# Mechanisms of the intracellular localization of the SUMO-activating enzyme Aos1/Uba2

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I herewith declare, that this thesis has been written independently and with no other sources and aids than explicitly quoted.

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# CONTENT

# ABSTRACT

Dynamic posttranslational modification with ubiquitin related proteins of the SUMO (small ubiquitin-related modifier) family is an important cellular mechanism to alter the activity, abundance or localization of proteins. Reversible covalent attachment of SUMO to target proteins requires the catalytic activities of an E1-activating enzyme, an E2-conjugating enzyme, one of several E3 ligases as well as specific isopeptidases. Consistant with the existence of nuclear and cytoplasmic SUMO substrates, the components of the enzymatic machinery are also found in both compartments. This raises the interesting question how these pools are generated.

The presented work aimed to identify the mechanisms underlying the intracellular localization of the SUMO E1 complex. Since both subunits, Aos1 and Uba2, are predominantly localized in the nucleus, I initially determined the generation of the nuclear pool. I demonstrated that nuclear import can occur in two ways: Aos1 and Uba2 can be imported independently by distinct nuclear localization signals (NLSs), and the assembled complex can be imported by the NLS of Uba2. In both cases the import is mediated by the receptor importin $\beta$ and the adaptor protein importin $\alpha$.

Functional studies concerning the generation of the minor cytoplasmic pool of Aos1/Uba2 indicated that the proteins are not actively exported from the nucleus into the cytoplasm. Since the nuclear and cytoplasmic pools of E1 are not subjected to frequent exchange and the cytoplasmic fraction of E1 is very small I reasoned that the SUMO activating acitvity in the cytoplasm would be constantly low. This raised the question whether the E1's intracellular localization is at all of general importance for SUMOylation in different cellular compartments.

With yeast *UBA2* shuffle strains, in which the endogenous *UBA2* gene is deleted and cells are kept alive by a removable exogenous copy, I studied the ability of cytoplasmic E1 to substitute for endogenous E1. Surprisingly, deletion of endogenous E1 can be rescued both by exogenous cytoplasmic or nuclear E1. Yeast strains with either cytoplasmic or nuclear E1 exhibit similar modification patterns, indicating that the localization of E1 might not determine compartment specific SUMOylation. These findings suggest that thioester charged Ubc9 may shuttle to and allow efficient SUMOylation both in the nuclear and cytoplasmic compartment.

# INTRODUCTION

## 1. Posttranslational protein modification

Posttranslational modifications of proteins have crucial roles in biological systems since they participate in virtually all cellular processes. The enzymatic covalent modification of amino acid side chains within target proteins can affect protein function, activity, abundance or localization. Depending on the type of modification a distinction is drawn between the attachment of proteins, e.g. ubiquitylation or ubiquitin-like modifications, versus organic and anorganic molecules, for example acetylation, methylation, phosphorylation and glycosylation (reviewed in Walsh et al. 2005).

### 1.1. Ubiquitin and ubiquitin-like modifiers

Ubiquitin is the first discovered and best-characterized representative of a whole family of modification pathways – the ubiquitin-like (UBL) modifications. To date, 9 phylogenetic classes with at total of 17 human UBLs have been described, amongst them FAT10, ISG15, URM1, UFM1, ATG12 and the well-studied NEDD8 and SUMO (small ubiquitin-related modifier protein) pathways. All UBLs share the conserved ubiquitin or β-grasp fold exemplified in Fig.1 and are attached to other proteins by similar pathways (Hochstrasser 2000; Welchman et al. 2005; Kerscher et al. 2006). Most UBLs are expressed as inactive precursor proteins and therefore have to be matured by C-terminal hydrolases. The mature modifier usually contains two glycine residues at the C-terminus (Amerik et al. 2004; Love et al. 2007). Despite these similarities each UBL system uses a discrete enzymatic cascade of E1 activating enzyme, E2 conjugating enzyme, and frequently E3 ligases, for modification. UBL-specific isopeptidases are required for maturation and for deconjugation of the according modification (for details see chapter *INTRODUCTION – Molecular mechanism of SUMOylation*).

The ubiquitin system comprises two E1 enzymes, Uba1 and Uba6, 37 E2 enzymes and more than 600 E3 ligases (for references see Komander 2009). E3 ligases can be subdivided into two functionally different groups: HECT E3 ligases form a thioester with ubiquitin and thereby directly participate in the transfer reaction. In contrast, RING

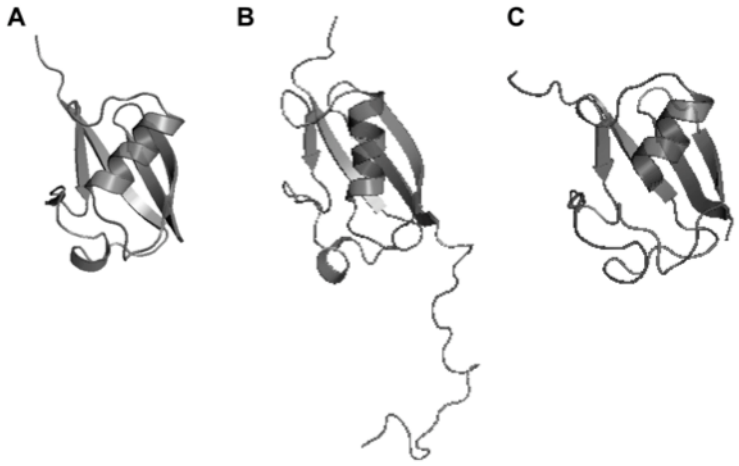


**Figure 1: Conserved β-grasp fold of ubiquitin and UBLs.** Cartoon of the structures of mature human ubiquitin (Vijay-Kumar et al. 1987) (A), SUMO1 (Bayer et al. 1998) (B) and NEDD8 (Whitby et al. 1998) (C). Structures were oriented with the helix in front of the β-sheets and the C-terminal GlyGly-motifs on top. Files were obtained from the Protein Data Bank (PDB) and images were generated with PyMOL v0.99 (DeLano Scientific LLC).

finger E3 ligases facilitate the conjugation reaction by serving as bridging factors for the interaction between E2 enzymes and the target proteins (Pickart 2001). The ubiquitin system modifies thousands of substrates and is thereby involved in most, it not all, biological processes. Depending on the target protein and the set of enzymes used for modification, ubiquitylation can result in mono-, multiple mono- or poly-ubiquitylation, each of which exerts different effects on the same target. Monoubiquitylation is involved in many different intracellular processes, e.g. endocytosis, endosomal sorting, histone regulation, DNA repair and nuclear export (Haglund et al. 2003; Hicke et al. 2003; Mosesson et al. 2006), whereas multiple mono-ubiquitylation primarily participates in endocytosis (Haglund et al. 2003). Due to the presence of 7 internal lysine-residues targets can be modified with polyubiquitin chains (reviewed in Ikeda et al. 2008; Komander 2009). Well-known examples for polyubiquitylation are lysine48-linked chains that target substrates for proteasomal degradation (Hershko et al. 1998; Thrower et al. 2000; reviewed in Finley 2009) and lysine63-linked chains that are involved in endocytosis (Geetha et al. 2005; Duncan et al. 2006), DNA-damage repair (reviewed in Moldovan et al. 2007; Panier et al. 2009), and signal transduction (reviewed in Skaug et al. 2009). The biological signficance of other polyubiquitin chains have been less well investigated: While lysine11-linked chains have been reported to serve as proteasomal degradation signal (Baboshina et al. 1996; Xu et al. 2009) and to play a role in cell cycle of mammalian cells (Jin et al. 2008), the physiologal roles of lysine6, 27, 29, 33 linkages are largely unknown. To complicate matters, recent studies report the formation of mixed and branched ubiquitin linkages (Ben-Saadon et al. 2006; Kim et al. 2007; Kim et al. 2009). However the *in vivo* abundance and relevance of these chains requires further analysis.

## 1.2. The SUMO family

Next to ubiquitin, the SUMO family is the best-characterized representative of UBL modifiers. SUMOylation has been found to be an essential process in many organisms (Johnson et al. 1997; Fraser et al. 2000; Nacerddine et al. 2005; Saracco et al. 2007) and SUMO proteins are ubiquitously expressed throughout the eukaryotic kingdom. Some organisms including *S. cerevisiae*, *C. elegans* and *D. melanogaster* have only one SUMO gene, whereas plants and vertebrates posses several SUMO genes. The human genome encodes for four SUMO proteins (Melchior 2000; Guo et al. 2004). Three of these paralogs, SUMO1 (also known as human Smt3c, PIC1, GMP1, sentrin and Ubl1), SUMO2 (also known as Smt3a and Sentrin3) and SUMO3 (also known as Smt3b and Sentrin2), are ubiquitously expressed in vertebrates (Guo et al. 2004). The mature forms of SUMO2 and SUMO3 are 97 % identical, and can therefore functionally not be differentiated (Saitoh et al. 2000; Tatham et al. 2001). Consequently they are designated as the SUMO2/3 subfamily. SUMO2/3 and SUMO1 significantly differ in their primary sequences and are only 47 % identical. Furthermore, the SUMO paralogs have different cellular functions as they are attached to different target proteins (Saitoh et al. 2000; Vertegaal et al. 2006). Whether the fourth paralog SUMO4 is also covalently attached to target proteins or acts via non-covalent mechanisms still remains to be completely clarified (Owerbach et al. 2005; Wei et al. 2008).

SUMOylation of target proteins predominantly results in conjugation of monomeric SUMO proteins. However, like ubiquitin, SUMOs can also form multimeric chains (reviewed in Ulrich 2008; Vertegaal 2010). While recent mass spectrometry studies give direct *in vivo* evidence for SUMO2/3 chains, attachment of SUMO1 on multimeric SUMO2/3 is suggested to limit chain extension (Matic et al. 2008; Hsiao et al. 2009),

In contrast to the ubiquitin system, the enzymatic SUMOylation machinery is much less complex. To date only a single E1, human Aos1/Uba2, a single E2, human Ubc9, about ten E3 ligases and six SUMO-specific isopeptidases have been identified (reviewed in Geiss-Friedlander et al. 2007).

## 2. Molecular mechanisms of SUMOylation

The capability of the SUMO system to modify and de-modify target proteins makes SUMOylation a reversible and highly dynamic posttranslational modification. The underlying molecular mechanisms resemble ubiquitination and other ubiquitin-like modifications, with an enzymatic cascade of E1, E2 and E3 causing conjugation of SUMO and isopeptidases catalyzing the de-conjugation (Fig.2). However, the set of enzymes involved in SUMOylation is distinct from the enzymes of other UBL systems (reviewed in Johnson 2004; Hay 2005; Geiss-Friedlander et al. 2007).

### 2.1. Activation of SUMO

SUMO proteins are translated as precursors and have to be processed by C-terminal hydrolases (SUMO specific cysteine proteases) prior to activation. Mature SUMO ends with the characteristic GlyGly-motif that is necessary for the attachment to target proteins. In the first step of the E1-E2-E3 cascade mature SUMO is activated by the SUMO specific E1 activating enzyme. The SUMO E1 consists of two subunits, Aos1

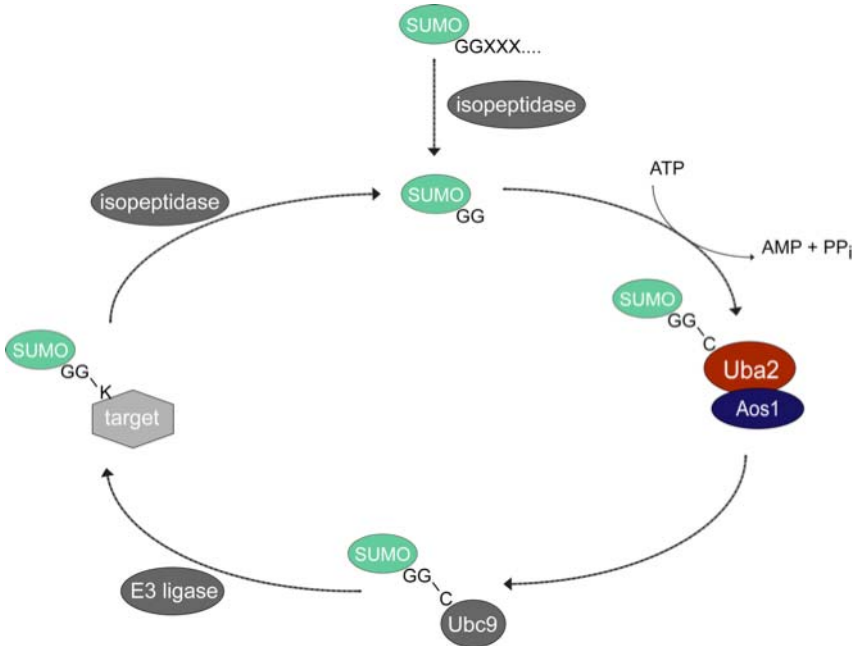


**Figure 2: The enzymatic cascade of SUMOylation.** Maturation of the SUMO precursor by hydrolases results in mature SUMO with a double glycin motif at the C-terminal end. The E1 activating enzyme, heterodimeric Aos1/Uba2 complex, activates the C-terminal carboxy group of SUMO by forming a high-energy thioester bond with the E1's active site cysteine residue. Activated SUMO is then, in a transesterification reaction, transferred to a cysteine in the E2 conjugating enzyme Ubc9. Assisted by SUMO E3 ligases, Ubc9 conjugates SUMO to a variety of target proteins. The resulting isopeptide bond is stable, wherefore desumoylation of the targets requires the activity of specific isopeptidases.

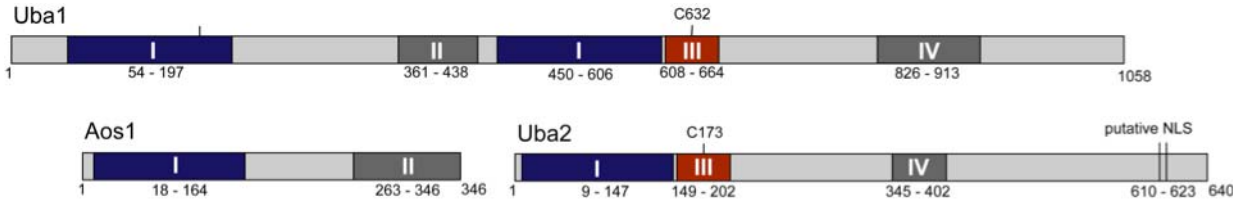


**Figure 3: Schematic representation of homologous domains in the primary sequences of Ub-like E1 enzymes.** Domains of high sequence similarity in the human ubiquitin E1 Uba1 and the SUMO E1 Aos1/Uba2 are highlighted. Domain I (blue) includes a potential nucleotide-binding motif and is found in a number of proteins unrelated to ubiquitin-like activating enzymes. Domain III (red) contains the active site cysteine of E1 enzymes that forms a thioester bond with the C-terminal carboxy-group of mature Ub-like proteins. The domains II and IV (dark grey) are found in several other proteins than E1 enzymes, but their function is so far unknown. Domains shown correspond to those described by Johnson et al. (Johnson et al. 1997). A putative NLS in Uba2 was described previously for the yeast homolog of Uba2 (Dohmen et al. 1995).

(also known as Sae1 or ULE1A) and Uba2 (also known as Sae2 or ULE1B) (Johnson et al. 1997; Desterro et al. 1999). The sequence of the SUMO E1 is highly similar to the ubiquitin E1 (Uba1). While Aos1 resembles the N-terminal half of Uba1, the second E1 subunit Uba2 resembles the C-terminal half (Fig. 3). Aos1 and Uba2 both contain a conserved nucleotide binding motif, *GxGxxG* (Wierenga et al. 1983) in domain I and a conserved region of so far undetermined function (domains II and IV) (Johnson et al. 1997; Desterro et al. 1999). The active cysteine, Cys-173 in Uba2, is located in an active-site consensus sequence KxxPzCTxxxxP (z is an apolar residue) (Hatfield et al. 1992) within the conserved domain III. A striking difference in the primary sequences of Uba1 and Aos1/Uba2 is the C-terminal region of Uba2, which is absent from the sequence of Uba1. This domain contains a putative consensus nuclear localization signal (NLS) and has been reported to be important for the nuclear localization of Uba2 (Dohmen et al. 1995; Desterro et al. 1999).

Similarities of Uba1 and Aos1/Uba2 are not only found in the primary sequences but also in the tertiary domain structure of the enzymes (Fig. 4). Comparison of the structures reveals similarities in the presence and position of the adenylation, the UbL (ubiquitin-like domain) and the catalytic cysteine domain (Lois et al. 2005). Interaction of Aos1 and Uba2 occurs via a relatively small surface in the adenylation domain of Uba2. While the catalytic cysteine domain harbours the active-site Cys173 that forms the thioester with SUMO, the UbL domain has an essential role in the recruitment of the E2 enzyme.

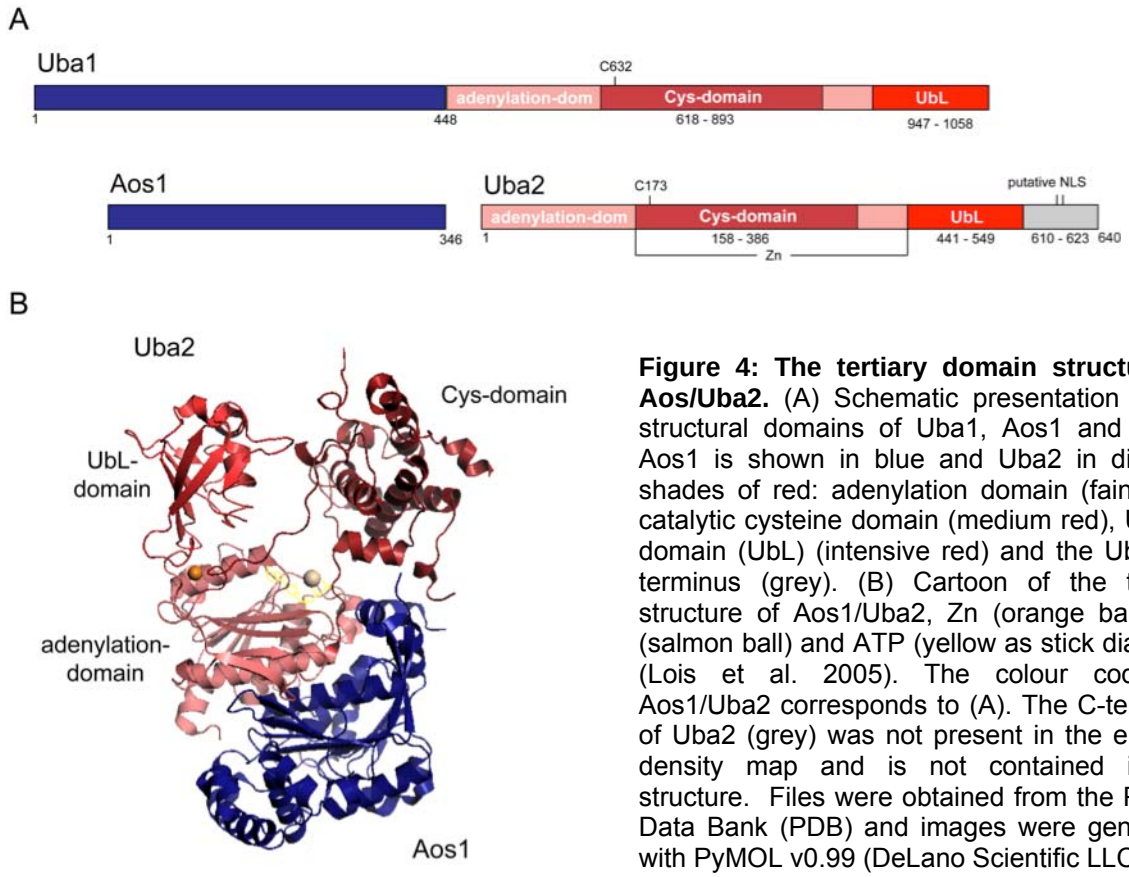


**Figure 4: The tertiary domain structure of Aos/Uba2.** (A) Schematic presentation of the structural domains of Uba1, Aos1 and Uba2. Aos1 is shown in blue and Uba2 in different shades of red: adenylation domain (faint red), catalytic cysteine domain (medium red), Ub-like domain (UbL) (intensive red) and the Uba2 C-terminus (grey). (B) Cartoon of the tertiary structure of Aos1/Uba2, Zn (orange ball), Mg (salmon ball) and ATP (yellow as stick diagram) (Lois et al. 2005). The colour code for Aos1/Uba2 corresponds to (A). The C-terminus of Uba2 (grey) was not present in the electron density map and is not contained in the structure. Files were obtained from the Protein Data Bank (PDB) and images were generated with PyMOL v0.99 (DeLano Scientific LLC).

