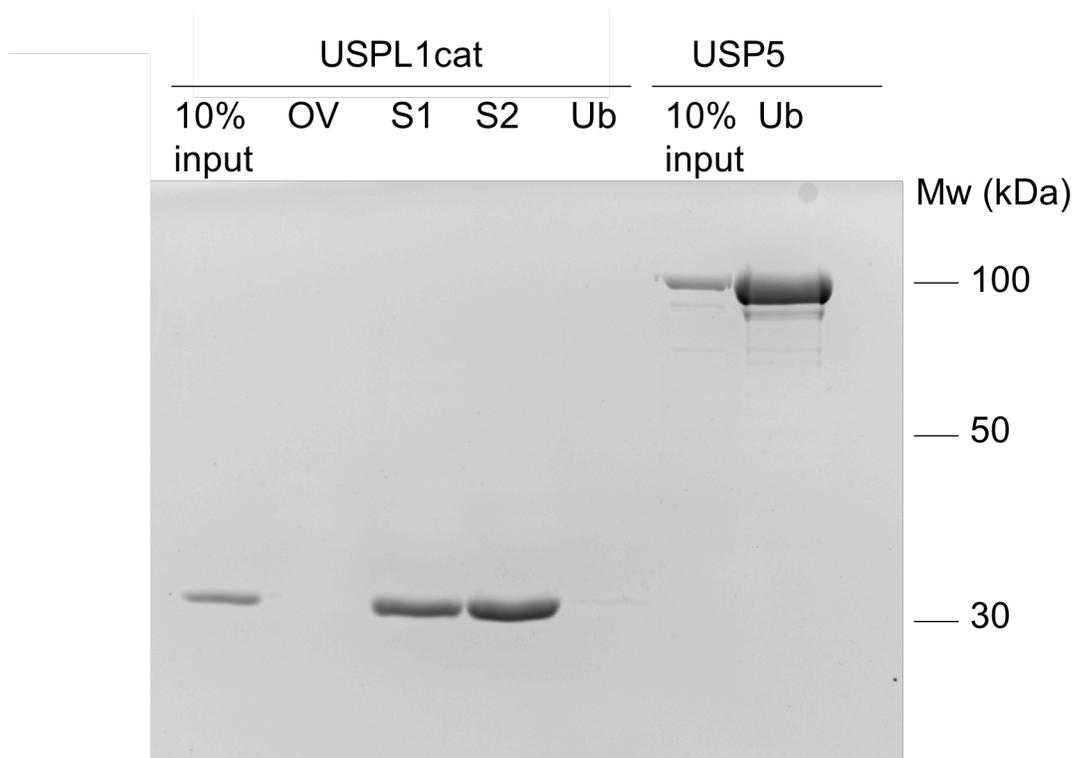


Fig. 18 USPL1 binds non-covalently to SUMO but not to Ubiquitin

10 μ g of recombinant USPL1cat was incubated with 20 μ g of either SUMO-1 (S1), SUMO-2(S-2), Ubiquitin (Ub) or ovalbumin (OV) immobilized on sepharose beads. After extensive washing of beads, the bound proteins were eluted with SDS sample buffer. The eluates were analyzed by SDS-PAGE and visualized by Coomassie staining. (sepharose beads with immobilized proteins and USP5 were kindly provided by Sarah Shulz)

Finally, USPL1's ability to cleave Ubiquitin was directly analyzed using Ubiquitin-AMC as a substrate. Ubiquitin-AMC is a fluorogenic substrate that releases fluorescent AMC upon treatment with Ubiquitin protease. The fluorescence is proportional to the amount of the released AMC and can be used to monitor