Analogous to activation of ubiquitin, the ATP-dependent activation of SUMO catalyzed by human Aos1/Uba2 involves two distinct steps. First, the C-terminal carboxyl group of SUMO attacks the $\alpha$-phosphate of the ATP, forming a SUMO adenylate and releasing pyrophosphate. Then, the catalytic cysteine in Uba2 attacks the adenylate whereby a high-energy thioester bond between E1 and SUMO is formed and AMP is released (Johnson et al. 1997).

These events require dramatic rearrangements in the E1 enzyme: While adenylation of SUMO is performed in an opened conformation, thioester-bond formation with Uba2 occurs in a closed conformation (Olsen et al. 2010). The structural changes from the opened to the closed state include a 130 degree rotation of the whole Cys-domain of Uba2 towards the adenylation site of the E1.

To complicate matters, activation of ubiquitin by Uba1 has been shown to involve asymmetric double loading of the E1 with two ubiquitin molecules (Haas et al. 1982; Haas et al. 1982; Schulman et al. 2009). Since a similar mechanism was found for the NEDD8 E1 complex NAE1/UBA3 (Bohnsack et al. 2003; Walden et al. 2003; Huang et al. 2007) it is very likely that a related mechanism is involved in the activation of

SUMO by Aos1/Uba2. Thus, after thioester-bond formation, SUMO transfer from the E1 to the E2 is probably accompanied by the binding of a second SUMO molecule.

## 2.2. Conjugation to SUMO targets

After activation by the E1 enzyme SUMO is transferred in a transesterification reaction to the catalytic cysteine of the SUMO E2 enzyme Ubc9 (Fig. 2). This transfer reaction results in a thioester linkage between the thiol group of Ubc9's active cysteine and the C-terminal carboxy group of SUMO (Desterro et al. 1997; Johnson et al. 1997; Lee et al. 1998). In contrast to the ubiquitin system for which more than 20 E2 enzymes have been described, only a single SUMO E2 is known.

In the last step of the SUMOylation cascade SUMO is transferred to a target protein, by formation of an isopeptide bond between SUMOs C-terminal carboxy group and the ε–amino group of a lysine residue in the target protein. This step is mostly performed by the concerted action of the E2 enzyme and a SUMO E3 ligase that facilitates the conjugation.

Unlike HECT ubiquitin E3 ligases, which directly participate in the ubiquitin-conjugation by forming a thioester with ubiquitin, all known SUMO E3 ligases promote the direct transfer from the E2 enzyme to the target. Three types of SUMO ligases have been described in some detail (reviewed in Hay 2005; Geiss-Friedlander et al. 2007): SP-RING ligases (Hochstrasser 2001), RanBP2 (Ran binding protein 2) (Pichler et al. 2002) and Pc2 (polycomb group protein 2) (Kagey et al. 2003).

SP-RING (Siz/PIAS-RING) ligases are the SUMO specific counterparts of the RING type ligases from the ubiquitin system. By direct interaction with thioester-charged Ubc9 and a target protein they position the SUMO-loaded E2 in a favorable position for the transfer of SUMO. The group of SP-RING ligases is composed of MMS21 (also known as NSE2) (Andrews et al. 2005; Potts et al. 2005; Zhao et al. 2005,) the meiosis specific yeast protein Zip3 (Cheng et al. 2006) and the PIAS family proteins (protein inhibitors of activated STAT). Recent chrystallographic analyis of the yeast PIAS-homolog Siz1 revealed that the central zinc-containing RING-like SP-RING domain and the SP-CTD (C-terminal domain) are required for the activation of the SUMO-E2 thioester, whereas the conserved N-terminal PINIT domain is essential for redirecting SUMO-conjugation to the acceptor lysine in the substrate (Takahashi et al. 2005; Yunus et al. 2009). Two PIAS family members, Siz1 and Siz2, have been described in *S. cerevisiae* and five in mammals, PIAS1, PIAS3, PIASxα, PIASxβ and

PIASy (Hochstrasser 2001; Jackson 2001). While the commonly shared SP-RING motif is essential for interaction with Ubc9, regions N- and C-terminal of the SP-RING motif mediate substrate recognition (Hochstrasser 2001).

In contrast to the family of SP-RING ligases, the SUMO specific ligases RanBP2 and Pc2 are unrelated to the ubiquitin E3s. The minimal catalytic domain of the nuclear pore protein RanBP2 is natively unfolded and assumes its structure upon folding around Ubc9 (Pichler et al. 2002; Pichler et al. 2004; Reverter et al. 2005). As RanBP2s minimal catalytic domain has not been detected to interact with any substrate, it is suggested to facilitate SUMO conjugation by positioning the Ubc9-SUMO thioester for an optimal attack by the acceptor lysine residue in the modified target protein (Pichler et al. 2002; Tatham et al. 2005).

Pc2 is a member of the human polycomb group (PcG) proteins that form large multimeric complexes. It has been shown to stimulate SUMOylation of the transcriptional co-repressor CtBP (Kagey et al. 2003; Kagey et al. 2005). While the exact mechanisms of how Pc2 functions are not yet fully unterstood, a very recent study indicates that the SUMO interaction motifs of Pc2 play in important role in its E3 acitvity (Merrill et al. 2010).

The attachment of SUMO to target proteins typically involves specific lysine residues within the targets. Preferred regions of SUMOylation were defined as consensus sequence $\psi$KxE ($\psi$ is a bulky hydrophobic residue) (Desterro et al. 1998; Melchior 2000). If the motif is accessible, Ubc9 can bind to it and subsequently transfer SUMO to the acceptor lysine in the consensus site of the target (Sampson et al. 2001; Bernier-Villamor et al. 2002). However, SUMOylation at consensus sites is not the only mechanism, as some targets are known to be modified at non-consensus lysines, e.g. PCNA (Hoege et al. 2002), E2-25K (Pichler et al. 2005) or USP25 (Meulmeester et al. 2008).

## 2.3. Deconjugation of SUMOylated targets

SUMO isopeptidases can remove SUMO from a modified target protein and thereby make SUMOylation a reversible and highly dynamic posttranslational modification. In addition to their isopeptidase activity, these enzymes exhibit a C-terminal hydrolase activity by which they can remove a short C-terminal peptide from the SUMO precursor protein generating mature SUMO (Fig. 2). By means of these two activities, SUMO isopeptidases are required for providing free conjugatable SUMO.

All known SUMO isopeptidases are members of the family of Ulp cysteine proteases (ubiquitin-like modifier protease). Two SUMO-specific isopeptidases, Ulp1 and Ulp2, have been identified in yeast (Li et al. 1999; Li et al. 2000) and, to date, six mammalian Ulp homologs are known, SENP1, 2, 3, 5, 6 and 7 (sentrin-specific isopeptidase). The SENP isoforms differ from each other by their catalytic activities in maturation and deconjugation of SUMO, in their specificity towards the SUMO paralogs (Di Bacco et al. 2006; Gong et al. 2006) and also in their intracellular localization (Mukhopadhyay et al. 2007) (for details see chapter *INTRODUCTION – Localization of SUMO targets and enzymes*). However, regarding the large number of ubiquitin specific proteases, it can be speculated that the list of identified SUMO isopeptidases might not yet be complete.

## 3. Functions of SUMO modification

The reversible and highly dynamic modification of target proteins with the small modifier SUMO can affect the characteristics of the target protein in three mechanistically different ways (Fig. 5): Attachment of the SUMO moiety can prevent or enable interactions with other macromolecules (proteins, DNA or RNA) or it can induce intramolecular structural rearrangements. Depending on the target and on the site at which the specific substrate is modified, SUMOylation can alter its function by influencing its activity, stability or localization in the cell. Due to the large number of known SUMO targets, SUMOylation contributes to a large and still growing number of pathways including transcriptional regulation, maintenance of genome integrity, signal transduction and nucleocytoplasmic transport (reviewed in Geiss-Friedlander et al. 2007; Zhao 2007). In the following, the function of SUMOylation is exemplified for some selected pathways.

### 3.1. SUMO and transcriptional regulation

One of the first discovered and probably best-known roles of SUMO modification is the regulation of transcription. The huge number of transcriptional regulators that have been shown to be SUMOylated indicates the importance of SUMOylation in transcription.

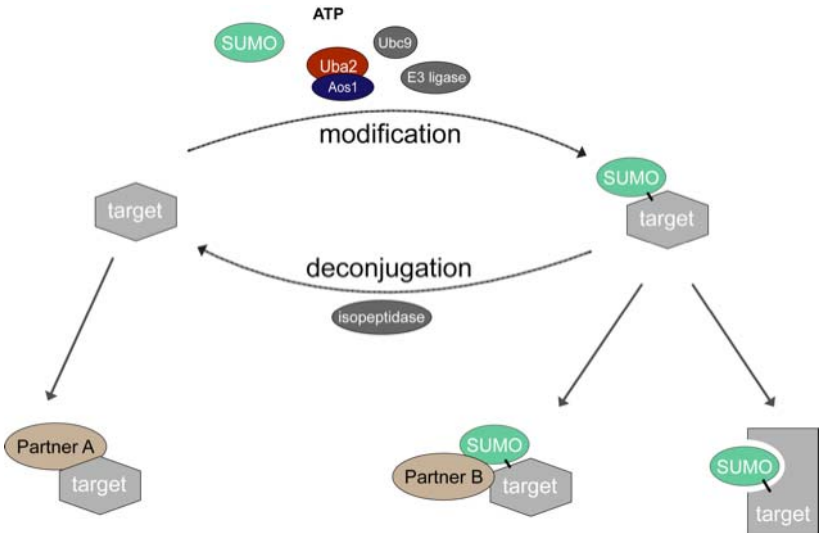


**Figure 5: Molecular consequences of reversible SUMOylation:** (A) SUMOylation can prevent the interaction of the target with partner A, only enabling the interaction in the absence of the SUMO modification. (B) SUMOylation can facilitate the interaction between the target and its partner B by providing an additional binding site for the interaction partner B. (C) SUMOylation can induce intramolecular conformational changes of the modified target protein. Adopted from (Geiss-Friedlander et al. 2007).

SUMOylation of transcription factors mostly causes transcriptional repression by inhibiting the activity of transcriptional activators (Gill 2005). This can occur by different mechanisms, e.g. inhibition of the DNA-binding capability (Anckar et al. 2006) or altered intracellular localization of transcription factors like LEF1 (Sachdev et al. 2001) and lipin1-$\alpha$ (Liu et al. 2009).

In addition, SUMO modification of transcription factors can lead to local formation of heterochromatin-like silenced DNA, as has been shown for the transcription factor Sp3 (Stielow et al. 2008). Sp3 SUMOylation recruits chromatin remodelling factors and histone methyltransferases leading to repressive modification of histones and attachment of heterochromatic proteins.

Modification with SUMO has in some cases also been shown to cause transcriptional activation. This is exemplified by the transcription factor Oct4 which has been reported to show increased DNA-binding upon SUMOylation (Wei et al. 2007) or the transcriptional repressor Tel whose association with DNA has been found to be impaired by its SUMOylation (Roukens et al. 2008).

## 3.2. SUMO and the maintenance of chromosome stability

Repair mechanisms ensure the proper propagation of the cellular genome during several cycles of cell division. A very intriguing example for the role of SUMOylation in postreplication repair processes is the modification of PCNA (proliferating cell nuclear antigen) (reviewed in Watts 2006; Ulrich 2009). PCNA serves as a processivity clamp for replicative DNA polymerases, whose ubiquitylation upon DNA damage promotes the bypass of replication-blocking lesions and thereby leads to DNA damage tolerance (reviewed in Lehmann et al. 2007). In contrast, SUMOylation of PCNA has been shown to negatively affect the bypass of lesions (Stelter et al. 2003). SUMOylation of PCNA recruits the anti-recombinogenic DNA helicase Srs2 to the replication forks where the helicase prevents spontaneous recombination of the single-stranded DNA (Papouli et al. 2005; Pfander et al. 2005). Consequently, PCNA SUMOylation prevents increased recombination between sister chromatids (Robert et al. 2006).

Another example for SUMOylation influencing DNA repair is the base excision repair enzyme thymidine DNA glycosylase (TDG). SUMO modification reduces the affinity of TDG for DNA and thereby helps to release the enzyme from mismatch lesions after removal of U or T (Hardeland et al. 2002; Baba et al. 2005; Steinacher et al. 2005; Fitzgerald et al. 2008).

## 3.3. SUMO and nucleocytoplasmic transport

SUMOylation has been implicated in the regulation of nuclear transport of cargo proteins. Interestingly, it has been shown by the laboratory of Frauke Melchior that the nuclear pore protein RanBP2 (Ran binding protein 2, also referred to as Nup358) contains SUMO E3 ligase activtiy (Pichler et al. 2002). RanBP2 is the main component of the cytoplasmic filaments of nuclear pores complexes (NPC) (Wilken et al. 1995; Wu et al. 1995; Yokoyama et al. 1995) and specifically interacts with the SUMO-modified form of RanGAP1, the first identified SUMO-substrate, which by itself is a soluble cytoplasmic protein (Matunis et al. 1996; Mahajan et al. 1997). In addition, the SUMO E2 enzyme Ubc9 has been shown to interact with the RanBP2/RanGAP1-SUMO complex (Saitoh et al. 1997; Lee et al. 1998). Thereby SUMO modifying activity of Ubc9 and RanBP2 is combined with components of the nucleocytoplasmic tranport machinery, RanBP2 and RanGAP1. Furthermore, the isopeptidase SENP2 has been shown to localize to the nuclear site of the NPCs via interaction with the nucleoporin Nup153 (Hang et al. 2002; Zhang et al. 2002). These data indicate that SUMOylation

and deSUMOylation can occur at the entry and exit sites of NPCs, pointing to an interesting role of dynamic SUMOylation in nucleocytoplasmic transport (Pichler et al. 2002).

Direct evidence for a role of SUMOylation in nuclear import has been obtained in *S. cerevisiae*. Mutants of the SUMO conjugation machinery have been shown to cause nuclear accumulation of the yeast importin $\alpha$-homolog Srp1 and thereby lead to inhibition of cNLS-dependent nuclear import (Stade et al. 2002).

In addition, some examples provide indirect indications for a role of SUMOylation in the nucleocytoplasmic distribution of proteins: On the one hand nuclear enrichment of SUMOylated forms has been described for targets like FAK (Kadare et al. 2003), caspase-7, -8 and procaspase-2 (Besnault-Mascard et al. 2005; Shirakura et al. 2005; Hayashi et al. 2006) and the centrosome-associated protein ninein (Cheng et al. 2006). On the other hand SUMOylation has been reported to induce nuclear export of *Dictyostelium* Mek1 Sobko et al. 2002 and to enhance the interaction between the bovine papilloma virus (BPV) E1 and its export receptor CRM1 (Rosas-Acosta et al. 2008). Although it is not always clear whether SUMOylation directly alters the transport process or anchors proteins in a compartment, the examples demonstrate a role of SUMOylation in the nucleocytoplasmic distribution of target proteins.

## 4. Nucleocytoplasmic transport

Millions of proteins and ribonucleoprotein particles have to enter and/or leave the nuclear compartment during the lifetime of a cell. Nuclear pore complexes (NPCs), anchored in the nuclear envelope, form the connection between the nucleus and the cytoplasm and enable a controlled exchange between these compartments. NPCs are huge complexes composed of several copies of about 30 different proteins, the nucleoporins (Cronshaw et al. 2002). Small molecules and proteins of a size below the threshold of approximately 50 kDa can diffuse through NPCs between cytoplasm and nucleoplasm. However, transport cargoes have to be actively transported in and out of the nucleus. Specific transport receptors bind to signal sequences within the cargo protein and mediate the translocation through the nuclear pore by interacting with nucleoporins (Fig. 6). Depending on the direction of transport these transport signals are designated as nuclear import signals (NLSs) or nuclear export signals (NESs) (reviewed in Fried et al. 2003; Weis 2003).

The direction of transport is defined by differential affinities of import or export receptor for their cargoes depending on the presence of RanGTP (Fig. 6). Import receptors bind their cargo only in the absence and release the cargo in the presence of RanGTP. In contrast, export receptors bind the cargo in the presence and release it in the absence of RanGTP. This system functions due to the compartment specific localization of Ran's key regulators, the guanine nucleotide exchange factor RCC1 and Ran's GTPase activating protein RanGAP1. While RCC1 is restricted to the nucleus and ensures a high concentration of RanGTP inside of the nucleus, RanGAP1 is localized in the cytoplasmic assuring that most cytoplasmic Ran is bound to GDP instead of GTP (for a collection of detailed reviews see Kehlenbach 2009).

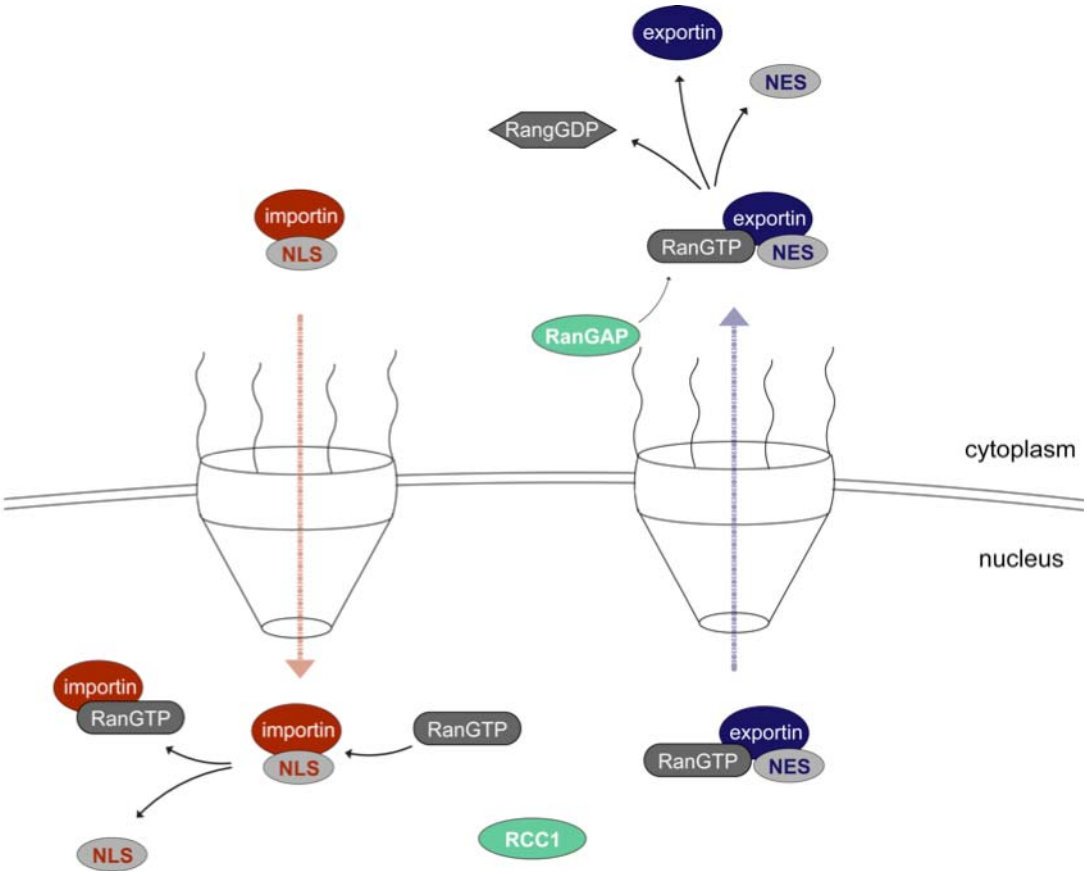


**Figure 6: Simplified model of the mechanism of nucleocytoplasmic transport across the nuclear envelope**. Schematic presentation of nuclear import (left) and export (right) depending on the status of Ran. The nuclear localization of the RanGEF (guanine nucleotide exchange factor) RCC1 and the cytoplasmic localized RanBP1, RanBP2 and RanGAP1 (GTPase activating enzyme) determine the compartment-specific formation or disassembly of import and export complexes.

## 4.1. Import

Nuclear import begins in the cytoplasm, where import receptors bind to the NLS of cargo proteins. Most import receptors, amongst them transportin, importin β, importin 5, 7 and 9, belong to the importin β family and vary in their preference for different NLSs. For example, transportins preferentially recognizes glycine-rich NLSs (M9-sequences) present for example in hnRNPs (Pollard et al. 1996; Siomi et al. 1997) or RS domain containing SR proteins (Kataoka et al. 1999; Lai et al. 2001). Other family members directly bind to stretches of basic amino acids found within ribosomal proteins (Jakel et al. 1998), core histones (Mosammaparast et al. 2001; Muhlhausser et al. 2001; Mosammaparast et al. 2002) and many other proteins. However, the best-characterized import signals within cargo proteins are classical NLSs (cNLSs). Monopartite cNLS, such as the SV40 large T antigen NLS (Kalderon et al. 1984), consist of one cluster of basic amino acids, whereas bipartite cNLSs, first found in nucleoplasmin (Dingwall et al. 1982; Robbins et al. 1991), are composed of two basic clusters separated by approximately 10 random amino acids. Nuclear import via a cNLS involves an adaptor protein of the importin α family, which binds to the cargo NLS and simultaneously, by its IBB domain (importin β binding domain), to the import receptor importin β via its IBB (importin β binding) domain.