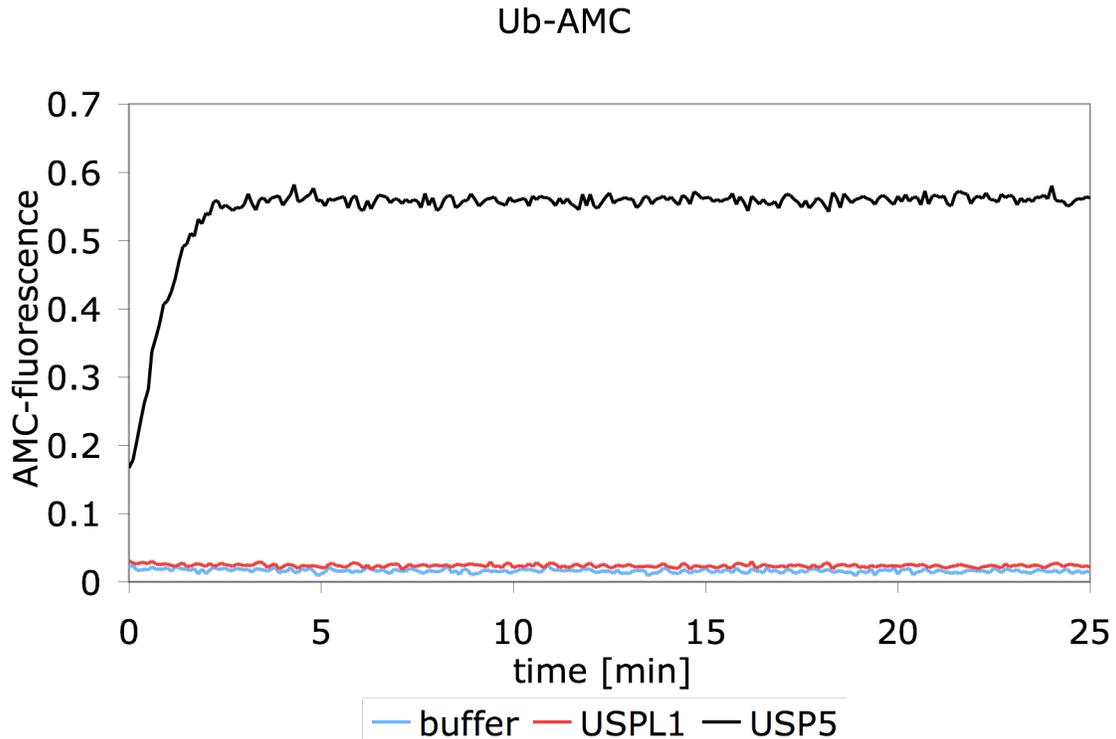


Figure 19. USPL1 does not exhibit specificity towards Ubiquitin-AMC. 5 μ M Ubiquitin-AMC was incubated with either 5 nM USP5, 50 nM USPL1cat or buffer. AMC fluorescence was measured and shown as a function of time. (USP5 kindly provided by Sarah Shulz)

enzymatic activity of Ubiquitin proteases. USPL1 showed no activity at all, while the positive control USP5 showed the expected activity. (Figure 19).

The findings presented here demonstrate that USPL1 is a SUMO, but not Ubiquitin, specific isopeptidase. It shows a strong preference for SUMO-2 conjugates and is able to cleave SUMO-2 chains.

4. DISCUSSION

Here, I describe the identification and initial biochemical characterization of a novel SUMO specific isopeptidase, USPL1. This enzyme was previously classified as an inactive Ubiquitin isopeptidase based on sequence homology. We also show that USPL1 prefers SUMO-2 to SUMO-1 and does not exhibit an enzymatic activity towards Ubiquitin.

4.1 USPL1 is an atypical member of the USP family.

Alignment of the catalytic domain of USPL1 with those of other USPs shows that the similarity between them is predominantly restricted to two sequence motifs, the Cys-box and the His-box (Figure 20). These motifs, which are present in all members of the USP family, contain residues that are responsible for catalysis and residues participating in binding of the Ubiquitin C-terminus (Hu *et al.*, 2002, Renatus *et al.*, 2004, Nijman *et al.* 2005,) (Figure 21). In USPL1 the residues of the catalytic triad are conserved (C236, H456, D473) but the residues binding the C-terminus of ubiquitin are not. The most striking example is the absence of the non-catalytic histidine important for binding of the C-terminus of Ubiquitin within the His-box. This residue is present in almost all other USPs (Quesada *et al.*, 2004). Lack of this residue was a reason for suggestion that USPL1 is an inactive Ubiquitin protease (swiss-prot/Q5W0Q7).

A prominent difference between USPL1 and other members of the USP family is the lack of the QQD-box in USPL1. Residues of this box form a loop that participates in binding of the Ubiquitin C-terminus (Hu *et al.*, 2002, Renatus *et al.*, 2004). Instead, USPL1 has a stretch of residues that are highly conserved in USPL1 from different species but absent from other USPs (Figure 20). The conservation of this region and its putative positioning close to the QQD-box in other USPs makes it a good candidate for a binding site for the C-terminus of SUMO.

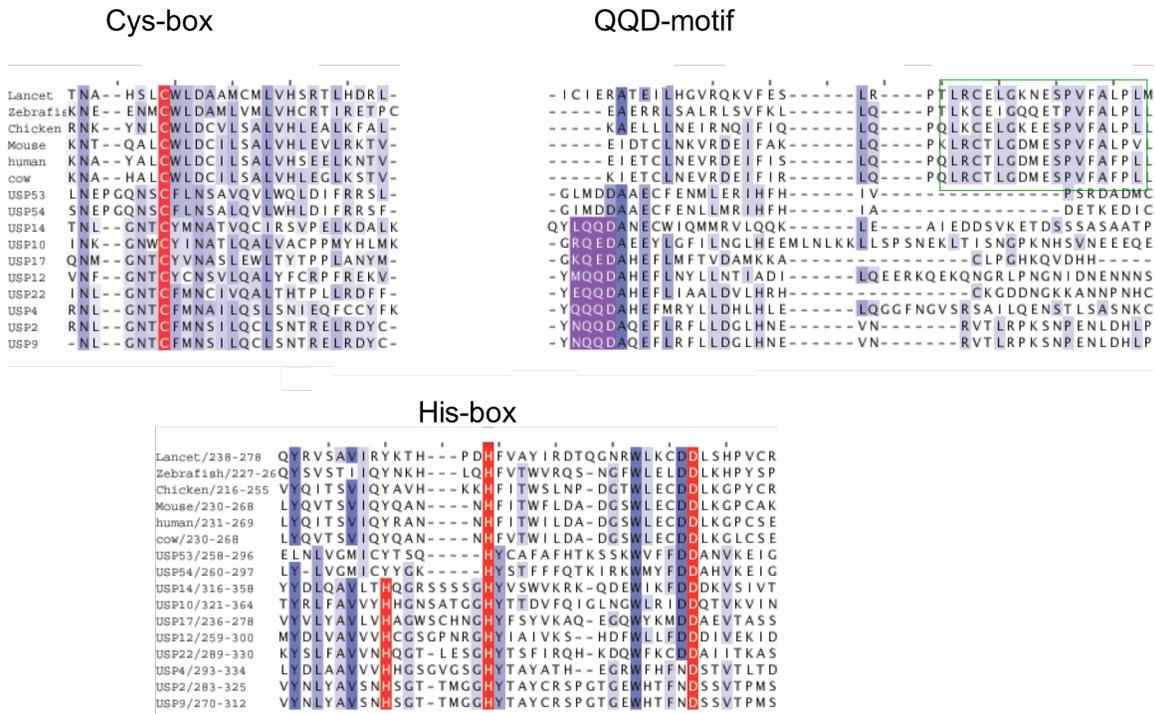


Figure 20. Catalytic domain of USPL1 has conserved Cys-box and His-box but the QQD-motif is absent from it. Catalytic domains of USPL1 from several species were aligned with catalytic domains of several members of the USP family. Conserved residues are shaded, the residues of catalytic triad are shown in red, the residues within QQD-motif in violet and the conserved region of USPL1 that may participate in SUMO binding is marked with a green box. (the alignment was prepared using MUSCLE online application at www.ebi.ac.uk, and visualized with Jalview online editor at www.jalview.org)