The typical import complex of cargo/importin α/β is formed in the cytoplasm in the absence of RanGTP (Fig. 6, left). After translocation through the NPC the import complex reaches the nucleoplasm where a high concentration of RanGTP is present. RanGTP interacts with importin β whereby the importin α/β complex and consequently the whole transport complex disassembles and the cargo is released into the nucleoplasm (Rexach et al. 1995; Gorlich et al. 1996). Subsequently importin α and importin β have to enter the cytoplasm to be available for another round of import. While importin β/RanGTP directly translocates back to the cytoplasm, importin α interacts with its own specific export receptor CAS and enters the cytoplasm in form of importin α/CAS/RanGTP complex (Kutay et al. 1997; Hood et al. 1998; Kunzler et al. 1998). Finally, in of the cytoplasm, GTP is hydrolyzed to GDP and the receptors are released in the cytoplasm, available for another import cycle.

## 4.2. Export

Nuclear export of most proteins is mediated by the export receptor CRM1 (chromosome region maintenance 1) (Fornerod et al. 1997; Stade et al. 1997; Hutten et al. 2007). CRM1 binds to leucine-rich NESs within export cargos, first described for the viral HIV-1 protein Rev (Fischer et al. 1995) and the cAMP-dependent proteinkinase inhibitor PKI (Wen et al. 1995). From these classical targets a consensus leucine-rich NES L-$X_{2-3}$-(L,I,V,M,F)-$X_{2-3}$-L-X-(L,I,V) was deduced. In addtition CRM1, CAS (Kutay et al. 1997) and the exportins t, 1, 4, 6, 7 (Arts et al. 1998; Kutay et al. 1998; Lipowsky et al. 2000; Brownawell et al. 2002; Stuven et al. 2003; Lund et al. 2004) have been shown to mediate nuclear export. Furthermore, the receptors importin13 and transportin, primarily known for their function in nuclear import, have also been shown to mediate export of selected cargoes (Gallouzi et al. 2001; Mingot et al. 2001; Shamsher et al. 2002).

Inside the nucleus, in the presence of the GTP-bound form of Ran, CRM1 forms a trimeric export complex with the NES-containing cargo protein and RanGTP (Fig. 6, right). The CRM1/RanGTP/cargo complex translocates through the NPC into the cytoplasm. At the cytoplasmic side of the NPC, the action of RanGAP1 stimulates Ran GTP-hydrolysis and thereby causes the disassembly of the export complex and the release of the export cargo into the cytoplasm. The Ran molecule that has been exported by this action is subsequently re-imported by its own import receptor NTF2 (nuclear transport factor 2) (Ribbeck et al. 1998; Smith et al. 1998).

As nuclear import or export require the presence and the accessibility of a NLS or a NES, conformational changes or posttranslational modifications of the cargo protein can sterically hinder the receptor-cargo interaction, preventing nuclear transport. These mechanisms allow the specific regulation of nuclear import and export and can thereby determine the intracellular distribution of a particular target (Fabbro et al. 2003; Terry et al. 2007).

# 5. Localization of SUMO enzymes

SUMOylation affects hundreds of proteins at different intracellular localizations. Based on proteomics studies and on immunofluorescence analysis, the majority of SUMOylated proteins are found in the nuclear compartment. Amongst those are

nuclear body proteins such as PML and SP100 (reviewed in Seeler et al. 2001), as well as many proteins involved in transcription (reviewed in Lyst et al. 2007; Garcia-Dominguez et al. 2009; Ouyang et al. 2009), chromatin remodelling (reviewed in Ouyang et al. 2009) and genome integrity (reviewed in Bergink et al. 2009). However, SUMOylated proteins are also found in the cytoplasmic compartment. Famous examples are mammalian RanGAP1 at the cytoplasmic side of the nuclear pore (Matunis et al. 1996; Mahajan et al. 1997), yeast septines located at the bud neck (Johnson et al. 2001; Takahashi et al. 2001; Takahashi et al. 2008), the ER-associated tyrosine phosphatase PTP1B (Dadke et al. 2007) and a number of proteins in the plasma-membrane, e.g. the voltage-gated potassium channel Kv1.5 (Benson et al. 2007), the kainate-receptor subunit GluR6 (Martin et al. 2007), the metatropic glutamate receptor mGluR8 (Tang et al. 2005), the glucose transporters GLUT1 and 4 (Giorgino et al. 2000) and the type I TGF-β receptor ALK5 (Kang et al. 2008).

A prerequisite for the reversible modification of SUMO targets is the presence of the enzymatic SUMO machinery. As some of the identified substrates are restricted to the cytoplasm, the enzymes should also be present in that compartment. And indeed, although most of the enzymes are enriched in the nucleus, most components have also been found in the cytoplasm (Fig. 7).

Both subunits of the SUMO E1 Aos1/Uba2 have been shown to predominantly localize to the nucleoplasm of mammalian and yeast cells (Dohmen et al. 1995; Azuma et al. 2001), but have also been detected in mammalian cytosol using fractionation studies (Pichler et al. 2002; Bossis et al. 2006). Analysis of the intracellular localization of Uba2 during embryogenesis of *D. melanogaster* revealed changes of the distribution of Uba2 between nucleus and cytoplasm during embryogenesis (Donaghue et al. 2001; Shih et al. 2002).

A mostly nuclear localization has been reported for the E2 enzyme Ubc9 (Seufert et al. 1995), but fractions of the enzyme have also been detected in the cytoplasm and associated with cytoplasmic filaments of the NPC (Lee et al. 1998; Pichler et al. 2002; Zhang et al. 2002; Bossis et al. 2006). Notably, the cytoplasmic pools of E1 and E2 have been shown to be specifically regulated by reactive oxidative species (Bossis et al. 2006): Macrophage activation causes the production of $H_2O_2$ close to the plasma-membrane, which in turn causes an inactivating crosslink of cytoplasmic Uba2 and Ubc9.

Members of the PIAS family of SUMO E3 ligases have been shown to be enriched in intranuclear dots, at least in part PML bodies, and additionally localize to the nucleoplasm and at low levels also to the cytoplasm (Sachdev et al. 2001; Kotaja et al. 2002; Miyauchi et al. 2002). However, the intracellular localization of the E3 ligases RanBP2 and Pc2 is restricted to a specific compartment, as RanBP2 is a component of the cytoplasmic filaments of the NPC (Wu et al. 1995; Yokoyama et al. 1995) and Pc2 localizes to intranuclear PcG bodies (Kagey et al. 2003; Roscic et al. 2006).

Finally, SUMO isopeptidases, necessary for the deconjugation and maturation of SUMO, are also found in both compartments. For example, *S. cerevisiae* Ulp1 and mammalian SENP2 are enriched at NPCs (Hang et al. 2002; Zhang et al. 2002; Li et al. 2003), SENP5 is enriched in the nucleolus (Nishida et al. 2000; Di Bacco et al. 2006) although a small fraction is found and required in the cytoplasm (Zunino et al. 2007), SENP1 can shuttle between nucleus and cytoplasm (Gong et al. 2000; Bailey et al. 2004) and SENP6 is found in both nucleus (Mukhopadhyay et al. 2006) and

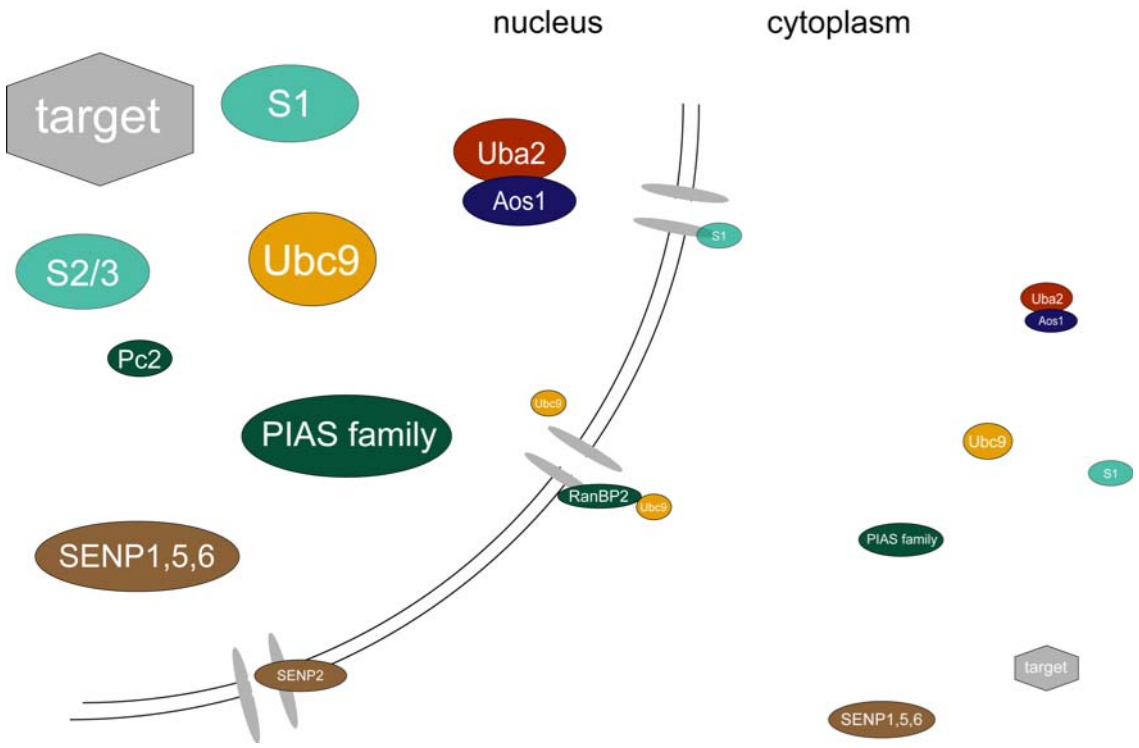


**Figure 7: Schematic illustration of the estimated intracellular localization of the components of the SUMO system**. Illustration of the distribution of SUMO proteins and components of the enzymatic machinery between nucleus and cytoplasm. The sizes of the icons rougly represent the relative amount of the enzymes, displaying the overall differences between the nuclear and cytoplasmic pools of the enzymes. Classes of enzymes are assigned to the following colourcode: the E1 enzyme Aos1/Uba2 in blue/red, the E2 Ubc9 in organge, E3 ligases in green and isopeptidases in brown. SUMO proteins are coloured turquoise and SUMO targets are shown in grey.

cytoplasm (Kim et al. 2000).

In conclustion, members of all classes of SUMO enzymes are present in the nucleus and in the cytoplasm, which allows dynamic SUMOylation in both compartments. However, to date only little is known about the mechanisms generating these enzymatic pools, not to mention their regulation.

## 6. Aim of this work

The SUMO E1 enzyme Aos1/Uba2 is predominantly localized in the nuclei of cells, with small amounts also present in the cytoplasm. The aim of this work was to analyze the molecular mechanisms and significance of the intracellular distribution of the SUMO-activating enzyme.

Key questions to be addressed were if and how the individual subunits and/or the assembled complex are imported into the nucleus and if the E1 enzyme can shuttle between nucleus and cytoplasm. In addition, I aimed to address the question whether the intracellular localization of the E1 complex is critical for SUMOylation.

# MATERIAL AND METHODS

## Material

### 1. Chemicals, reagents and enzymes

Standard chemicals and buffer substances were obtained from AppliChem, CARL ROTH, Serva, Sigma-Aldrich and Merck. The sources of special chemicals, reagents and enzymes are listed below:

| | |
|---|---|
| [$\gamma^{32}$P]GTP | Amersham |
| Acrylamid (30 %) | AppliChem |
| Aprotinin | Biomol |
| ATP | Sigma-Aldrich |
| BSA, fraction V | AppliChem |
| Creatinphosphate | Calbiochem |
| Creatinphosphate kinase | Calbiochem |
| Digitonin | Calbiochem |
| DMEM (high glucose) | Gibco |
| DNA marker (1 kb) | Fermentas |
| dNTPs | Fermentas |
| sorbitol | Fluka |
| ECL | Pierce |
| FCS | Gibco |
| Ficoll 400 | MP Biomedicals |
| Fluorescence Mounting Medium | DakoCytomation |
| GDP and GTP | Sigma-Aldrich |
| Hoechst 33258, Hoechst 33342 | Sigma-Aldrich |
| IPTG | Fermentas |
| Leptomycin B (LMB) | present of Dr. M. Yoshida (Tokio, Japan) |
| Leupeptin | Biomol |
| Oligonucleotides | MWG; Operon; Sigma-Aldrich |
| OptiMEM | Invitrogen |
| Pepstatin | Biomol |
| Phusion polymerase | New England Biolabs |

| | |
|---|---|
| PMSF | Sigma-Aldrich |
| Polyfect | Qiagen |
| Restriction enzymes | Fermentas, New England Biolabs |
| Rnase A | AppliChem |
| Iodoacetamide | Sigma |
| Methyl methanesulfonate (MMS) | Fluka |
| Sodium fluoride (NaF) | Sigma |
| N-Ethylmaleimide (NEM) | Sigma |
| Sequencing mix and buffer | Applied Biosystems |
| T4 DNA ligase | Fermentas |
| Trypane Blue | Fluka |
| Vent polymerase | New England Biolabs |
| Zymolyase 20T | MP Biomedicals |

## 2. Reaction kits

| | |
|---|---|
| NucleoBond® PC 100, PC500 | Macherey & Nagel |
| NucleoSpin® RNAII | Macherey & Nagel |
| NucleoSpin® Extract II | Macherey & Nagel |
| QIAquick® Gel Extraction Kit | Qiagen |
| QIAquick® PCR Purification Kit | Qiagen |
| BCA Protein Assay kit | Pierce |
| BCA Protein Assay kit – Reducing Agent Compatible | Pierce |

## 3. Consumables

| | |
|---|---|
| Autoradiography films (Amersham Hyperfilm™ECL) | GE Healthcare |
| Cell culture consumables | Sarstedt, TPP |
| Centrifugal filter units | Millipore, Vivaspin |
| Cyanogen bromide-activated sepharose 4B | Sigma-Aldrich |
| Dialysis tubing Spectra-Por | Roth |
| Disposable plastic columns Bio-Spin, Poly-Prep, Econo-Pac | Bio-Rad |
| Filter paper 2MM Whatman | Whatman |
| Gloves (Rotiprotect-LATEX, -NITRIL) | Roth |

| | |
|---|---|
| Microscope cover glasses (12 mm diameter, 18x18 mm) | Marienfeld |
| Microscope slides (76x26x1 mm) | Marienfeld |
| ProBond™ Nickel-chelating resin | Invitrogen |
| PROTRAN nitrocellulose | Schleicher & Schuell |
| Reaction tubes | Sarstedt, Eppendorf |
| Sterile filters and membranes (0.22 – 0.45 μM) | Millipore, Pall, Renner, Sartorius |

## 4. Buffers, media and stock solutions

Buffers and stock solutions were prepared in deionized water and media were sterilized by autoclaving unless noted otherwise. Buffers were titrated with sodium hydroxide or hydrochloride. Stock solutions were prepared freshly or stored at -20°C if possible.

*Buffers and media*

| | |
|---|---|
| 5-fluoroorotic acid (5-FOA) | 0.67 % (w/v) bacto yeast nitrogen base without amino acids, 0.2 % (w/v) SC-Ura drop out mix, 2 % (w/v) glucose, 0.01 % (w/v) uracil, 0. 1 % (w/v) 5-FOA, medium was filter sterilized, for plates 5-FOA medium was supplemented with autoclaved solution of bacto agar to 2 % (w/v) bacto agar final |
| DNA loading dye (6x) | 10 mM Tris/HCl pH8, 50 mM EDTA, 1 % (w/v) SDS, 30 % (v/v) glycerol, 0.1 % (w/v) bromophenol blue, 0.1 % (w/v) xylencyanol |
| Ficoll buffer | 18 % (w/v) Ficoll-400, 10 mM Tris/HCl pH 7.5, 20 mM KCl, 5 mM $MgCl_2$, 3 mM DTT, 1 mM EDTA |
| Lämmli running buffer (10x) | 250 mM Tris, 1,92 M glycine, 0,1% (w/v) SDS |
| LB medium | 1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 1 % (w/v) NaCl, pH 7, for agar plates LB medium was supplemented with 1.5 % (w/v) bacto agar |
| PBS-Tween | PBS supplemented with 0.2% (v/v) Tween 20 |
| Phosphate buffered saline (PBS) | 140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, pH 7.5 |
| pull-down buffer | 50 mM Tris pH 7.4, 200 mM NaCl, 1 mM $MgCl_2$, 5 % (v/v) glycerol and 2 mg/ml BSA or ovalbumine |

| | |
|---|---|
| SC complete | 0.67 % (w/v) bacto yeast nitrogen base without amino acids, 0.2 % (w/v) SC complete mix, 2 % (w/v) glucose, 0.005 % (w/v) adenine, SC complete medium for fluorescence analysis of cells was filter sterilized |
| SC-X (X = Ura, Leu, His or Trp) | 0.67 % (w/v) bacto yeast nitrogen base without amino acids, 0.2 % (w/v) SC-X drop out mix, 2 % (w/v) glucose, 0.005 % (w/v) adenine, SC-X medium was filter sterilized, for plates SC-X medium without adenine was supplemented with autoclaved solution of bacto agar to 2 % (w/v) bacto agar final |
| SDS sample buffer | 50 mM Tris/HCl pH 6.8, 2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue, 10 % (v/v) glycerol, 100 mM DTT, final, prepared as 1x, 2x and 4x stock solutions |
| SOC medium | 2% (w/v) tryptone, 5% (w/v) yeast extract, 50 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ |
| Spheroblasting buffer | 50 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 M sorbitol, 1 mM DTT |
| Sumoylation assay buffer (SAB) | transport buffer supplemented with 0.2 mg/ml ovalbumine, 0.05 % (v/v) Tween 20, 1 mM DTT, aprotinin, leupeptin, pepstatin |
| TAE | 40 mM Tris acetate pH 7.7, 1 mM EDTA |
| Transport buffer (TB) | 20 mM Hepes pH 7,3, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM EDTA, 2 mM DTT, 1 μg/ml of each aprotinin, leupeptin, pepstatin |
| Western blot transfer buffer | 25 mM Tris/HCl, 193 mM glycine, 20% (v/v) methanol, 0,036% (v/v) SDS |
| YPD | 1 % (w/v) bacto yeast extract, 2 % (w/v) bacto peptone, 2 % (w/v) glucose, for plates YPD medium was supplemented with 2 % (w/v) bacto agar |

*Stock Solutions*

| | |
|---|---|
| Ampicillin | 100 mg/ml |
| Aprotinin (1000x) | 1 mg/ml |
| ATP | 100 mM in 100 mM $Mg(OAc)_2$, 20 mM HEPES, pH 7.4 |
| Chloramphenicol | 30 mg/ml |
| Coomassie destainer | 50 % (v/v) methanol, 10 % (v/v) acetic acid |
| Coomassiestaining solution | 50 % (v/v) methanol, 10 % (v/v) acetic acid, 2.5 % (w/v) Brill. Blue R250 |
| Digitonin | 10 % (w/v) in DMSO |
| Dithiothreitol (DTT) | 1M |
| Gel drying solution | 20 % (v/v) ethanol, 1 % (v/v) glycerol |
| Hoechst 33258 | 0.1 mg/ml |
| Kanamycin | 50 mg/ml |
| Leupeptin/Pepstatin (1000x) | 1 mg/ml each in DMSO |
| PMSF | 100 mM in 2-propanol |
| Ponceau-S | 0,5 % (w/v) Ponceau-S, 1 % (v/v) acetic acid |
| P1 | 50 mM Tris/HCl pH 8.0, 10 mM EDTA, 100 μg/ml RNase A |
| P2 | 200 mM NaOH, 1 % (v/v) SDS |
| P3 | 3 M KOAc pH 5.5 |
| Silver gel fixing solution | 50 % (v/v) ethanol, 12 % (v/v) acetic acid, 0.018 % (w/v) formaldehyde |
| Silver gel developing solution | 3 % (w/v) $Na_2CO_3$, 0.018 % (v/v), formaldehyde, 0.0005 % (w/v) $Na_2S_2O_3$ |
| TAE (50x) | 2 M Tris acetate pH 7.7, 0,05 M EDTA |
| TFB-I | 100 mM RbCl, 15 % (v/v) glycerol, 0.5 mM LiCl, pH 5.8 |
| TFB-II | 10 mM MOPS pH 7, 10 mM RbCl, 75 mM $CaCl_2$, 15 % (v/v) glycerol |