The afore mentioned differences between USPL1 and other USPs are easy to explain, since the C-termini of SUMO and Ubiquitin differ significantly. Consequently, it is reasonable to suspect that they are recognized by the proteases in a different way.

USPs bind to the β -grasp domain of Ubiquitin and the recognition is based on the complementary shapes of Ubiquitin and USP and is mediated by numerous weak interactions (Hu *et al.*, 2002, Renatus *et al.*, 2004). Most probably USPL1 binds to the β -grasp domain of SUMO, however it is not possible to predict the amino

acid residues in USPL1 or SUMO that are essential for binding. Structural analysis of a USPL1/SUMO complex and mutagenesis studies will be needed to elucidate the specificity of USPL1 for SUMO.

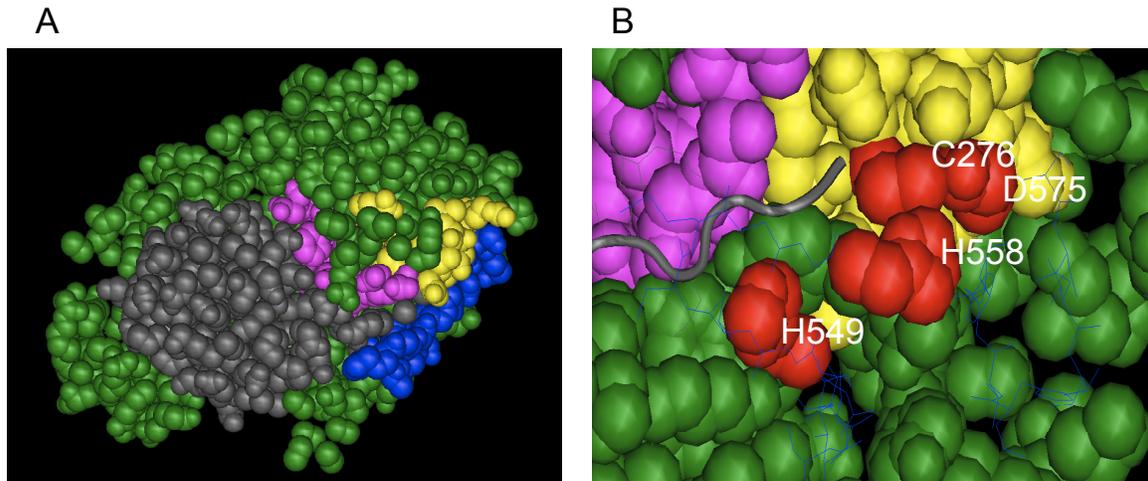


Figure 21. Structural elements of USP2 participating in Ubiquitin recognition.

A) Overall arrangement of the USP2/Ubiquitin complex. Ubiquitin is shown in grey, QQD motif of USP2 in magenta, Cys-box in yellow and His-box in blue. Remaining residues of USP2 are shown in green. B) Catalytic residues of USP2 as well as the non catalytic histidine of the His-box are shown in red. (Prepared using Cn3D application and PBD file 1TDZ)

USPL1 is not the only member of the USP family that exhibits specificity towards a Ubl other than Ubiquitin. However, other Ubls are much more similar to Ubiquitin than SUMO is, especially at their C-termini. Therefore it is not surprising that the QQD motif is present in these USPs. A recent screen for ISG15 cross-reactive isopeptidases revealed that USP2, 5, 13, 14 and 18 are able to react with ISG15-VS, an ISG-15 derivative functioning in analogic way to SUMO-VME (Catic *et al.*, 2007). ISG15 is a close homologue of Ubiquitin and contains two β -grasp domains arranged in a tandem manner. The last six C-terminal residues are identical between ISG15 and Ubiquitin. Some of the ISG-15 proteases have dual specificity (USP2,14), whereas others like USP18 cannot cleave ubiquitin

containing conjugates. Another closely related Ubl protein Nedd8 is cleaved by USP21, a protease that can also work on Ubiquitin (Gong *et al.*, 2002).

Similar to USPL1 another Ubl protease's specificity was previously misattributed. SENP8/DEN1/NEDP1, which belongs to the Ulp/SENp family, was predicted to be a SUMO specific protease based on its sequence. However, biochemical assays revealed that it works on Nedd8 instead (Gan-Erdene *et al.*, 2003, Mendoza *et al.*, 2003). DEN1 is the least related to the other members of the Ulp/SENp family and together with its homologues forms a separate branch of the Ulp/SENp family. Recently obtained structural data provided insight into the mode that DEN1 uses to recognize Nedd8. The motifs utilized by DEN1 for Nedd8 recognition are found in similar places as motifs through which SENPs recognize SUMO, however the identity of these residues participating in binding is different (Reverter *et al.*, 2005).

4.2 Conservation of USPL1

USPL1 homologues can be found in all vertebrates and in lower invertebrates like sea anemone, however it is absent from higher invertebrates like *D. melanogaster* or *C.elegans*, which suggests that USPL1 is an old gene that was lost during evolution of invertebrates.

USPL1

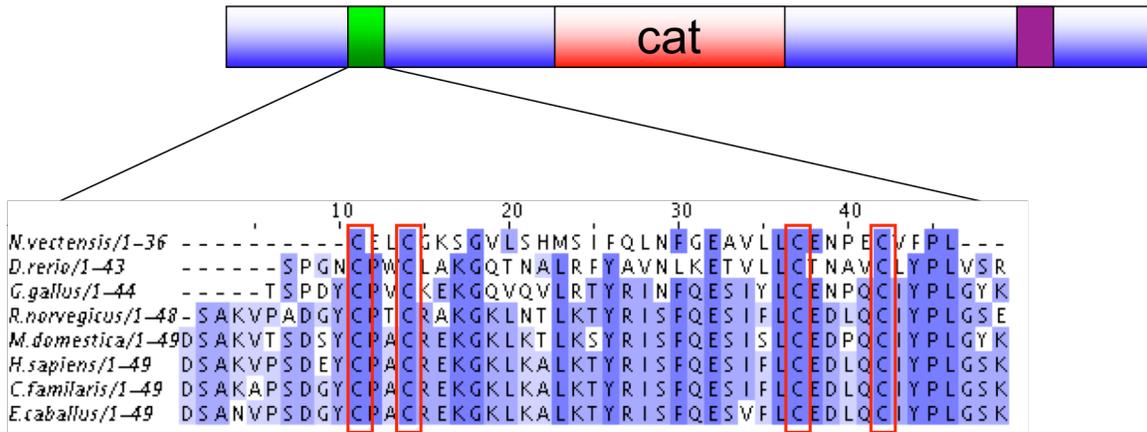


Figure 22. Domain organization of USPL1. The catalytic domain is shown in red, The putative N-terminal Zinc Finger domain is shown in green and the stretch of positive residues within C-terminus is shown in violet. Alignment of the putative Zinc finger is shown with conserved residues shaded and cysteines marked with red boxes. (the alignment was prepared using MUSCLE online application at www.ebi.ac.uk, and visualized with Jalview online editor at www.jalview.org)

A sequence alignment of USPL1 from different species performed by Dr. Kay Hoffman (Miltenyi Biotec, Köln) suggests the presence of three domains: an N-terminal domain, a catalytic domain localized in the middle and the C-terminal domain (Figure 22). Conservation is most prominent within the catalytic domain, especially for the catalytic residues and the region described previously that may mediate interaction with the C terminus of SUMO. Within the N-terminal domain a forty amino acid region containing four completely conserved cysteines is present. Presence of these cysteines suggests that this may be a Zinc Finger domain. Within the C-terminal part short conserved stretches can be found, including a region rich in positively charged residues.