## 5. Cell lines

*Bacterial strains*

| | |
|---|---|
| DH5α | F- Φ80lacZ ΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17($r_K$-, $m_K$+ ) supE44 thi-1 gyrA96 relA1 λ- |
| BL21 (DE3) | F- ompT hsd$S_B$($r_B$-$m_B$-) gal dcm λ(DE3) |
| BL21 (DE3) pLysS | F- ompT hsd$S_B$($r_B$-$m_B$-) gal dcm λ(DE3) pLysS($Cm_R$) |

*Yeast strains*

| | |
|---|---|
| ESM356-1 (from Elmar Schiebel*) | (Spore2.1) MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63 (ADE2) Ura- Trp- His- Leu- $G418^S$ |
| ESM356-1/pRS316*UBA2*/*uba2*Δ::nat | *UBA2* shuffle strain based on ESM356-1 |

*Mammalian cell lines*

| | |
|---|---|
| adherent HeLa cells | human cervix carcinoma cell line |
| HeLa suspension cells (CSH HeLa strain) | human cervix carcinoma cell line |
| NIH3T3 | mouse embryonic fibroblast cell line |

* Prof. Dr. Elmar Schiebel, ZMBH in Heidelberg

## 6. Oligonucleotides

*Oligonucleotides for cloning*

| *amplicon* | *acceptor* | | *#* | *sequence* |
|---|---|---|---|---|
| CFP | pET28a | for | 327 | ATTTAAGCTAGCATGGTGAGCAAGGGCGAGGAGC |
| | | rev | 328 | CGGGATCCCTTGTACAGCTCGTCCATGCCG |
| YFP | pET28b | for | 995 | TGCTAGCAACATGGTGAGCAAGGGC |
| | | rev | 996 | CGGATCCCCCTTGTACAGCTCGT |
| GFP | p413-Uba2, p413-*S.c.*Uba2 | for | 1541 | AA CTCGAG ATGGTGAGCAAGGGCGAGGAGC |
| | | rev | 1542 | TTGGTACCCTACTCGTCCATGCCGAGAGTGATC |

| | | | | |
|---|---|---|---|---|
| Aos1 | pET28a-CFP | for | 991 | AGAATTCATGGTGGAGAAGGAGGAGGC |
| | | rev | 992 | GTGCCTTGGCCCCAAGTGAAAGCTTAAA |
| Uba2 | pET28b, pET28-YFP | for | 1091 | AACCATGGGGATGGCACTGTCGCGGGGGCTG |
| | | rev | 994 | TGCTAGCTCCATCTAATGCTATGACATCATCAA G |
| Uba2-YFP | pcDNA3.1(-) | for | 1410 | AACTCGAGGAAATGGCACTGTCGCG |
| | | rev | 1413 | TTGAATTCCTACAGCTCGTCCATGC |
| Uba2 | p413 | for | 1527 | AAACTAGTATGGCACTGTCGCGGGGGCTG |
| | | rev | 1528 | TTGTCGACTCAATCTAATGCTATGACATCATC |
| *S.c.*Uba2 | p413 | for | 1532 | AAACTAGTATGCCAAGGGAAACAAGTTTGG |
| | | rev | 1533 | TTGTCGACTCAGTCTAATTCAACAATATCAGAA |
| *S.c.*Uba2 | p413-GFP | for | 1532 | AA ACTAGTATGCCAAGGGAAACAAGTTTGG |
| | | rev | 1534 | TTGTCGACGTCTAATTCAACAATATCAGAA |
| *S.c.*Uba2 | p413-NES | for | 1532 | AAACTAGTATGCCAAGGGAAACAAGTTTGG |
| | | rev | 1535 | TTGTCGACTCATAGTGCTAGTGCTGCTAGTGC TAG GTCTAATTCAACAATATCAGAA |
| *S.c.*Uba2 | p413-NES-GFP | for | 1532 | AA ACTAGTATGCCAAGGGAAACAAGTTTGG |
| | | rev | 1536 | TTGTCGACTAGTGCTAGTGCTGCTAGTGCTAG GTCTAATTCAACAATATCAGAA |
| *natNT2* | *UBA2*-deletion | for | 1543 | CTTCAACAGACACACAAGATCAGGAGCGCTAC GCCAAACAAGAAAAGAAAATGCGTACGCTGCA GGTCGAC |
| | | rev | 1544 | ACTGCGAACCACGATTAAATAAATATATAGATA CCTTTTCTTATTTATTAATCGATGAATTCGAGC TCG |
| *UBA2* | pRS316 | for | 1539 | AAACTAGTGAGCTTTTTCTTTCCCCCCTTCAAG |
| | | rev | 1540 | TTGTCGACGAAATTCAAGATCACGCAAGAGGG |

*Oligonucleotides for mutagenesis*

| *mutation(s)* | | *#* | *sequence* |
|---|---|---|---|
| Aos1-P192A | for | 1118 | CCAAGGAGTAGAAGATGGGGCCGACACCAAGAGAGC |
| | rev | 1119 | GCTCTCTTGGTGTCGGCCCCATCTTCTACTCCTTGG |
| Aos1-KR195,196$A_2$ | for | 1067 | GAAGATGGGCCCGACACCGCGGCAGCAAAACTTGATT CTTCTGAG |
| | rev | 1068 | CTCAGAAGAATCAAGTTTTGCTGCCGCGGTGTCGGGC CCATCTTC |
| Aos1-KRAK195-198$A_4$ | for | 1069 | GGGCCCGACACCGCGGCAGCAGCACTTGATTCTTCT GAGACAACG |
| | rev | 1070 | CGTTGTCTCAGAAGAATCAAGTGCTGCTGCCGCGGTG TCGGGCCC |

| | | | |
|---|---|---|---|
| Aos1-LD199,200A$_2$ | for | 1120 | CGACACCAAGAGAGCAAAAGCTGCTTCTTCTGAGACAACG |
| | rev | 1121 | CGTTGTCTCAGAAGAAGCAGCTTTTGCTCTCTTGGTGTCG |
| Aos1-KK207,208A$_2$ | for | 1071 | CTTCTGAGACAACGATGGTCGCAGCGAAGGTGGTCTTCTGCCCTGC |
| | rev | 1072 | GCAGGGCAGAAGACCACCTTCGCTGCGACCATCGTTGTCTCAGAAG |
| Aos1-KKKV207-210A$_4$ | for | 1073 | GAGACAACGATGGTCGCAGCGGCGGCGGTCTTCTGCCCTGTTAAAGAAGC |
| | rev | 1074 | GCTTCTTTAACAGGGCAGAAGACCGCCGCCGCTGCGACCATCGTTGTCTC |
| Uba2-RK610,611A$_2$ | for | 1059 | CGTCAGTGAAGAAGAGAAGCGCCGCGAGGAAATTAGATGAGAAAGAG |
| | rev | 1060 | CTCTTTCTCATCTAATTTCCTCGCGGCGCTTCTCTCTTCTTCACTGACG |
| Uba2-RKRK610-613A$_4$ | for | 1061 | GAAGAGAGAAGCGCCGCGGCGGCATTAGATGAGAAAGAGAATCTC |
| | rev | 1062 | GAGATTCTCTTTCTCATCTAATGCCGCCGCGGCGCTTCTCTCTTC |
| Uba2-KR623,624A$_2$ | for | 1063 | GATGAGAAAGAGAATCTCAGTGCAGCGGCGTCACGTATAGAACAGAAGGAAGAGC |
| | rev | 1064 | GCTCTTCCTTCTGTTCTATACGTGACGCCGCTGCACTGAGATTCTCTTTCTCATC |
| Uba2-KRSR623-626A$_4$ | for | 1065 | GAGAAAGAGAATCTCAGTGCAGCGGCGGCAGCTATAGAACAGAAGGAAGAGC |
| | rev | 1066 | GCTCTTCCTTCTGTTCTATAGCTGCCGCCGCTGCACTGAGATTCTCTTTCTC |
| *S.c.*Uba2-KRTK619-622AATA | for | 1551 | GAGGCGCCCAGTAACGCAGCGACAGCGTTAGTTAATGAACCG |
| | rev | 1552 | CGGTTCATTAACTAACGCTGTCGCTGCGTTACTGGGCGCCTC |

## 7. Vectors and plasmids

*Vectors*

| *name* | *designated use* | *source* |
|---|---|---|
| pECFP-C1 | amplification of ECFP | Clontech |
| pEYFP-C1 | amplification of EYFP | Clontech |
| pET11d | bacterial expression | Novagen |
| pET28a | bacterial expression | Novagen |
| pET28a-ECFP | bacterial expression | this work |
| pET28b | bacterial expression | Novagen |

| | | |
|---|---|---|
| pET28b-EYFP | bacterial expression | this work |
| pcDNA3.1(-) | mammalian expression | Invitrogen |
| pGBT9 | yeast expression | Manfred Kögl* |
| p413 | yeast expression | Elmar Schiebel# |
| pRS316 | yeast expression | Elmar Schiebel# |

* Dr. Manfred Kögl, DKFZ, Genomics and Proteomics Core Facilities, Protein Interaction Screenings in Heidelberg

# Prof. Dr. Elmar Schiebel, ZMBH in Heidelberg

*Plasmids*

All sequences, except for GFP-derivates, are coding for human proteins unless noted otherwise. Tags are always located at the respective terminus shown in the plasmid construct.

| *name* | *source* |
|---|---|
| pET28a-His-Aos1 | (Pichler et al. 2002)* |
| pET28a-His-ECFP-Aos1 | this work |
| pET28a-His-ECFP-Aos1-KR195,196$A_2$ | this work |
| pET28b-Uba2-EYFP-His | this work |
| pET28b-Uba2-KR623,624$A_2$-EYFP-His | this work |
| pET28b-Uba2-His | this work, (Werner et al. 2009) |
| pET11d-Uba2 | (Pichler et al. 2002)* |
| pcDNA3.1(-)-ECFP-Aos1 | this work |
| pcDNA3.1(-)-ECFP-Aos1-KR195,196$A_2$ | this work |
| pcDNA3.1(-)-ECFP-Aos1-KRAK195-198$A_4$ | this work |
| pcDNA3.1(-)-ECFP-Aos1-KK207,208$A_2$ | this work |
| pcDNA3.1(-)-ECFP-Aos1-KKKV207-210$A_4$ | this work |
| pcDNA3.1(-)-ECFP-Aos1-P192A | this work |
| pcDNA3.1(-)-ECFP-Aos1-LD199,200$A_2$ | this work |
| pcDNA3.1(-)-Uba2-EYFP | this work |
| pcDNA3.1(-)-Uba2-RK610,611$A_2$-EYFP | this work |
| pcDNA3.1(-)-Uba2-RKRK610-613$A_4$-EYFP | this work |
| pcDNA3.1(-)-Uba2-KR623,624$A_2$-EYFP | this work |
| pcDNA3.1(-)-Uba2-KRSR623-626$A_4$-EYFP | this work |
| pET11d-*S.c.*Uba2 | Erica Johnson# |

| | |
|---|---|
| pRS316-*S.c.UBA2* | this work |
| pKS134-pFA6a-natNT2 | Elmar Schiebel[+] |
| p413-Uba2 | this work |
| p413-*S.c.*Uba2 | this work |
| p413-*S.c.*Uba2-EGFP | this work |
| p413-*S.c.*Uba2-KRTK619-622AATA | this work |
| p413-*S.c.*Uba2-KRTK619-622AATA-EGFP | this work |
| p413-*S.c.*Uba2-KRTK619-622AATA-NES | this work |
| p413-*S.c.*Uba2-KRTK619-622AATA-NES-EGFP | this work |
| pET30a-His-S-Importin beta-1 | Larry Gerace° |
| pQE70-His-Importin alpha-2 (Rch1) | Dirk Görlich[§] |

* Dr. Andrea Pichler, Max-Planck Institute of Immunobiology, Freiburg

[#] Ph.D. Erica Johnson, Jefferson Medical College, Thomas Jefferson University, Pennsylvania

[+] Prof. Dr. Elmar Schiebel, ZMBH, Heidelberg

° Ph.D. Larry Gerace, The Scribbs Research Institute, La Jolla, California

[§] Prof. Dr. Dirk Görlich, Max-Planck Institute of Biophysical Chemistry, Göttingen

*Generation of plasmids and vectors within this work*

*pET28a-ECFP, pET28b-EYFP*: ECFP was PCR amplified (#327/328) from pECFP-C1 and cloned into pET28a via NheI and BamHI sites. Likewise EYFP was amplified (#995/996) from pEYFP-C1 and cloned into NheI and BamHI sites of pET28b.

*pET28a-His-ECFP-Aos1 wt and* -KR195,196$A_2$*:* Human Aos1 was PCR amplified (#991/992) from pET28a-Aos1 (Pichler et al., 2002) or pcDNA3.1(-)-KR195,196$A_2$, respectively, and cloned into pET28a-ECFP via EcoRI and HindIII sites.

*pET28b-Uba2-His, pET28b-Uba2-EYFP-His, pET28b-Uba2-KR623,624A2-EYFP-His*: Human Uba2 wt and mutant were PCR amplified (#1091/994) from pET11d-Uba2 (Pichler et al., 2002) or *pcDNA3.1(-)-Uba2-KR623,624$A_2$-EYFP,* respectively, and cloned via NcoI and NheI sites into pET28b and / or pET28b-EYFP.

*pcDNA3.1(-)-ECFP-Aos1*: ECFP-Aos1 was swapped from *pET28a-His-ECFP-Aos1* into pcDNA3.1(-) via NheI and HindIII sites.

*pcDNA3.1(-)-ECFP-Aos1-P192A, -KR195,196$A_2$, -LD199,200$A_2$ and -KK207,208$A_2$*: Indicated amino acids were changed to alanines by site directed mutagenesis on

pcDNA3.1(-)-ECFP-Aos1 (P192 #1118/1119, KR195,196 #1067/1068, LD199,200 #1120/1121, KK207,208 #1071/1072).

*pcDNA3.1(-)-ECFP-Aos1-KRAK195-198A$_4$ and -KKKV207-210A$_4$*: The indicated amino acids were changed to alanines in two steps, performing site-directed mutagenesis on already mutated templates. KRAK195-198 were changed by mutagenesis (#1069/1070) on pcDNA3.1(-)-ECFP-Aos1-KR195,196A$_2$ and KKKV207-210 by further mutagenizing (#1073/1074) pcDNA3.1(-)-ECFP-Aos1-KK207,208A$_2$.

*pcDNA3.1(-)-Uba2-EYFP*: Human Uba2-EYFP was PCR amplified (#1410/1413) from pET28b-Uba2-EYFP-His and cloned into pcDNA3.1(-) via XhoI and EcoRI sites.

*pcDNA3.1(-)-Uba2-RK610,611A$_2$-EYFP and -KR623,624A$_2$-EYFP*: The indicated amino acids were changed to alanines by site directed mutagenesis (RK610,611 #1059/1060, KR623,624 #1063/1064) on pcDNA3.1(-)-Uba2-EYFP.

*pcDNA3.1(-)-Uba2-RKRK610-613A$_4$-EYFP and -KRSR623-626A$_4$-EYFP*: The indicated amino acids were changed to alanines in two steps, performing site-directed mutagenesis on already mutated templates. RKRK610-613 were changed by mutagenesis (#1061/1062) on pcDNA3.1(-)-Uba2-RK610,611A$_2$-EYFP and KRSR623-626 by mutagenizing (#1065/1066) pcDNA3.1(-)-Uba2-KR623,624A$_2$-EYFP.

*pRS316-S.c.UBA2*: The *UBA2* gene from yeast, including approximately 500 bp in the 5'- and 3'-prime untranslated region, was PCR amplified (#1539/1540) from isolated genomic yeast DNA provided by the lab of Elmar Schiebel (ZMBH, University of Heidelberg) and cloned into pRS316 via SpeI and SalI sites.

*p413-Uba2, p413-S.c.Uba2*: Human and yeast Uba2 were PCR amplified (human #1527/1528, yeast #1532/1533) from pET28b-Uba2 or pET11d-*S.c.*Uba2 and cloned via SpeI and SalI sites into p413.

*p413-S.c.Uba2-KRTK619-622AATA:* The change of the indicated amino acids to alanines was performed by PCR amplification in two steps. First, two independent PCRs were performed to introduce the mutation, with reaction one (#1532/1552) producing an amplicon from the 5'- start to the mutation site and reaction two (#1551/1533) producing an amplicon from the mutation site to the 3'-end. Then, these amplicons were used as template DNA in a PCR (#1532/1533) resulting in the complete final insert which was cloned into p413 via SpeI and SalI sites.

*p413-S.c.Uba2-KRTK619-622AATA-NES:* S.c.Uba2-KRTK619-622AATA was PCR amplified (#1532/1535) from p413-S.c.Uba2-KRTK619-622AATA introducing a nuclear export sequence (NES) by the primer for the 3'-end. Then, the amplicon was cloned via SpeI and SalI into p413.

*p413-S.c.Uba2-EGFP:* In the first step, a plasmid of p413 with yeast Uba2 not containing a stop-codon in the ORF was constructed. Therefore, yeast Uba2 was PCR amplified (#1532/1534) from pET11d-*S.c.*Uba2 and cloned into p413 via SpeI and SalI. Secondly, EGFP was introduced into the created construct via SalI and XhoI sites after PCR amplification (#1541/1542) from pET28a-CFP.

*p413-S.c.Uba2-KRTK619-622AATA-EGFP*: This plasmid was created in a similar way to p413-S.c.Uba2-EGFP. First, a plasmid of yeast Uba2-KRTK619-622AATA without stop-codon was constructed by PCR amplification (#1532/1534) and subsequent cloning of *S.c.*Uba2-KRTK619-622AATA via SpeI and SalI in p413. Then, PCR amplified (#1541/1542) EGFP was inserted into the created construct via SalI and XhoI sites.

*p413-S.c.Uba2-KRTK619-622AATA-NES-EGFP*: First, yeast Uba2-KRTK619-622AATA-NES was PCR amplified (#1532/1535) from p413-S.c.Uba2-KRTK619-622AATA and cloned into p413 via SpeI and SalI, generating a construct whose ORF did not contain a STOP-codon. Then, EGFP was PCR amplified (#1541/1542) from pET28a-CFP and cloned via SalI and XhoI into the before generated plasmid.

## 8. Recombinant proteins

All recombinant proteins are, except for GFP-derivate tags human proteins unless noted otherwise. Tags are always located at the respective terminus indicated by the given name.

| *name* | *source* |
|---|---|
| His-Aos1 | this work |
| His-ECFP-Aos1 | this work |
| His-ECFP-Aos1-KR195,196$A_2$ | this work |
| His-Aos1/Uba2 | this work |
| Uba2-His | this work |
| Uba2-EYFP-His | this work |
| Uba2-KR623,624$A_2$-EYFP-His | this work |

| | |
|---|---|
| Ubc9 (mouse) | common stock* |
| SUMO1ΔC4 | common stock* |
| RanGAP1 (mouse) | common stock* |
| YFP-SUMO1ΔC4 | common stock* |
| CFP-GAPtail (mouse) | common stock* |
| His-S-Importin beta 1 | this work |
| His-Importin alpha (Rch1) | this work |
| His-Transportin | Ralph Kehlenbach# |
| His-Importin 5 | Ralph Kehlenbach# |
| His-Importin 7 (xenopus) | Ralph Kehlenbach# |
| His-Importin 9 | Ralph Kehlenbach# |
| His-Importin 13 | Ralph Kehlenbach# |
| His-Crm1 | Ralph Kehlenbach# |
| Ran, RanQ69L | Ralph Kehlenbach# |
| His-YFP-M9 | Ralph Kehlenbach# |

*These proteins were purified alternatingly by members of the Melchior lab and are available as common protein stocks in the lab.

# Dr. habil. Ralph Kehlenbach, University of Göttingen

## 9. Antibodies

*Primary antibodies*

| *name* | *source* | *concentr.* | *working dilution* |
|---|---|---|---|
| goat α-Aos1 (affinity purified) | Melchior lab | 0.5 mg/ml | IF 1:100, WB 1:1000 |
| goat α-Uba2 (affinity purified) | Melchior lab | 0.5 mg/ml | IF 1:100, WB 1:1000 |
| goat α-Ubc9 (affinity purified) | Melchior lab | 1.5 mg/ml | WB 1: 500 |
| goat α-GM130 clone 35 | BD Biosciences | 250 µg/ml | WB 1:250 |
| goat α-LDH | Santa Cruz Biochtechnology | 200 µg/ml | WB 1:500 |
| goat α-Nop1 | Santa Cruz Biotechnology | 200 µg/ml | WB 1:100 |
| rabbit α-R6IP2 (ELKS) | Melchior lab | 0.5 mg/ml | WB 1:1000 |

| | | | |
|---|---|---|---|
| rabbit α-smt3 | Helle Ulrich* | unknown | WB 1:4000 |
| mouse α-NMDAR1 | Nils Brose# | unknown | WB 1:1000 |
| mouse α-NTF97 clone 3E9 | Abcam | unknown, ascites | inhibiton studies 1:2 |
| mouse α-Pgk1 clone 22C5D8 | Molecular Probes | 1 mg/ml | WB 1:4000 |
| mouse α-RCC1 clone 9 | BD Biosciences | 250 µg/ml | WB 1:1000 |

* Prof. Dr. Helle Ulrich, London Research Institue, London, UK

# Prof. Dr. Nils Brose, Max-Planck Institute for Experimental Medicine, Göttingen

*Secondary antibodies*

Horseradish peroxidase-conjugated antibodies raised in donkey against IgGs from goat, mouse or rabbit were obtained from Dianova. They were used for western blot analysis in dilutions of 1:5,000 – 1:10,000.

Alexa488- and Alexa594-conjugated antibodies from donkey against goat IgG were obtained from Molecular Probes and used in an dilution of 1:500 – 1:1,000 for immunofluorescence analyses.

## 10. Technical equipment and software

*Technical equipment*

| | |
|---|---|
| Bacterial incubator Kelvitron t | Heraeus |
| Cell culture incubator Heracell | Heraeus |
| Cell culture incubator Incucell | MMM Medcenter |
| Centrifuges 5415C, 5424, 5415, 5430, 5417R | Eppendorf |
| Chromatography system Äkta Purifier | GE Healthcare |
| Confocal microscope CLSM510meta | Zeiss |
| Electrophoresis Power Supply EPS300 | Pharmacia Biotech |
| Elektrophoresis and blotting chambers | Workshop MPI of Biochemistry, Martinsried |
| EmulsiFlex-C5 | Avestin |
| Film developing machine Curix 60 | Agfa |
| Fluoroskan Ascent microtiter plate reader | Thermo Scientific |
| Live cell imaging system *CellObserver HighSpeed* | Zeiss |

| | |
|---|---|
| Microinjector | Eppendorf |
| NanoDrop ND1000 | Thermo Scientific |
| Rotors TLA-100.3, JS-5.2, Type45Ti, Type60Ti, JA-20, Type70.1Ti | Beckman Coulter |
| Scanner 4990 Photo | Epson |
| Sterile cell culture hood Herasafe | Heraeus |
| Thermocycler Primus | MWG Biotech |
| Thermocycler T3000 and Tprofessional | Biometra |
| Thermomixer Compact | Eppendorf |
| Ultracentrifuge Optima Max, Optima L-80 XP | Beckman Coulter |
| Vacuum pump LABOPORT N480.3FTP | KNF Neuberger |
| Vortex Genie 2 | Scientific Industries |

*Software*

| | |
|---|---|
| Adobe acrobat 9 pro | Adobe |
| Adobe creative suite 4 | Adobe |
| Axiovision software 4 | Zeiss |
| BioMath Calculators | http://www.promega.com/biomath/calc11.htm, Promega |
| ClustalW2 | http://www.ebi.ac.uk/Tools/clustalw2/index.html, EMBL |
| Endnote X2 | Thomson Reuters |
| PyMOL v0.99 | DeLano Scientific LLC |
| Rotor Calculator | Beckman Coulter |
| SigmaPlot 8.02 | Systat Software Inc. |
| Vector NTI | Invitrogen |

## Methods

Standard procedures in molecular biology, biochemistry and cell biology were performed according to basic methods described in *Molecular Cloning: A Laboratory Manual* (Maniatis et al. 1989), *Current Protocols in Protein Science* (Coligan et al. 2003), *Current Protocols in Cell Biology* (Bonifacino et al. 2000), and *Cells: A Laboratory Manual* (Spector et al. 1998).

### 1. Molecular biology methods

*1.1. Preparation of chemical competent bacteria*

Chemical competent *E.coli* were prepared from a 200 ml growing culture with an optical density $OD_{600nm}$ of 0.5. After 10 minutes incubation on ice, bacteria were harvested by centrifugation with 5,000 g at 4°C. The cell pellet was resuspended in 200 ml ice-cold TFB-I buffer and incubated on ice for 2 hours. The cells were again collected with 5,000 g at 4°C and resuspended in 8 ml of sterile cold TFB-II buffer. Aliquots of 100 µl were frozen in liquid nitrogen and stored at -80°C.

*1.2. Transformation of competent bacteria*

Chemical competent *E.coli* were thawed on ice and incubated with DNA for 30 minutes on ice. Incorporation of the DNA was achieved by heat-shock at 42°C for 90 seconds, followed by incubation on ice for 2 minutes. For regeneration the bacteria were supplemented with 500 µl LB medium and incubated in a shaker at 37°C for 1 hour. Finally the transformed cells were plated on LB agar plates supplemented with the respective antibiotic for the transformed plasmid.

*1.3. Plasmid DNA purification*

Small amounts of plasmid DNA were prepared by alkaline lysis (Birnboim et al. 1979) and subsequent precipitation. 2 ml overnight cultures of *E.coli* DH5α were harvested for 5 minutes with 3,000 g at room temperature and resuspended in 300 µl resuspension buffer P1. After mixing with 300 µl alkaline lysis buffer P2 the lysate was incubated for 5 minutes at room temperature before proteins and chromosomal DNA were precipitated by the addition of 300 µl neutralization buffer P3. The lysates were cleared from unsoluble components by centrifugation for 30 minutes with

13,000 g at 4°C and the plasmid DNA was precipitated from 800 µl supernatant with 640 µl 2-propanol. After collecting DNA by centrifugation for 15 minutes with 13,000 g at room temperature the DNA was washed with 70 % (v/v) ethanol, dried and resuspended in 30 – 50 µl water.

High quality isolation of DNA was necessary for plasmids used for transfection of cultured cells. Purification of small or large quantities of DNA was performed using NucleoBond® PC 100 or PC500 Kit from Macherey & Nagel according to the manufactorer's instructions.

*1.4. Measurement of DNA concentration*

The concentration of DNA was quantified by measuring the $OD_{260nm}$, with an appropriate dilution ensuring that the OD is in the dynamic range of the spectrometer. The ratio of $OD_{260nm}$ to $OD_{280nm}$, ideally 1.8, revealed information about the purity of the DNA.

*1.5. Agarose gel electrophoresis*

DNA fragments of different size were separated by agarose gel electrophoresis. Depending on the fragment sizes 0.5 – 3 % (w/v) agarose were dissolved in 50 ml TAE buffer by heating and the solid gel was formed by cooling down in a gel chamber. The gel was loaded with 8 µl of 1 kb DNA marker and with DNA samples supplemented with an appropriate amount of 6x DNA loading dye. After seperation at 80 Volt DNA was stained in a bath with 1 µg/ml ethidiumbromide and was visualized with UV light of 365 nm.

*1.6. Isolation of DNA from agarose gels*

After separation of DNA fragments by agarose gel electrophoresis bands were cut out of the gel and DNA fragments were extracted using NucleoSpin® Extract II Kit from Macherey & Nagel according to the manufacturer's instructions. DNA was eluted in 20 - 40 µl elution buffer.

*1.7. Restriction of DNA by endonucleases*

The buffers and enzymes of Fermentas were used for DNA restriction. Reaction conditions were chosen according to the manufactorer's instructions for the

respective enzymes used. In preparative restrictions 1 μg DNA was converted in 100 μl reactions containing approximately 2 – 10 units of enzyme for 2 – 4 hours at 37 °C, analytical reactions were performed in 20 μl reactions containing 200 – 500 ng DNA. To prevent star-activity the amount of enzymes never exceeded 1/10 of the total reaction volume.

*1.8. Ligation of DNA fragments*

Ligations were set up with vector to insert at a molar ratio of 1:3 – 1:5 in 10 μl reactions containing 1 unit T4 DNA ligase (Fermentas), ligation buffer and some additional ATP. Ligation was performed for 2 h at 30 °C or at 16 °C overnight and the reaction was transformed into DH5α.

*1.9. Sequencing of DNA*

All plasmids constructed via PCR amplification were verified by DNA sequencing based on the chain-teminating method in principle established by Sanger, Nicklen and Coulsen (Sanger et al. 1977). The sequencing reactions were performed with BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) and contained 50 – 200 ng plasmid DNA, 10 pmol primer, 2 μl sequencing buffer and 1 μl sequencing mix in a final volume of 10 μl. PCR was performed according to the following program: initial denaturation 2 minutes at 96°C, denaturation during cycling 10 second at 96 °C, annealing for 15 seconds at 55 °C, elongation at 60 °C for 4 minutes, 25 cycles.

After stopping the reaction with 1 μl of each 3 M Na(OAc) pH 5.2 and 125 mM EDTA the PCR product was precipitated by addition of 50 μl ice cold absolute ethanol and centrifugation for 15 minutes with 13,000 g. The pellet was washed with 70 % (v/v) ethanol, was dried and was resuspended in 30 μl sterile water. Read-out was performed in a Genetic Analyzer 3100 (Applied Biosystems) at the Göttinger Zentrum für Molekulare Biowissenschaften (GZMB) of the University of Göttingen.

Alternatively sequencing was carried out by GATC Biotech from 30 μl of 30 – 100 ng/μl plasmid DNA and 10 pmol/μl primer.

The sequences were analyzed using Lasergene Gene Quest (DNAstar) or a free of charge version of the software Vector NTI (Invitrogen).

*1.10. Polymerase chain reaction (PCR)*

The principle of the polymerase chain reactions traced back to K.B. Mullis (Mullis 1990). Amplification of specific DNA fragments was performed by PCR with two primers defining beginning and end of the amplicon. Reactions were set up in a final volume of 50 µl with 50 – 100 ng template DNA or 1 – 2 µl cDNA, 20 pmol of each forward and reverse primer, 200 µl of each dNTP and 1 unit Phusion (Finnzymes) or Vent (NEB) polymerase. In the case of GC-rich DNA 3 % (v/v) DMSO were added to the reaction. The annealing temperature for the appropriate oligonucleotide was calculated according to a web-based calculator (http://www.promega.com/biomath/calc11.htm). 3 °C were added to the calculated temperature when phusion polymerase was used, whereas 5 – 6 °C were substracted when DMSO was added to the reaction. Amplification time was chosen in dependence of the processivity of the used polymerase. In general, amplification was performed according the following program:

initial denaturation 1 minute 95 °C, denaturation during cycling 30 seconds 95 °C, annealing for 30 seconds at the calculated temperature, elongation at 72 °C for the calculated time, standardwise 35 cycles and an additional final elongation step at 72 °C for 2 – 5 minutes.

*1.11. Site directed mutagenesis*

PCR reactions for site directed in vitro-mutagenesis of plasmids were based on the protocol QuickChange Site-Directed Mutagenesis Kits from Stratagene. The used oligonucleotides were reverse-complementary to each other and comprised the desired mutations. Reactions of 50 µl final volume contained 25 ng plasmid DNA, 5 pmol of each primer, 5 nmol of each dNTP, 2.5 µl 10fold Pfu buffer and 1.5 units Pfu DNA polymerase. Amplification was performed according to the following program: initial denaturation 2 minutes 95 °C, denaturation during cycling 30 seconds 95 °C, annealing for 1 minute at the calculated temperature (see above), elongation at 72 °C for the calculated time, standardwise 28 cycles and an additional final elongation step at 72 °C for 2 – 5 minutes. Subsequently the reaction was cooled to 4 °C, incubated with 10 units DpnI for 1 h at 37 °C for selective degradation of the methylated template DNA and transformed into competent bacteria.

## 2. Biochemical methods

### *2.1. Measurement of protein concentration*

The concentration of protein solutions was determined with a BCA-test essentially established by Smith et al. (Smith et al. 1985). In alkaline solution proteins form a complex with $Cu^{2+}$ ions (Biuret-reaction) and the reduced ions subsequently form a violet complex with bicinchinoninic acid (BCA) that can be measured at 562 nm. Concetrations were measured using BCA Protein Assay kit (Pierce) according to the manufacturer's instructions. As standard the result was confirmed by comparison to different defined amounts of BSA analyzed in the same Coomassie stained gel.

### *2.2. SDS PAGE and detection of proteins*

SDS polyacrylamide gel electrophoresis (SDS PAGE)

Separation of proteins was performed by SDS polyacrylamide gel electrophoresis essentially according to the system described by Laemmli (Laemmli 1970). In most cases 10 % gels or continous 5 – 20 % gradient gels were used. Gels were prepared in blocks of eight gels, first casting the separation gel containing 10 % (w/v) polyacrylamide (for 10 % gels) in 0.4 M Tris-HCl pH 8.8, 0.1 % (w/v) SDS, 0.001 % (w/v) (v/v) of each APS and TEMED; an overlay of 2-propanol assured an even surface. Gradient gels were prepared using a double-cylindrical gradient mixer, each cylinder filled with equal amounts of 5 % and 20 % (w/v) polyacrylamide solutions. The mixture of these solutions was filled into the block resulting in a continous gradient of 5 % at the top and 20 % towards the bottom of the gels. After polymerization of the separation gel the 2-propanol was removed and the stacking gel consisting of 4 % (w/v) polyacrylamide, 50 mM Tris-HCl pH 6.8, 0.1 % (w/v) SDS, 0.001 % (w/v) APS and 0.001 % (v/v) TEMED was poured on top of the separation gel. After insertion of combs the stacking gel polymerized for approximately 2 hours. The gels were run with Laemmli buffer at 20 mAmp/300V per gel at room temperature.

Before loading the samples were adjusted to approximately 50 mM Tris-HCl pH 6.8, 2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue, 10 % glycerol, 100 mM DTT with one-, two-, or four-fold concentrated SDS sample buffer and were boiled at 95 °C for a few minutes. DTT sensitive samples like thioester bonds were mixed 1:1 with TLB

(50 mM Tris pH 6.8, 2 % SDS, 4 M Urea, 10 % glycerol) supplemented with 0.1 % (w/v) bromophenol blue and were incubated for 10 minutes at 60 °C.

*Coomassie staining*

For Coomassie staining, gels were stained for at least 1 hour in Coomassie-staining solution and then incubated in Coomassie destaining solution until all excess dye was removed from the gel.

Alternatively gels were fixed in a solution of 40 % ethanol and 10 % acetic acid for 20 – 30 minutes, the gels were rehydrated for some minutes in water and were stained in a solution of 10 % acetic acid and 0.005 % Coomassie R-250. In this case, destaining of the gel due to high background was not necessary.

For documentation gels were incubated for 30 – 60 minutes in gel drying solution, mounted between cellophane sheets and clamped between two plastic frames for drying.

*Silver staining*

Detection of small amounts of proteins in gels was achieved by silver staining, a method principally developed by Merril et al. (Merril et al. 1981). For this, the gel was fixed for at least 1.5 hours in fixing solution, washed three times for 20 minutes in 50 % (v/v) ethanol, incubated in fresh 0.01 % (w/v) disodium-thiosulfate $Na_2S_2O_3$ for 1 minute, washed three times for 20 seconds in water and stained for 20 minutes in fresh 0.1 % (w/v) silver nitrate $AgNO_3$. The stained gels were developed by incubation in developing solution for up to 10 minutes. When the protein bands became visible, the gel was washed with water and the reaction was stopped with 10 mM EDTA.

*Immunoblotting*

Prior to immunological detection, the proteins were transferred in a semi-dry western blot apparatus from the gel onto a nitrocellulose membrane with a pore size of 0.2 μm (Kyhse-Andersen 1984). Three layers of Whatman 3MM paper, a nitrocellulose membrane, the gel and again three layers Whatman paper soaked in transfer buffer were prepared from anode to kathode. Protein transfer was executed with 20 volt for 1 – 2 hours depending on the size of the protein of interest. The transfer was controlled by staining protein with Ponceau-S solution and excess dye was removed by washing with 1 % (v/v) acetic acid.

Unspecific binding sites on the membrane were blocked by incubation with blocking buffer (5 % (w/v) skim milk in PBS-Tween) for 1 hour at room temperature. Appropriate dilutions of primary and HRP-coupled secondary antibody in blocking buffer were prepared; the blot was incubated with primary antibody for at least 1 hour at room temperature or overnight at 4 °C, washed three times for 15 minutes with PBS-Tween and then incubated with secondary antibody for 1 – 2 hours at room temperature. After removing unbound antibody by washing with PBS-Tween, bound antibody was detected by chemiluminescence using ECL kits from Pierce or Millipore. Exposure times of the films were chosen according to the strength of the signal; films were developed using an automatic developing machine.

*2.3. Protein precipitation with TCA*

To concentrate protein solutions for SDS PAGE, proteins were precipitated and redissolved. Precipitation was performed by mixing the protein solution with an equal volume of 20 % (w/v) trichloroacetic acid (TCA), followed by incubation for 30 minutes on ice. The precipitated proteins were collected by centrifugation with 14,000 x g for 30 minutes at 4 °C, the TCA solution was completely removed and the protein was dissolved in 4 x SDS sample buffer facilitated by sonication.

*2.4. Expression and purification of recombinant proteins*

Recombinant Aos1, Uba2 or E1 complexes were expressed and purified according to the detailed protocol of Werner et al. (Werner et al. 2009); the following paragraphs briefly describe the applied procedures.

*Expression and purification of recombinant Aos1*

For recombinant expression of N-terminally His- or His-CFP-tagged Aos1 the respective pET28a expression construct was transformed into *E. coli* strain BL21(DE3) and directly used for inoculation of LB medium containing 30 μg/ml kanamycin. After growth over-night at 37 °C, bacteria were harvested by centrifugation, resuspended in fresh medium and grown at 37 °C until they reached an $OD_{600nm}$ of 0,6. Then, protein expression was induced by addition of a final concentration of 1 mM IPTG and bacteria were grown for another 6 hours at 25 °C.

After harvesting by centrifugation at 4000 rpm in a Beckmann JS-5.2 rotor, bacteria were resuspended in 50 ml E1 lysis buffer (50 mM Na-phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole), flash frozen in liquid nitrogen and stored at – 80 °C.
Once thawed, 1 mM β-mercaptoethanol, 0.1 mM PMSF and 1 μg/mL each of aprotinin, leupeptin and pepstatin were added before cells were lyzed using an emulsion flex. Bacterial debris was removed by centrifugation at 100,000 x g at 4 °C for 1 h in a Beckman Type 45 Ti rotor. Per 1 l bacterial culture 1 ml pure ProBond Nickel-chelating resin (Invitrogen) was transferred into a column and equilibrated in E1 lysis buffer supplemented with 1 mM β-mercaptoethanol and 1 μg/mL AP and LP. The protein supernatant was applied to the column at 4 °C and His-Aos1 was collected on the beads by gravity flow. The column was extensively washed with E1 wash buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol, 1 μg/ml AP and LP) until no more proteins were detected in the flow-through. Bead-bound proteins were eluted with at least 5 column volumes elution buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol, 1 μg/ml AP and LP) and collected in 1.5 ml fractions. Protein-containing fractions were combined and concentrated to approximately 5 ml. After filtration of the concentrate through a 0.2 μm low protein binding filter, the solution was injected onto a HiLoad 26/60 Superdex 200 pg column (GE Healthcare) equilibrated in S200 buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 μg/ml AP and LP). 5 ml fractions were collected, analyzed by SDS-PAGE and fractions containing His-Aos1 were combined and dialyzed against TB. If necessary, the purified protein was concentrated and aliquots were flash frozen in liquid nitrogen and stored at -80 °C. Using to this protocol approximately 5 – 7 mg His-Aos1 or His-CFP-Aos can be purified per liter of *E. coli* culture.

*Expression and purification of recombinant Uba2*

For expression of C-terminally His- or YFP-His-tagged Uba2 the respecitve pET28b expression construct was tranformed into chemically competent BL21(DE3) and streaked on LB agar plates containing 30 μg/ml kanamycin. LB medium supplemented with kanamycin was inoculated with a single colony and grown over-night for approximately 18 hours at 37 °C. Protein induction and bacterial lysis as well as the purification step via ProBond resin and preparative Superdex 200 gel filtration column (GE Healthcare) exactly corresponded to the description of the purification of

Aos1. However, purification of Uba2 requires one additional purification step. Therefore, Uba2 containing fractions after gel filtration chromatography were combined and applied to a MonoQ anion-exchange column (GE Healthcare) equilibrated with S200 buffer. Elution from the MonoQ was performed by a linear gradient of 50 mM to 500 mM NaCl in S200 buffer. Fractions of 0.5 ml were collected and analyzed for Uba2 content by SDS-PAGE and Coomassie staining. Despite a predicted size of 72 kDa, untagged Uba2 migrates at 90 kDa in SDS PAGE and tagged protein correspondingly slower. Combined fractions were concentrated, dialyzed against TB and aliquots were flash-frozen in liquid nitrogen and stored at -80 °C. Purification results in a yield of approximately 1.5 – 3 mg Uba2-His or Uba2-YFP-His per liter *E. coli* culture.

*Expression and purification of recombinant SUMO E1 enzyme*

SUMO-E1 complex can be obtained by two different approaches. The classical protocol is based on co-expression of N-terminally His-tagged Aos1 and untagged Uba2, whereas an alternative approach developed as part of this thesis N-terminally His-tagged Aos1 and C-terminally His-tagged Uba2 are independently purified as single subunits and E1 complex is subsequently reconstituted.