4.3 Functions of USPL1

Our current knowledge on USPL1 is still insufficient to allow prediction of its physiological function, however information found in databases provides some interesting clues. The Zebra fish homologue of USPL1 was identified in a screen designed to find genes necessary for embryonic development (Amsterdam *et al.*, 2004). The phenotype of the mutant strain is characterized by strong necrosis in the central nervous system and head, leading to death.

The chicken homolog of USPL1 is called *retinovin* as it is selectively expressed in early stages of retina development (Itami *et al.*, 2002). Both of these studies suggest that USPL1 has a role in development.

One interesting observation comes from the GEO profiles database (www.ncbi.nlm.nih.gov/sites/entrez?db=geo). In HeLa cells, levels of USPL1 mRNA are upregulated in response to heat shock. It is well known that SUMO-2/3 is predominantly present in the unconjugated form, but after heat shock a rapid increase of SUMO-2/3 conjugates is observed. After the temperature comes back to normal, a recovery phase takes place during which high-molecular weight SUMO-2/3 conjugates disappear (Saitoh and Hinchey 2000). Upregulated level of USPL1 after heat shock could be a mechanism that cells use to remove SUMO-2/3 conjugates. The putative Zinc finger present in the N-terminus gives another hint about possible USPL1 function. Many members of the USP family have Ubiquitin binding domains, some of which are Zinc fingers, localized N-terminally from their catalytic domains. The exact role of these domains is still unclear, but in some cases they are important for binding and the effective processing of Ubiquitin chains. By analogy, the putative Zinc finger of USPL1 may be important for SUMO-2/3 chain cleavage. USPL1 is related to the Ubiquitin proteases, which are known to play a role in protein degradation. Recently SUMO-2/3 chains were also implicated in this process (Tatham *et al.*, 2008, Lallemand-Breitenbach V *et al.*, 2008). A very attractive hypothesis is therefore the involvement of USPL1 in SUMO-2/3 mediated protein degradation.

Another intriguing possibility is an interaction between USPL1 and HCF-1. Similar to SENP5, USPL1 posses HCF-1 binding motif (1023-1026 DHNY). Although it is more probable that in our experiment HCF-1 was copurified with SENP5, due to SENP5's abundance and specific reaction with SUMO-2-VME, it is still possible that HCF-1 attracts USPL1 to desumoylate transcription factors or/and histones. HCF-1 is a target for SUMO-2 modification and SENP5 or USPL1 can be responsible for its desumoylation. The levels of sumoylated HCF-1 increase after inhibition of proteasome, which suggests that it may be a target for SUMO-2 mediated protein degradation (Schimmel *et al.*, 2008).

4.4 Open questions and further work

The USPL1 studies described in this work can go in at least two directions: structural and functional. First it would be very interesting to obtain a crystal structure of USPL1 in complex with SUMO. This would address the question of how USPL1 structurally differs from other USPs and how it recognizes SUMO. Towards this goal we have established a collaboration with Prof. Titia Sixma (NKI, Amstardam). Characterization of the putative Zinc finger would allow us to address whether it is a new SUMO binding domain and if so, whether it has a role in USPL1 catalytic activity. My attempts to investigate the role of the putative Zinc Finger were unsuccessful as I could not obtain soluble recombinant protein containing this region (data not shown). Functional studies should allow understanding the role of USPL1 and its possible involvement in the heat shock response. Initial data obtained by overexpression of YFP-USPL1 fusion protein suggest that USPL1 is a nuclear protein (data not shown), however this still needs to be confirmed for the endogenous protein. Knocking down USPL1 using siRNA could be used to address its role during heat shock in cell culture, whereas a knock out mouse model would be a good option to study its possible role during development. The most interesting question that arises is that for USPL1 specific substrates. Their identification should significantly contribute to

understanding its role. To do this, several approaches can be used. Immunoprecipitation of USPL1 or a yeast-two-hybrid approach could identify binding partners, some of which may be USPL1 targets. Another possibility is a proteomic approach in which proteins that show increased sumoylation in the absence of USPL1 can be identified. These would also be putative targets of USPL1.