For the classical E1 purification pET28a-His-Aos1 and pET11d-Uba2 expression constructs are co-transformed into BL21(DE3) and immediately used for inocculation of LB medium supplemented with 50 μg/ml ampicillin and 30 μg/ml kanamycin. The subsequent procedure exactly matches the previously described protocol for expression and purification of Uba2. The resulting fractions of the MonoQ purification step are analyzed by SDS-PAGE and Coomassie staining and fractions containing equal amounts of His-Aos1 and Uba2 were combined and processed as described.

In the alternative protocol equimolar amounts of purified His-Aos1 and Uba2-His were incubated in TB on ice for 1 – 2 h in order to form heterodimeric E1 complex. To separate assembled complex from any excess of one subunit or of partially unfolded protein His-Aos1/Uba2-His was subsequently purified by a preparative Superdex 200 gel filtration, flash frozen in liquid nitrogen and stored at -80 °C.

*Expression and purification of recombinant import receptors*

His-Importin α was expressed and purified essentially as described by Hu et al. (Hu et al. 1996). Briefly, BL21 cells were transformed with the expression construct and streaked on LB agar containing ampicillin. Subsequently LB supplemented with 50

μg/ml ampicillin was inocculated with all colonies of the plate of BL21 cells. The bacterial culture was grown at 37 °C until an $OD_{600nm}$ of 0.6 was reached and recombinant protein was expressed in the presence of 1 mM IPTG at 25 °C for 5 – 6 hours. Cells were harvested, resuspended in lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl) and stored at -80 °C. After addition of 5 mM ®-mercaptoethanol, 1 mM PMSF and 1 μg/ml AP, LP bacteria were lyzed using an Emulsion flex and subjected to ultracentrifugation. After addition of 10 mM imidazol the supernatant was transferred onto an equilibrated column of 0.5 ml Ni-beads per liter of bacterial culture, washed with 12 mM imidazol in lysis buffer and eluted with S200 buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1mM DTT) containing 250 mM imidazol. Protein-containing fractions were combined, concentrated and injected onto a Superdex 200 preparative column equilibrated and run with S200 buffer. Fractions containing importin α were combined, concentrated, flash-frozen in liquid nitrogen and stored at -80 °C.

Purification of His-S-Importin β was performed according to Chi et al. (Chi et al. 1997). The expression construct was transformed into BL21(DE3)-pLysS, streaked on a LB agar plate containing chloramphenicol and kanamycin and LB medium supplemented with kanamycin was inocculated with all colonies from the fresh plate. The culture was grown at 37 °C to $OD_{600nm}$ of 0.3 – 0.4, temperature was adjusted to 20 °C and at $OD_{600nm}$ of 0.6 protein expression was induced by addition of 1 mM IPTG. After expression for 4 hours, bacteria were harvested, resuspended in lysis buffer (20 mM Tris, pH 8.0, 2 mM $MgCl_2$, 250 mM NaCl, 10 % (v/v) glycerol) and stored at -80 °C. The cells were lyzed with an Emulsion flex in the presence of 4 mM β–mercaptoethanol, 1 mM PMSF and 1 μg/ml AP, LP and centrifuged for 1 hour with 100,000 g at 4 °C. After addition of 10 mM imidazol the supernatant was transferred to 0.5 ml Ni-beads per liter of bacterial culture equilibrated in lysis buffer containing protease inhibitors, washed with 10 mM imidazol in S200 buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM DTT) and eluted with S200 buffer supplemented with 300 mM imidazol. Protein-containing fractions were combined, concentrated and subjected to a Superdex 200 preparative gel filtration in S200 buffer. Fractions of importin ® were dialyzed against MonoQ buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT) and subsequently purified further by a MonoQ anion-exchange column. Proteins were eluted with a gradient of 50 mM – 500 mM NaCl. After combining and concentrating importin ® fractions the solution was flash frozen and stored at -80 °C.

Purified recombinant His-transportin (Baake et al. 2001), His-importin 5 (Jakel et al. 1998), His-importin 7 (Wohlwend et al. 2007), His-importin 9 (Muhlhausser et al. 2001) His-importin 13 (Jakel et al. 1998; Mingot et al. 2001) and His-CRM1 (Hutten et al. 2006) were kindly provided by Dr. habil. Ralph H. Kehlenbach, University of Göttingen, purified as described.

*2.5. Interaction experiments*

*Pull-down experiments*

For pulldown experiments with import receptors recombinant His-CFP-Aos1 or Uba2-YFP-His were immobilized on cyanogenbromide-activated sepharose (Sigma Aldrich) according to the manufacturer's instructions, with final concentrations of 0.014 nmol Aos1 or 0.01 nmol Uba2 per 1 μl pure beads. 15 μl beads were incubated for 1.5 h at 4 °C in 500 μl pull-down buffer (50 mM Tris pH 7.4, 200 mM NaCl, 1 mM $MgCl_2$, 5 % (v/v) glycerol) with 2 mg/ml BSA or ovalbumin and 0.05 nmol recombinant import receptor. Where indicated, reactions contained 1 μM RanQ69L loaded with GTP as described by Kehlenbach et al. (Kehlenbach et al. 1999). After three times washing with pulldown buffer, bound proteins were eluted with SDS-sample buffer and subjected to SDS-PAGE followed by silver staining.

Pulldown experiments with the export receptor CRM1 were carried out in 100 μl pulldown buffer containing 20 μl His-CRM1 immobilized on CNBr-activated sepharose beads with 0.5 μg protein/ml beads and 2.5 μg His-Aos1, 2.5 μg His-Uba2 or 3 μg His-Aos1/Uba2 cargo in the absence or presence of GTP-loaded RanQ69L (Kehlenbach et al. 1999). Reactions with empty beads served as control for the detection of unspecific background binding of the tested cargo proteins to the beads.

*Analytical gel filtration*

Different combinations of 1 μM purified recombinant His-CFP-Aos/Uba2-YFP-His complex (wt/wt, wt/mut, mut/wt, mut/mut) were incubated with 5 μM of each recombinant importin α and importin β in a total volume of 100 μl transport buffer with 1 mM DTT rotating for 1 hour at 4 °C. Analytical gel filtration was performed with a Superose6-HR10/30 column in an Äkta purifier system (GE Healthcare). After equilibration of the column with 2 column volumes of transport buffer with 1 mM DTT the sample was injected onto the column. The analytical filtration was performed with a flow of 0.2 ml/min and fractions of 0.3 ml were collected. For evaluating the protein

composition 50 µl of every second fraction was loaded on a 5 % - 20 % gradient SDS gel and visualized by Coomassie-staining. Calculation of the corresponding elution profile from the Äkta (GE Healthcare) was done with sigma plot 8.02 (Systat Software Inc.).

*RanGAP1 assay*

Labeling of recombinant, purified Ran with [$\gamma^{32}$P]GTP and RanGAP1 assays were essentially performed as described (Askjaer et al. 1999; Kehlenbach et al. 2001). The final reactions contained 1 µM CRM1, 30 nM [$\gamma^{32}$P]RanGTP , 60 nM RanGAP1, 200 nM GTP and 3 or 5 µM Aos1, Uba2 or E1 complex in a total volume of 50 µl TB. Reactions with 1 or 3 µM nuclear export sequence (NES; NS2 protein of minute virus of mice, CVDEMTKKFGTLTIHDTEK; Askjaer et al. 1999) served as positive controls. All components except for [$\gamma^{32}$P]RanGTP and RanGAP were mixed, after addition of [$\gamma^{32}$P]RanGTP and incubation for 10 minutes at room temperature RanGAP1 was added. 10 minutes after incubation at room temperature, the reaction was stopped with stop solution (7% charcoal, 10% ethanol, 0.1 M HCl, 10 mM $NaH_2PO_4$), the charcoal was collected by centrifugation for 4 minutes at 14,000 x g and the released [$^{32}$P]phosphate in the supernatant was measured by scintillation counting. Background counts from a reaction without RanGAP were subtracted and GTP hydrolysis was expressed as the percentage of the value of radioactivity measured in a reaction with RanGAP and without CRM1. A reaction with 1 or 3 µM nuclear export signal (NES) served as positive control.

*2.6. Cell fractionation*

*Cell fractionation by differential centrifugation*

A 3 l culture of cycling HeLa suspension cells was harvested at a density of 5 – 8 x $10^5$ cells/ml by centrifugation with 70 x g for 10 minutes at room temperature. The cells were washed two times with cold HBS (10 mM HEPES pH 7.4, 145 mM NaCl), the resulting pellet was resuspended in 3.5 volumes of ice cold hypotonic lysis buffer (15 mM KCl, 1.5 mM MgOAc, 1 mM DTT, 10 mM HEPES-KOH pH 7.5, 1 mM PMSF, 1 µg/ml of each aprotinin, leupeptin, pepstatin) and then subjected to swelling on ice for 10 minutes. Subsequently the cells were lyzed with 10 strokes in a tight-fitting glass dounce homogenizer. Effective lysis was confirmed by microscopic analysis of 5 µl cell suspension gently mixed with 5 µl trypan blue revealing a blue staining for

| *k*-factor | $g_{average}$ | rpm | time [min] | $S_{20,w}$ | organelle | fraction |
|---|---|---|---|---|---|---|
| 42,639.3 | 376 | 2,300 | 5 | $5 \times 10^5$ | $>10^7$ whole cells<br>$>10^5$ intact nuclei | 1 |
| 12,199.1 | 1,313 | 4,300 | 10 | $7 \times 10^4$ | nuclear debris | 2 |
| 4,119.1 | 3,888 | 7,400 | 20 | $1 \times 10^4$ | $>10^4$ mitochondria, lysosomes | 3 |
| 1,219.5 | 13,134 | 13,600 | 37 | $2 \times 10^3$ | $>2 \times 10^3$ lysosomes, peroxisomes | 4 |
| 122.0 | 131,294 | 43,000 | 15 | $5 \times 10^2$ | $>5 \times 10^2$ endoplasmic reticulum | 5 |
| 97.9 | 163,602 | 48,000 | 55 | $1 \times 10^2$ | $>10^2$ polysomes | 6 |
| | | SN | | | cytosol | SN |

**Table 1: Centrifugation steps for fractionation of HeLa cells by differential centrifugation.** Based on the S20,w values (Svedberg constants at 20°C in water) described by Sheeler Sheeler 1981 and chosen appropriate centrifugation times, the k-factors for the sedimentation of the according organelles were calculated according to *k*-factor = $S_{20,w} \cdot$ time [h]. Subsequently the corresponding speed for a Beckman Type 60 Ti rotor was calculated using Beckman's online rotor calculations tool.

most nuclei. The resulting homogenate was mixed with 1/5 volume of ice cold hypertonic buffer (375 mM KCl, 22.5 mM MgOAc, 1 mM DTT, 220 mM HEPES-KOH pH 7.5, 1 mM PMSF, 1 μg/ml of each aprotinin, leupeptin, pepstatin) and centrifuged at 4 °C with 2,300 rpm in a Beckman Type 60 Ti rotor for 5 minutes. The resulting supernatant was subjected to the next centrifugation step as listed in table 1 and this procedure was repeated until the last centrifugation step of 50,000 rpm for 1 h. Calculations were carried out based on $S_{20,w}$ values (Svedberg-constants at 20 °C in water) for different organelles as described by Sheeler (Sheeler 1981). Using the equation *k*-factor = $S_{20,w} \cdot$ time [h] and a chosen centrifugation time, k-factors for specific sedimentation of a certain organelle were calculated. The required centrifugation speed, expressed in rpm and $g_{average}$ were computed for the used rotor from the resulting *k*-factors by using Beckman's rotor calculations online tool (www.beckmancoulter.com/resourcecenter/labresources/centrifuges/rotorcalc.asp).
Resulting pellets from the different steps were resuspended in an appropriate volume of tranport buffer supplemented with 1 mg/ml of each aprotinin, leupeptin and pepstatin. Protein concentration of all samples, including the homogenate and the final supernatant, was determined and then adjusted to a final protein concentration

of 1 mg/ml with 2 x SDS sample buffer. 40 µl of the resulting samples were loaded on a 5 % - 20 % gradient SDS gel and subsequently analyzed by immunoblotting.

*Fractionation of murine hippocampal neurons*

Samples of subcellular fractions of hippocampal neurons from mouse were kindly provided by Prof. Dr. Nils Brose, Max-Planck Institute for Experimental Medicine, Göttingen. The fractionations were prepared essentially as described previously (Jones et al. 1974) and designated as follows: H, homogenate; P1, nuclear pellet; S1, supernatant after nuclear sedimentation; P2, crude synaptosomal pellet; P3, light membrane pellet; S3, cytosolic fraction; LP1, heavy membrane pellet; LS1, intermediate fraction; SMP, synaptic plasma membranes; LP2, crude synaptic vesicle fraction; LS2, cytosolic synaptosomal fraction. 20 µg of total protein per fraction were analyzed by SDS PAGE followed by immunoblotting.

*2.7. In vitro SUMOylation reaction*

*Detection via SDS-PAGE*

For analysis of E1 activity in *in vitro* SUMOylation reactions by SDS-PAGE, 79 nM substrate (RanGAP1) was incubated with 225 nM SUMO-1ΔC4, 27 nM Ubc9 and 1.6 nM E1 in a total volume of 20 µl SAB buffer in the presence or absence of 1 mM ATP at 30 °C for different periods of time. Reactions were stopped by addition of an equal volume 2 x SDS sample buffer. 6 µl of each sample was separated on a 5 – 20 % gradient SDS gel and the substrate was subsequently detected by immunoblotting.

*Detection via FRET*

Fluorescence resonance energy transfer (FRET) based SUMOylation assays allows to measure kinetic, quantitative and high-throughput SUMOylation reactions (Bossis et al. 2005; Stankovic-Valentin et al. 2009). Briefly, the assay is set up as a standard *in vitro* SUMOylation reaction using a CFP-tagged substrate and YFP-tagged SUMO. If the CFP-substrate is modified with YFP-SUMO, the proximity of the fluorescent molecules is often close enough (less than 10 nm) to enable efficient energy transfer from the excited CFP to YFP and thereby lowering the detected emission of CFP and enhancing the detected YFP emission. Therefore, the ratio between YFP emission (527nm) / CFP emission (485nm) increases.

For the purpose of comparing kinetics of different concentrations of E1 enzymes, 100 nM CFP-RanGAPtail, 100 nM YFP-SUMO and 44 nM Ubc9 were incubated with 22 nM, 1.4 nM or 86 pM E1 in SAB buffer in a final reaction volume of 25 µl in a 384-well fluorescence microtiter plate. The reaction was preincubated in a microplate fluorometer (Fluoroskan Ascent from Thermo Scientific) to reach the desired reaction temperature of 30 °C and subsequently started by automatical addition of 5 µl of 5 mM ATP. Simultaneous analysis of the fluoresecent signals was performed with a 430 nm excitation filter and 485 nm and 527 nm emission filters.

*2.8. Affinity purification of polyclonal antibodies*

The goat sera of polyclonal α-Aos1 and α-Uba2 antibodies, raised against His-tagged human full-length proteins, were available in the lab. Prior to use these antibodies had to be affinity purified. After depletion of the serum from antibodies against the His-tag by pre-adsorption against an unrelated immobilized His-tagged protein, antibodies were affinity purified from the serum using affinity columns containing the corresponding antigens. Briefly, the recombinant protein was extensively dialyzed against carbonate buffer (0.2 M $NaHCO_3$ pH 8.9) overnight with at least two buffer changes. CNBr beads (Sigma) were prepared according to the manufacturer's instructions and incubated for 4 h at room temperature with the respective amount of protein to reach a final concentration of approximately 1 mg protein/ml beads. To determine the efficiency of coupling, the OD at 280 nm was checked before and after coupling. The beads were washed two times with carbonate buffer and incubated with 100 mM ethanolamine for 1 h at room temperature to block all remaining coupling sites. After three washing steps with 500 mM NaCl in PBS the beads were used for His-depletion and affinity purification of antibodies from the serum. For this, 1 – 2 ml of the His-tag affinity matrix (1 mg protein per 1 ml pure beads) was incubated with 25 ml serum and 25 ml PBS in a falcon tube rotating at 4 °C for 3 h and subsequently the resulting supernatant was incubated with 2.5 ml of the antigen affinity matrix (1 mg protein per 1 ml pure beads) rotating at 4 °C over night. The beads were transferred into a column and washed two times with at least 50 ml PBS supplemented with 0.5 M NaCl. Washing was continued until no more protein was detected in the flow through by spotting on nitrocellulose followed by ponceau staining. When the column was completely emptied by gravity flow, the elution buffer (0.2 M acetic acid pH 2.7, 500 mM NaCl)

was carefully applied onto the column and antibodies were eluted with approximately 10 column volumes. Fractions of 500 µl were collected and the pH was immediately neutralized by addition of 100 µl of 1 M Tris base. The antibody content of the fractions was determined by $OD_{280nm}$ and positive fractions were combined and concentrated to approximately 1 mg/ml. The buffer was changed to PBS, antibodies were mixed with 1 volume of 87 % (v/v) glycerol and stored in aliquots at -20 °C.
Before usage, purified antibodies were generally tested for detection of the recombinant antigen, the endogenous antigen in HeLa cell lysate and cross reactivity with other human proteins in an immunoblot of HeLa lysate. Finally the antibodies were titrated and tested in immunoblotting and immunofluorescence to determine appropriate dilutions for usage. Purification using the described protocol yielded in approximately 1.2 mg α-Aos1 or 0.7 mg α-Uba2 per 20 ml of serum from final blood.

## 3. Cell biology methods for mammalian cells

### *3.1. Cultivation of adherent and suspension cells*

Adherent HeLa cells were cultured in Dulbecco's modified Eagle Medium (DMEM, Gibco) supplemented with 10 % (v/v) FBS, maintained in a humidified incubator with 5 % (v/v) $CO_2$ at 37 °C. In general, cells were split at a 1/10 ratio just before reaching confluency. For this purpose the cells were washed with sterile PBS, detached from the culture dishes with trypsin/EDTA and diluted with fresh medium to the designated confluency.
HeLa suspension cells were propagated in Jokliks medium,10 mM HEPES, pH 7.1 and 24 mM $NaHCO_3$, supplemented with 5 % (v/v) NCS, 5 % (v/v) FBS and, if the medium was older than two weeks, 2 mM glutamine. The cells were cultured in spinner flasks at 100 rpm at 37 °C at 3 – 10 x $10^5$ cells/ml. The cell density was adjusted daily after determining the density using a Neubauer counting chamber.
For long term storage, exponentially growing cells were trypsinized and diluted into serum-containing medium to inactivate the protease. The cells were collected by centrifugation for 5 minutes at room temperature with 70 x g, resuspended in FBS or NCS in case of suspension cells and were then supplemented dropwise with 7 – 10 % (v/v) DMSO under gentle agitation. Aliquots were slowly frozen at -80 °C in box insulated with 2-propanol and for long-term storage transferred to liquid nitrogen tanks.

*3.2. Transient transfection*

Adherent HeLa cells were seeded in 24-well plates 24 hours prior to transfection. Transient transfection was usually performed with 0.75 μg DNA of the designated expression construct or of the corresponding empty vector using Polyfect transfection reagent (Qiagen) according to the manufacturer's instructions. Analysis of expression was carried out with fluorescence microscopy after fixation and processing as described for general detection of fluorescent proteins.

*3.3. Fluorescence based detection of intracellular proteins*

*Overexpression and detection of fluorescent proteins*

HeLa cells were seeded in 24-well plates on autoclaved 12 mm glass coverslips 24 hours prior to transfection. Transient chemical transfection was performed with 0.75 μg DNA of each expression construct using Polyfect transfection reagent (Qiagen) according to the manufacturer's instructions. 24 hours after transfection cells were washed three times with PBS to remove the medium. Cells were generally fixed with 3.7 % (v/v) formaldehyde in phosphate buffered saline (PBS) for 15 minutes, subjected to two washing steps in PBS and were then mounted onto glass slides with fluorescent mounting medium (Dako). If not stated differently, fluorescence was analyzed by confocal microscopy using a confocal LSM 510meta microscope (Zeiss) with a LCI Plan-Neofluar 63x/1.3 Imm Korr DIC objective and appropriate filter settings.

*Indirect immunofluorescence on cells*

HeLa cells grown on 12 mm glass coverslips were fixed for 15 minutes in 3.7 % (v/v) paraformaldehyde in PBS, washed with PBS and permeabilized for 5 minutes with icecold 0.2 % (v/v) Triton X-100 in PBS. After washing, cells were blocked with 2 % (w/w) BSA in PBS for 30 minutes. The coverslips were incubated for one hour at room temperature in 1:1,000 diluted affinity-purified anti-Aos1- or anti-Uba2 antibody from goat (approximately 0.5 μg/ml final concentration) in 2 % (w/v) BSA/PBS, washed with PBS and then incubated in a 1:1,000 dilution of Alexa488-coupled donkey anti-goat-antibody (2 μg/ml final concentration) (Molecular Probes). After extensive washing with PBS the cells were mounted and analyzed as described for general detection of fluorescent proteins.

*Immunohistochemistry*

Staining of endogenous Uba2 in paraformaldehyde-fixed and paraffin-embedded slides of normal murine brain (Abcam). The manufacturer cut the tissue section to a thickness of 5 μm, mounted them on positively charged glass slides and stained one slide of each lot by haemotoxylin and eosin to ensure quality.

Slides were deparaffinized by incubating them twice for 10 minutes in xylene (Sigma), followed by hydration in 100 %, 95 %, 85 % and 75 % (v/v) ethanol in distilled water for 5 minutes each. After transfer to pure distilled water for 5 minutes, slides were incubated in TBS (50 mM Tris pH 7.6, 100 mM NaCl) three times for 5 minutes. For antigen retrieval, slides were boiled in 10 mM Na-citrate pH 6.0 supplemented with 0.05 % (v/v) Tween20 for 30 minutes in a microwave, cooled down to room temperature and transferred first into distilled water and then into TBS, three times for 5 minutes each. After 30 minutes blocking with 3 % (w/v) BSA in TBS at room temperature, the tissue section on the slide was covered with a 1:20 dilution of affinity purified goat anti-Uba2 antibody (25 μg/ml final concentration) in 1 % (w/v) BSA/TBS and incubated for 1 hour at room temperature. The slides were washed twice for 5 minutes with TBS and then stained for 1 hour at room temperature in the dark with 1 % (w/v) BSA/TBS containing 1:100 diluted Alexa488-coupled donkey anti-goat (Molecular Probes) and 0.25 μg/ml Hoechst33342. After extensive washing 3 times for 5 minutes with TBS, the tissue slides were mounted onto glass slides with 20 μl SLOW-FADE Gold mounting medium (Invitrogen) and fluorescent signals were detected with a CellObserver (Zeiss) using Colibri LED, Plan-Apochromat 20x/0,8 objective and appropriate filters.

*3.4. In vitro import assay*

Import reactions were performed based on the method established by Adam et al. (Adam et al. 1990). HeLa cells grown on sterile 12 mm glass coverslips to 40 – 70 % confluency were washed twice for 5 min on ice with transport buffer (20 mM Hepes pH 7,3, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM EDTA) supplemented with 1 mg/ml AP, LP and 2 mM DTT. The plasmamembrane was permeabilized with 0.01 % (w/v) digitonin in transport buffer on ice for 4 minutes. Permeabilized cells were washed three times for 5 minutes and were then incubated for 45 minutes at 37 °C with 30 µl of a transport reaction mix containing fluorescently labeled cargo protein (5 μM of

single E1 subunits or 1 μM of E1 complexes), 15 μl of cytosolic HeLa extract in transport buffer and either 1.5 μl of an energy-regenerating system (final concentrations 1 mM ATP, 5 mM creatinphosphate and 100 U/ml creatine phosphate kinase) or as a specificity control with 2 μl energy-depleting system (final concentration 16 U hexokinase and 5 mM glucose in TB). Import assays with recombinant import receptors were performed with reaction mixes containing 1 μM cargo protein (single E1 subunits or formed E1 complex), 1.5 μM importin α and/or 1 μM importin β and either 12 μM Ran-GTP or RanQ69L-GTP, loaded with GTP as previously described (Kehlenbach et al. 1999). After the import reactions, cells were washed three times for 5 minutes with TB and were subsequently fixed and processed as described for general detection of fluorescent proteins.

*Preparation of cytosolic HeLa extract*

Cycling HeLa suspension cells were harvested at a density of 5 – 8 x $10^5$ cells/ml by centrifugation at 250 x g for 10 minutes at 4 °C in a Beckman JS-5.2 rotor. After washing two times with ice cold PBS and once with ice cold washing buffer (10 mM HEPES pH 7.3, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT), the cell pellet was resuspended in an equal volume of hypotonic lysis buffer (5 mM HEPES pH 7.3, 10 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT,1 mM PMSF, 1 μg/ml of each leupeptin, pepstatin, aprotinin) and then subjected to swelling on ice for 10 minutes. The cells were lyzed by 5 strokes in a tight-fitting stainless steel dounce homogenizer and the resulting homogenate was centrifuged at 1,500 x g for 15 minutes at 4 °C to remove cell debris and nuclei. The supernatant was first centrifuged at 15,000 x g for 20 minutes in a Beckman JA-20 rotor and subsequently at 100,000 x g for 1 hour in a Beckman 70.1 Ti rotor. The final supernatant was dialyzed for 3 hours against 3 changes of transport buffer, concentrated to 10 mg/ml and aliquots were flash frozen in liquid nitrogen prior to storage at -80 °C.

*3.5. Microinjection into adherent HeLa cells*

HeLa cells were grown on autoclaved 12 mm coverslides and medium was changed from DMEM to $CO_2$-independent medium (Gibco) supplemented with 4 mM L-glutamine 1 hour prior to injection. Microinjection mixes were prepared as follows: microinjection of pre-assembled E1 complexes was performed with mixtures of different combinations (wt/wt, wt/mut, mut/wt or mut/mut) of at least 1.4 (- 2.4) μM

purified recombinant His-CFP-Aos1/Uba2-YFP-His complex and 1.5 μM Dextran-coupled TRITC, whereas injection mixtures of single subunits contained 4.5 μM TRITC-Dextran and either 17 μM His-CFP-Aos1 or 8 μM Uba2-YFP-His. Inhibition experiments with monoclonal anti-Importin ® antibody clone 3E9 (Abcam) were carried out with 2 : 1 : 1 mixtures of antibody, TRITC-Dextran (final 1.6 μM) and either His-CFP-Aos1 (final 5.6 μM), Uba2-YFP-His (final 3.3 μM), YFP-M9 (final 8.3 μM) or pre-assembled His-CFP-Aos1/Uba2-YFP-His (final 3 μM). After microinjecting cells using an eppendorf femtojet at room temperatur for approximately 20 – 30 minutes, cells were washed with PBS and incubated for 30 minutes at 37 °C and 5 % $CO_2$ atmosphere in DMEM (Gibco) supplemented with 10 % (v/v) fetal bovine serum. After extensive washing with PBS the cells were fixed and processed as described for general detection of fluorescent proteins.

*3.6. Analysis of nucleocytoplasmic shuttling*

*Interspecies heterokaryon assay*

Heterokaryon assays were performed essentially as described (Roth et al. 1998). Briefly, HeLa cells, seeded in 24-well plates, were transfected with the fluorescent expression construct pcDNA3.1(-)-CFP-Aos1 48 hours prior to the experiment. 24 hours before the assay, HeLa cells were mixed in a 2:3 ration with mouse 3T3 cells and seeded in one well of a 6-well plate containing 4 – 5 glass coverslips. The cells were subjected to medium supplemented with 100 μg/ml cycloheximide in order to inhibit protein biosynthesis and were incubated for 3 hours. The cells were then washed two times with PBS and fused in 50 % (v/v) PEG2000 in PBS for 3 minutes at room temperature. PEG was washed away by three wash steps with PBS and subsequently the cells were incubated in cycloheximide-containing medium. Samples were taken at different time points and processed and analyzed as described for detection of fluorescent proteins. As de novo protein biosynthesis was inhibited by the addition of cycloheximide any fluorescent signal in the blue spotted mouse nuclei would indicate shuttling of CFP-Aos1.

*Combined FRAP and FLIP in multinuclear cells*

Nucleocytoplasmic shuttling was analyzed performing combined FRAP (fluorescence recovery after photobleaching) and FLIP (fluorescence loss in photobleaching) experiments in multinuclear cells with modifications based on the described

techniques (Koster et al. 2005; Belaya et al. 2006). HeLa cells were grown in 4-well Lab-Tek™II coverglass chambers and transfected with the fluorescent expression construct pcDNA3.1(-)-CFP-Aos1 24 hours prior to the experiment. The cells were washed with PBS and fused to homopolykaryons in 50 % (v/v) PEG2000 in PBS for 3 minutes at room temperature. PEG was washed away by three wash steps with PBS and subsequently the cells were incubated in medium at 37 °C in the incubation chamber of the microscope. The experiments were performed using a confocal LSM 510meta microscope (Zeiss) with a LCI Plan-Neofluar 63x/1.3 Imm Korr DIC objective and appropriate filter settings. Due to a high mobility of nuclear CFP-Aos1, measured in preparatory experiments, the fluorescence in one nucleus was completely bleached by scanning an approximately 15 μm x 10 μm ROI (region of interest) within this nucleus 300 times with 100 % intensity of the 458 nm argon laser and a speed of 6.39 μm/sec. Pictures were taken before and at different time points after bleaching the nucleus as generally described in *Detection of fluorescent proteins*. To circumvent bleaching of the fluorescence signal by repeated image acquisition, the laser intensity was adjusted to 10 % and exposure time for detection was minimized resulting in low-resolution images. For data interpretation fluorescence intensities of different ROIs were calculated in percentage of the intensity of ROI 1, located within the bleached nucleus, before bleaching. Changes of the fluorescence intensity in ROI 1 within the bleached nucleus was compared to the intensity in ROI 2, 3 and 4 within unbleached nuclei in the same cell and the cytoplasmic background signal measured in ROI 5. Furthermore, the slopes of the signals after bleaching were calculated for ROI 1, average of the ROIs 2-4 and the cytoplasmic background to compare the speed of signal accumulation. A steeper slope of ROI 1 compared to ROIs 2-4 would indicate shuttling of the transporter protein CFP-Aos1.

## 4. Yeast methods

### *4.1. Culture and storage of yeast strains*

Yeast cells from glycerol stocks were first streaked on plates with a sterile glass pipette. For growth of cells in liquid culture, a preculture was inoculated with cells from plates and incubated over nigth. The main culture was inoculated with the preculture considering the growth rate of the strain and the growth temperature, with

an optimal growth temperature of wild type strains of 30 °C. Wild type strains were grown in YPD or SC-complete medium, whereas plasmid containing strains were selected for growth in SC medium lacking the amino acid or base whose synthesis was encoded by the selective marker on the plasmid. If yeast strains were manipulated by chromosomal integration of genes, the transformants were selected by growth on the respective selective medium.

Stocks for long term storage of yeast strains were obtained by transferring fresh cell material from plates into a sterile cryo-tube containing 1.5 ml 15 % (v/v) glycerol and subsequent dispersion of the cells by vortexing. To avoid the sedimentation of cells, the mixture was flash-frozen in liquid nitrogen prior to storage at -80 °C.

*4.2. Preparation and transformation of competent yeast cells*

The transformation of yeast cells was performed according to a protocol adapted from Schiebel and Rietz (Schiestl et al. 1989).

A 10 ml preculture, which was grown over night at 30 °C, was diluted to $OD_{600nm}$ of 0.1 in 50 ml and grown to an $OD_{600nm}$ of 0.6 - 0.8. The cells were harvested by centrifugation at 3,200 rpm for 2 minutes at room temperature followed by one wash with 25 ml sterile $ddH_2O$ and one wash in 12.5 ml LiSorb (10 mM Tris-HCl, pH 8.0, 1 M sorbitol, 100 mM LiOAc, 1 mM EDTA). Once all residual LiSorb was removed by an additional centrifugation step, cells were resuspended in 300 µl LiSorb and 50 µl carrier DNA (Salmon Sperm, Invitrogen) that had been denatured at 95 °C for 5 minutes and cooled on ice. Competent yeast cells were used immediately or stored at -80 °C in 110 µl aliquots.

For each transformation 50 µl of competent cells, thawed at room temperature, were mixed with DNA and incubated at room temperature. After 15 minutes 300 µl LiPEG (10 mM Tris-HCl, pH 8.0, 40 % (v/v) PEG3350, 100 mM LiOAc, 1 mM EDTA) were added, the mixture was briefly vortexed and incubated for 15 minutes at room temperature. 30 µl DMSO was added and mixed immediately by vortexing. The cells were subjected to heat-shock by incubation in a water bath at 42 °C for 10 minutes. Finally, the cells were centrifuged at 3,200 rpm for 3 minutes, resuspended in 200 µl PBS, plated onto selective plates and incubated at the appropriate temperature.

No recovery was necessary when auxotrophic markers were used. However, for chromosomal integration of a transformed deletion cassette and subsequent

selection with nourseothricin cells were centrifuged after heat shock, resuspended in 1 ml YPD and incubated with shaking for 6 hours before plating.

For transformation of PCR cassettes 5 µl of a standard cassette PCR reaction (Janke et al. 2004) was used with 50 µl of competent cells, whereas for transformation of plasmids 1 µg DNA was used.

*4.3. Processing of positive yeast transformants*

Positive transformants were picked with a sterile pipette tip and re-streaked on selective plates to isolate single colonies. Single colonies were then streaked as a patch onto a fresh plate and, in case of antibiotic selection, single colonies were again streaked on fresh selection plates to identify positive ones. For storage of newly generated yeast strains, glycerol stocks of two independent clones were prepared.

*4.4. Generation of yeast UBA2 shuffle strains*

For phenotypic analysis of the essential UBA2 gene (Dohmen et al. 1995), an *UBA2* shuffle strain was constructed. In a shuffle strain the wild type copy of a gene is deleted and replaced with a marker, while cells are kept alive by another copy of this gene on an *URA3* plasmid.

First, the original strain ESM356-1 was transformed with the URA3 plasmid containing the *UBA2* gene including 500 bp up- and downstream of the coding region (pRS316-*UBA2*), resulting in the yeast strain ESM356-1*/pRS316-*UBA2*. Subsequently, the genomic copy of the *UBA2* gene was deleted and replaced by a cassette containing a resistance marker for the antibiotic nourseothricin (for details see Cassette PCR). Disruption of the genomic copy of the gene in the constructed shuffle strain ESM356-1*/pRS316-*UBA2*/uba2Δ::nat was confirmed by colony PCR (for details see Yeast colony PCR). Subsequently, constructs of the HIS3 plasmid p413 containing ORFs for differents Uba2 variants were transformed into the shuffle strain ESM356-1*/pRS316-*UBA2*/uba2Δ::nat. Single colonies of these transformants were characterized.

*Cassette PCR*

For deletion of the *UBA2* gene in yeast the cassette module natNT2 was amplified with S1-/S2-primers, designed to anneal 500 bp up- and downstream of *UBA2*, using

pKS134:pFA6y-natNT2 as template as described by Janke and Knop (Janke et al. 2004). A standard 50 µl reaction contained 5 µl 10 x buffer 1 (500 mM Tris-HCl, pH 9.2, 160mM $(NH_4)_2SO_4$, 7.5 mM $MgCl_2$, 2 mM each dNTP), 10 µM of each primer, 100 ng cassette plasmid DNA and 2 U Taq polymerase mixed with 0.4 U Vent polymerase. Initial denaturation (97 °C - 5 minutes) was followed by 10 cycles of denaturation (97 °C – 30 seconds), annealing (52 °C – 30 seconds) and extension (68 °C – 1 min/kb) and 20 cycles of denaturation (97 °C – 30 seconds), annealing (52 °C – 30 seconds) and extension (68 °C – 1 min/kb + 20 ses/cycle). Products were purified after analysis on an agarose gel.

*Yeast colony PCR*

Colony PCR was used to directly confirm the disruption of the *UBA2* gene in yeast. The 5'-primer was designed to anneal 112 bp upstream and the 3'-primer annealed 109 bp downstream of the gene. A wildtype yeast strain was included as negative control. A small amount of yeast cells was resuspended in 50 µl 0.01 % N-Lauroyl sarcosine, 0.02 N NaOH and boiled for 10 minutes at 95 °C. 1 µl of the resulting extracts was used for a PCR reaction. The 25 ∝l reaction volume contained 2.5 µl Taq polymerase buffer, 2.5 µl dNTP-Mix (final 2 mM each), 1.5 µl of each primer (final 10 µM) and 1 µl Taq polymerase (1 U/µl). Initial denaturation (97 °C - 5 minutes) was followed by 35 cycles of denaturation (97 °C – 30 seconds), annealing (52 °C – 30 seconds) and extension (68 °C – 1 min/kb) and a final extension step (68 °C - 3 minutes). Products were analyzed on agarose gels.

*4.5. Phenotypic analysis of UBA2 shuffle strains*

For the phenotypic analysis of exogenous encoded Uba2 variants the transformed shuffle strains were subjected to selection on plates containing 5-FOA leading to the loss of the URA3 plasmid pRS316-*UBA2* containing the wild type copy of the *UBA2* gene and thereby selecting for cells with the protein encoded by the transformed overexpression construct. The *URA3* gene encodes orotidine-5'phosphate decarboxylase, an enzyme required for the biosynthesis of uracil. *URA3* cells convert 5-FOA into toxic 5-fluorouracil and thus *URA3*-positive cells die, whereas ura3-negative cells are resistant to the drug.

*Microscopic analysis of UBA2 shuffle strains*

Intracellular localization of C-terminally GFP-tagged Uba2 variants was determined by fluorescence microscopy. For this, precultures inoculated with colonies from 5-FOA plates and grown over night at 30 °C in filter-sterilized SC-complete medium were diluted to $OD_{600nm}$ of 0.05 and grown to an $OD_{600nm}$ of 0.3 - 0.5. 2.5 ml of the cultures were harvested by centrifugation at 3,200 rpm for 3 minutes at room temperature and the pellet was resuspended in 1 ml of 3.7 % (v/v) PFA in PBS. After incubation for 10 minutes at room temperature the cells were spun down, 500 µl PBS were added and the samples were kept on ice. DNA was stained with 1 ml of 1 µg/ml Hoechst33342 in PBS for 1 hour at 4 °C in the dark. The cells were washed two times for 5 minutes with PBS and the resulting pellet was resuspended in 20 µl antifade mounting medium (DakoCytomation). 3.5 µl of the suspension were applied onto a glass slide and covered with a coverslip. Fluorescent signals were detected with a Cell Observer (Zeiss) using Colibri LED with appropriate filters and a Plan-NeoFluar 100x/1.30 oil objective.

*Growth test of UBA2 shuffle strains*

Logarithmically growing cells were harvested and resuspended in PBS at a concentration of 1 $OD_{600nm}$ per ml corresponding to approximately 2 x $10^7$ cells/ml. Serial 1:5 dilutions in PBS were prepared and 2 – 3 µl of each dilution was spotted onto plates and incubated at appropriate temperatures.

For analysis of rescue of *UBA2* deletion by different Uba2 variants the same dilutions of cells were tested for growth on SC-His plates and 5-FOA plates in parallel. SC-His plates only selected for the presence of the p413 expression construct, thus allowing to compare the dilutions, whereas 5-FOA plates selected for cells without the *UBA2* gene encoded in the pRS316 construct and thereby allowed to compare rescue efficiency of the overexpressed Uba2 variant.

*Fractionation of UBA2 shuffle strains*

A 500 ml culture of logarithmically growing cells with $OD_{600nm}$ of 0.5 – 0.6 was harvested by centrifugation at 3,200 rpm for 3 minutes and resuspended in spheroblasting buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 M sorbitol, 1 mM DTT). After 15 minutes incubation at room temperature the cells were harvested, resuspended in 15 ml spheroblasting buffer supplemented with 0.75 mg/ml zymolyase 20T and incubated for 2 hours at 30 °C. Spheroblasts were spun down

with 3,200 rpm for 5 minutes and all following steps were performed on ice or at 4 °C. The suspension was washed 3 times with spheroblast buffer and the resulting pellet was resuspended in 5 ml Ficoll buffer (18 % Ficoll-400, 10 mM Tris-HCl, pH 7.5, 20 mM KCl, 5 mM $MgCl_2$, 3 mM DTT, 1 mM EDTA, 0.1 mM PMSF, 1 μg/ml AP and LP, 25 mM N-Ethylmaleimide (NEM), 25 mM iodoacetamide, 50 mM NaF). Cells were homogenized with approximately 50 strokes in a tight-fitting glass dounce homogenizer. After confirmation of efficient lysis with a light microscope the homogenate was centrifuged with 4,500 rpm for 5 minutes in a 5417 R refrigerated microcentrifuge (Eppendorf) to remove non-lyzed cells and cell debris. The supernatant was transferred into fresh tubes and subjected to repeated centrifugation in fresh tubes unitl no further pellet formation was detected. The final centrifugation step with 14,000 rpm for 20 minutes separated the nuclear fraction in the pellet from the cytosolic fraction in the supernatant. The nuclear fraction was resuspended in 2 x SDS sample buffer and proteins in the cytosolic fraction were enriched by TCA precipitation followed by resuspension in 4 x SDS sample buffer. All samples were stored at -80 °C. For analysis of the fractions the protein concentration was determined in the SDS samples using reducing agent-compatible BCA Protein Assay (Pierce). Equivalent amounts of protein from the samples were analyzed by SDS PAGE and subsequent immunoblotting.