4.5 Are there more SUMO specific proteases among the USP family?

USPL1 was described in the database as an inactive Ubiquitin protease which was attributed to the absence of the non-catalytic histidine within its His-box. A similar study reports the inactivity of USP53 and USP54. A number of human USPs were expressed in bacteria together with Ub- β -galactosidase fusion protein. The activity was judged by the cleavage of the fusion protein. In this kind of assay USP53 and closely related USP54 had no activity (Quesada *et al.*, 2004). Both these proteins lack the non-catalytic histidine residue, but a closer look reveals that all the catalytic residues are present in these proteins. Their QQD box is different from that of other USPs (Figure 20). Although those two proteases are believed to be inactive it is worthwhile to check whether they could be specific for SUMO or Ubls other than ubiquitin.

4.6 Further approaches to identify novel SUMO specific isopeptidases

It is possible that more SUMO specific proteases exist. To further investigate this the following approaches can be used.

1) Educated guess - USP53 and 54 were reported to be inactive due to the lack of the conserved noncatalytic histidine within the His-box. However, all the

residues important for catalysis are conserved in both proteins, therefore it is possible that they can work on SUMO or another Ubl.

2) Biochemical purification from biological material using SUMO-VME. The approach described here is based on lysates from HeLa cells as the source of proteins, which might not be optimal. Sumoylation is involved in meiosis, which does not normally take place in somatic cells. Therefore using lysates prepared from testes as a material may provide more relevant candidates. Also lysates prepared from brain tissue as a source would be a good idea as that is where many proteins are expressed. Also the cellular fraction that we focused on might be not the optimal one. Many SUMO targets are chromatin associated proteins, therefore it would make sense to also test the chromatin associated fraction.

3) Biochemical purification using SUMO derivatives other than SUMO-VME. Chemical probes used to study the Ubiquitin system were developed several years ago (Borodovsky *et al.*, 2001 and 2002). They were successfully used to identify new ubiquitin and other Ubl like proteases. They can also be used to study the enzymatic activity of proteases. The specificity of the probe towards the enzyme depends on the chemical moiety attached to the C-terminus. It was observed that a given Ubl derivative can differ significantly in specificity towards proteases of the same family (Borodovsky *et al.*, 2002). Therefore using chemical probes other than SUMO-VME could also be considered.

4) SUMO-4 is a very enigmatic protein and so far it is not known whether it is processed and therefore whether it can be conjugated to targets. This is believed to be caused by a proline residue in the C-terminus of SUMO-4 (Owerbach *et al.*, 2005). Preparing a SUMO-4VMe could help to answer the question whether there are proteases that can recognize the SUMO-4.

5) Screening bacterial expression libraries using the FRET based assay. Although the screen that we performed here did not result in identification of a SUMO protease, we have shown that a FRET based assay can be applied to a high-throughput screen. It would be worth to screen a library covering much

larger number of ORFs than the library that we used (only 4000). Introducing some modifications should also be considered. We used RanGAP1 modified with SUMO-1, however most of the known SUMO proteases exhibit specificity towards SUMO-2 (Dasso 2007). Therefore using RanGAP modified with SUMO-2 seems to be a good idea. Full length SUMO proteases exhibit specificity not only towards different SUMO isoforms but also towards substrate, therefore using a substrate different than RanGAP should also be considered.

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