# RESULTS

## 1. Generation and characterization of SUMO E1 variants

A number of protocols in the literature describe the generation and purification of recombinant SUMO E1 enzyme (Johnson et al. 1997; Desterro et al. 1999; Gong et al. 1999; Okuma et al. 1999; Long et al. 2000; Pichler et al. 2002; Werner et al. 2009). Many of these protocols, including the standart protocol from our laboratory, are based on co-expression and subsequent co-purification of the two subunits Aos1 and Uba (Okuma et al. 1999; Long et al. 2000; Pichler et al. 2002). Since a number of experiments whithin this thesis required the reconstitution of E1 complexes containing different combinations of Aos1- and Uba2-variants with or without fluorescent tag I established a system in which single subunits are purified and active E1 complex is subsequently reconstituted, allowing to flexibly combine different variants of Aos1 and Uba2 (Werner et al. 2009).

### 1.1. Reconstitution of E1 complex from singly His-tagged subunits

Preliminary data from our lab revealed that combination of His-Aos1 and His-Uba2 (N-terminally tagged) does not lead to a very active E1 complex (K. Chmielarska, PhD thesis), whereas previous studies based on recombinant SUMO E1 with Uba2-His demonstrated that E1 composed of C-terminally tagged Uba2 does exhibit E1 activity (Johnson et al. 1997; Okuma et al. 1999; Long et al. 2000). Therefore I established a protocol in which E1 is reconstituted from N-terminally His-tagged Aos1 and C-terminally His-tagged Uba2 (Werner et al. 2009).

As shown in Figure 8A, His-Aos1 and Uba2-His were independently expressed from pET28a- or pET28b-based constructs and the recombinant proteins were purified via nickel-pull-down and gel filtration and, in the case of Uba2, also anion-exchange chromatography. After combination of purified His-Aos1 and Uba2-His, the assembled E1 complex was separated from excess of any of the single subunits by gel filtration. Comparison of E1 purified according to the co-expression protocol versus the new reconstitution protocol (Fig. 8B) demonstrated that both ways of E1 purification resulted in complexes of comparable purity. Due to a higher expression

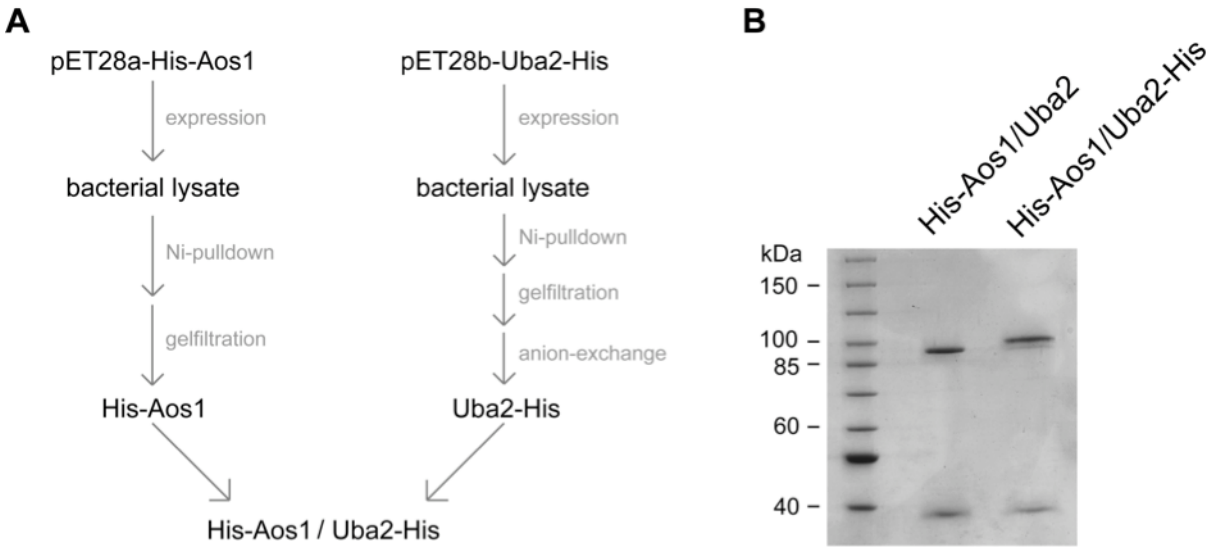


**Figure 8: Purification of His-Aos1/Uba2-His.** (A) Schematic illustration of E1 purification procedure according to the new reconstitution protocol. (B) Comparison of recombinant His-Aos1/Uba2 and His-Aos1/Uba2-His purified according to the old co-expression protocol or the new reconstitution protocol respectively. The Coomassie stained SDS gel reveals identical purity of E1 complex by means of both purification procedures.

level of singly expressed Uba2-His compared to untagged Uba2 during co-expression, the amount of E1 obtained by the standard co-expression protocol of 0.5 – 1.5 mg per liter *E. coli* culture was lower compared to 1.5 – 3 mg Uba2-His and 5 – 7 mg His-Aos1 per liter culture obtained by the new protocol.

## 1.1. Generation of fluorescently labeled E1 complex

Analysis of the intracellular localization of SUMO E1 complex required fluorescence microscopic detection of the single subunits within assembled E1 complexes. For this reason, the bacterial expression vectors pET28a and pET28b were modified by insertion of ECFP or EYFP (PCR amplified from pECFP-C1 or pEYFP-C1) into the multiple cloning sites, resulting in expression vectors that coded for a N-terminal His-CFP tag (pET28a-ECFP) or a C-terminal YFP-His tag (pET28b-EYFP). Expression of Aos1 and Uba2 from these constructs allowed purification of His-CFP-Aos1 and Uba2-YFP-His and subsequent reconstitution of His-CFP-Aos1/Uba2-YFP-His complex. The expression and purifciation procedures were identical to His-Aos1/Uba2-His and resulted in comparable amounts of equally pure E1 complex (demonstrated in Fig. 18).

## 1.2. Reconstituted His-Aos1/Uba2-His complex exhibits comparable specific activity to co-purified His-Aos1/Uba2

In contrast to the commonly used E1 that contained His-tagged Aos1 and untagged Uba2, E1 complex generated according to the new protocol included His-tagged Uba2. Since it has to date not been tested whether C-terminal tagging of Uba2 to some extent interferes with the catalytical E1 activity, I compared the E1 activities of recombinant purified His-Aos1/Uba2 and His-Aos1/Uba2-His in *in vitro* SUMOylation assays.

*In vitro* SUMOylation of RanGAP1 was set up with E1 activity as the rate-limiting factor of the reaction and the assay was performed with identical concentrations of His-Aos1/Uba2 or His-Aos1/Uba2-His (Fig. 8B) in parallel. Analysis by immunoblotting with α-RanGAP1 antibody (Fig. 9A) revealed that both SUMOylation reactions proceeded with approximately similar kinetics. For a more detailed analysis, I turned to a FRET-based *in vitro* SUMOylation assay developed in our lab (Bossis et al. 2005). This assay involves YFP-tagged SUMO and CFP-tagged model substrate (RanGAP-tail). Upon conjugation FRET can occur between CFP and YFP, which can be followed online using a fluorescence plate reader, by measuring emission at 480 and 527 nm after excitation of CFP at 430 nm. Figure 9B clearly shows that the reaction rates for different concentrations of E1 were very similar between His-Aos1/Uba2 and His-Aos1/Uba2-His.

Taken together, these data demonstrate that purification of Aos1/Uba2 according to

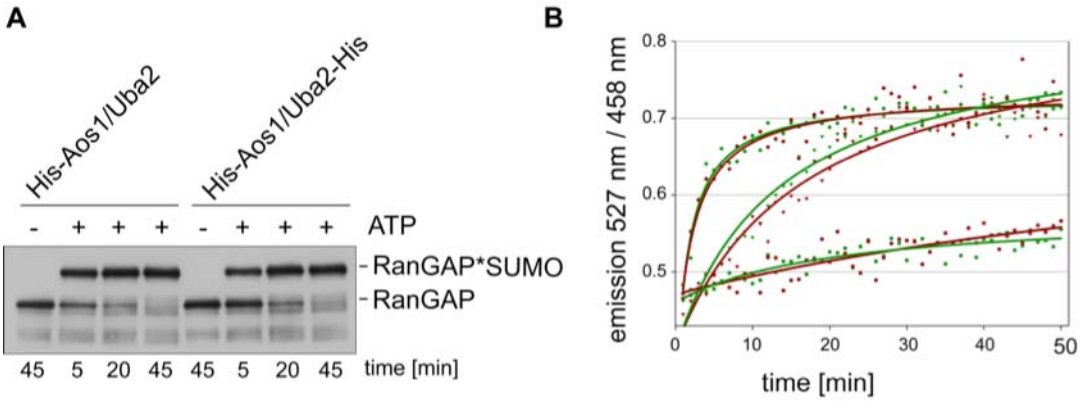


**Figure 9: Identical E1 activities of His-Aos1/Uba2 and His-Aos1/Uba2-His.** (A) *In vitro* SUMOylation of RanGAP with limiting amounts of E1 detected by SDS PAGE and α-RanGAP immunoblotting. (B) *In vitro* SUMOylation reaction detected by FRET reveals identical kinetics for His-Aos1/Uba2-His (green) and His-Aos1/Uba2 (red) at the tested concentrations of 22 nM (dots), 1.4 nN (triangles) and 86 pM E1 (quadrates) respectively.

the new protocol results in E1 enzye of the same specific activity as obtained by the standard protocol based on co-expression.

## 2. Characterization of the nuclear import of SUMO E1

Based on the predominantly nuclear localization of Aos1 and Uba2 in cells (Dohmen et al. 1995; Azuma et al. 2001; Pichler et al. 2002) it is obvious that the proteins have to be imported into the nucleus after synthesis in the cytoplasm as well as after nuclear breakdown in mitosis. Since the mechanisms underlying the generation of active nuclear SUMO E1 are to date largely unknown, I performed a detailed characterization of the nuclear import of Aos1/Uba2.

### 2.1. Aos1 and Uba2 contain distinct functional NLSs

To gain insights into the import mechanisms of Aos1/Uba2, I first addressed the question whether import of the single subunits can occur independently of complex formation. For the Uba2 homolog from *Saccharomyces cerevisiae* it has previously been shown by overexpression of a C-terminally GFP-tagged deletion fragment of the protein that the C-terminal region (amino acids 551 - 636) is necessary for nuclear localization (Dohmen et al. 1995). Sequence alignments of the C-termini of Uba2 from different species (Fig. 10) revealed a cluster of basic amino acids

```
H. sapiens          DVEFEVVGDAPEKVGPKQAEDA-AKSITNGSDDGAQPSTS--TAQEQDDVLIVDSDEEDS 57
M. musculus         DVEFEVVGDSPEKVGPKQAEDA-AKSIANGSDDGAQPSTS--TAQEQDDVLIVDSDEEGP 57
X. laevis           ---FEVVGDVPEKGPQKPPEES-VKNITNGSDDGAQPSTS--KAQDQDDVLIVDSDEESP 54
D. rerio            -----VVGDAPDKAPAPSAPEE-GKNIANGNKDSAQPSTSSKAAVEDDDVLLVDSDEEPS 54
S. cerevisiae       ---CNTCSLPDVEVPLIKANNSPSKNEEEEKNEKGADVVATTNSHGKDGIVILD-DDEGE 56
D. melanogaster     -------DDGPSTSKRSRPNEVVEEDDDDCLVIEEDEDQADVVVVATDKLSVQSPPKSGS 53
                           .          . :   :.  :          :       * : : .  ..

H. sapiens          SN-NADVSEEERSRKRKLDEKENLS-AKRSRIEQ-KEELDD--VIALD 100
M. musculus         SN-STDCSGDDKARKRKLEENEAAS-TKKCRLEQ-MEDPDD--VIALD 100
X. laevis           SSSNADVGMESASLKRKLPDEEAVSSTKRKRIEPPVEEDDD--IIALD 100
D. rerio            SS-TMDTESSNRKRKHHDAETDDAS-SKRKRLDQQPADDDDEDIIALD 100
S. cerevisiae       ITIDAEPINGSKKRPVDTEISEAPS-NKRTKLVN---EPTNSDIVELD 100
D. melanogaster     KRKPCEVIEDEDITEILESSDDEPAGPTKCKRSR-LDDSNPVAVISID 100
                         :    .          :  :  .: :      :     :: :*
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**Figure 10: Sequence alignment of the C-terminus of Uba2.** The C-terminal 100 amino acids of Uba2 from *H. sapiens, M. musculus, D. rerio, X. laevis, D. melanogaster and S. cerevisiae* were aligned using ClustalW2. Conserved clusters of basic amino acids potentially participating in nuclear import are underlined and highlighted in bold.

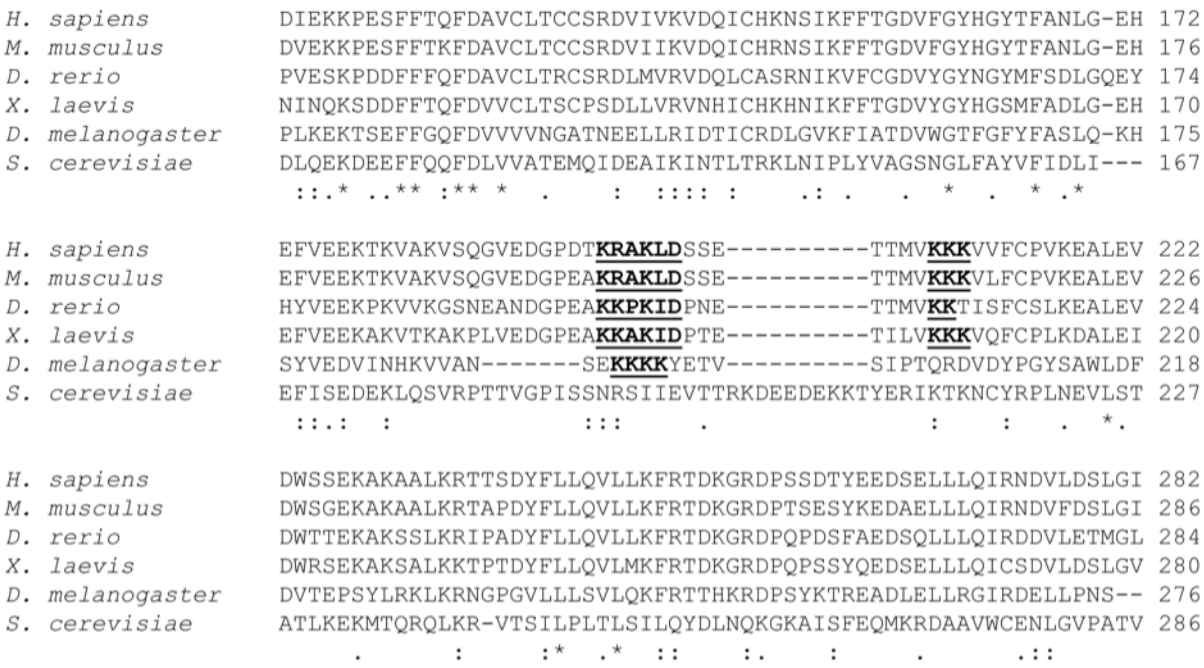


**Figure 11: Sequence alignment of Aos1.** Amino acid sequences of Aos1 from *H. sapiens, M. musculus, D. rerio, X. laevis, D. melanogaster and S. cerevisiae* were aligned using ClustalW2. Conserved clusters of basic amino acids potentially participating in nuclear import are underlined and highlighted in bold. This figure shows the section from amino acid 114 to 282 of of the alignment, for the complete alignment see Supplemantal Fig.1

conserved from human to fly that were reminiscent of a classical bipartite NLS. Before I started my investigations there was no evidence for a functional NLS in the E1 subunit Aos1. However, an alignment of Aos1 homologs from different species (Fig. 11) revealed two conserved clusters of basic amino acids matching the characteristics of classical NLSs.

To test whether these basic sequences are required for nuclear import I introduced mutations substituting amino acids of the potential NLSs by alanines (Fig. 12A) and compared the localization of wild type and mutant variants of CFP-Aos1 and Uba2-YFP upon transient transfection of HeLa cells (Fig. 12B). The analysis of Aos1 revealed that CFP-Aos1-KRAK195-198$A_4$ but not CFP-Aos1-KKKV207-210$A_4$ localized to the cytoplasm of transfected cells (Fig. 12B, upper panel). This result showed that only the first of the two basic clusters participates in mediating nuclear import of Aos1 and pointed towards a monopartite and not a bipartite classical NLS. Interestingly, a more detailed comparison with identified import sequences of other proteins revealed a striking similarity of the identified region of the Aos1 NLS with the conserved residues of the NLS of the oncoprotein c-Myc ($^{320}$PxxKRxKLD$^{328}$) (Stone et al. 1987; Dang et al. 1988). Since all required residues of the c-Myc NLS are found

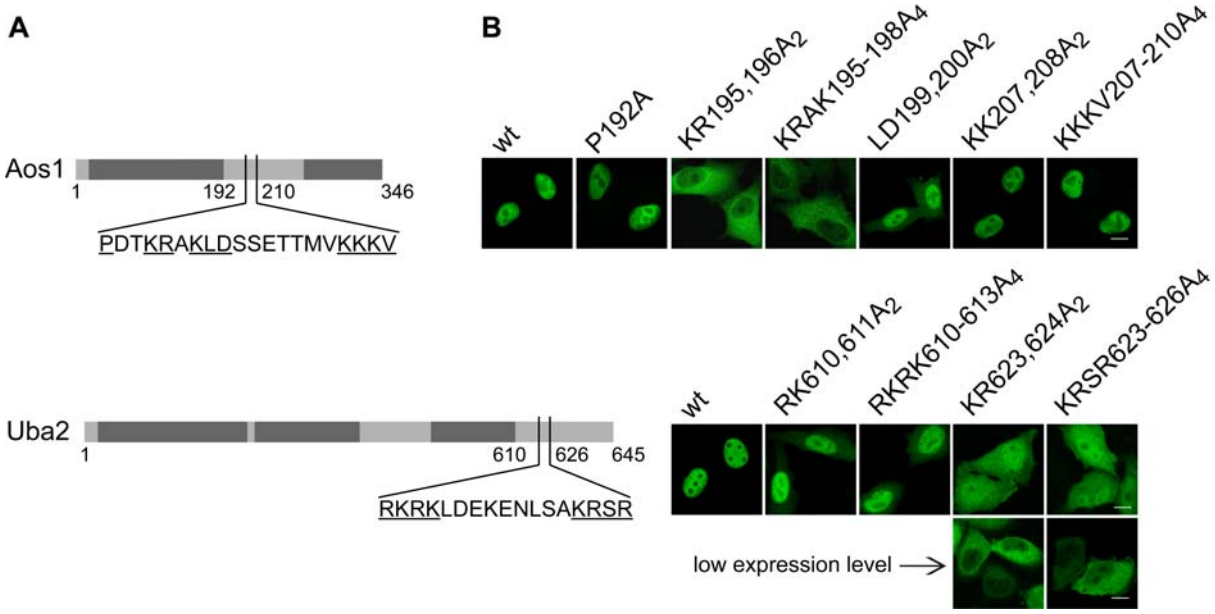


**Figure 12: Both Aos1 and Uba2 contain distinct NLSs.** (A) Schematic illustration of potential NLSs in Aos1 or Uba2. Amino acids changed by alanines for analysis of their participation in nuclear import are underlined. (B) HeLa cells were transiently transfected with pET28a-CFP-Aos1 (upper panel) or pET28b-Uba2-YFP (lower panel). 24 hours post transfection intracellular localization of overexpressed wild type and variant CFP-Aos1 or Uba2-YFP was detected by fluorescence microscopy. Localization of Uba2 variants upon low expression levels is indicated. Bar, 10 μm.

within Aos1 (Conti et al. 2000), I additionally performed a mutational analysis to verify the importance of these residues in mediating nuclear import of Aos1. Figure 12B demonstrates that Aos1-LD199,200$A_2$ partially mislocalized to the cytoplasm, whereas substitution of the proline did not alter the localization. Hence, the identified NLS of Aos1 consists of a cluster of basic residues directly followed by leucine and aspartate and thereby largely matches the conserved amino acids ($^{320}$PxxKRxKLD$^{328}$) of the unconventional NLS of c-Myc (Stone et al. 1987; Dang et al. 1988; Makkerh et al. 1996).

Mutational analysis of Uba2-YFP revealed an important role of two clusters of basic amino aicds, RKRK610-613 and KRSR623-626, in mediating nuclear import (Fig. 12B, lower panel). While replacement of the first cluster by alanines resulted in only partial mislocalization of Uba2, Uba2-KRSR623-626$A_4$ showed a strong cytoplasmic accumulation. The observed effect was most apparent in cells expressing lower levels of the protein. Thus, these results confirm the suggestion by Dohmen et al. that a basic cluster of amino acids in the C-terminal region of Uba2 participates in nuclear import (Dohmen et al. 1995). However, whether the import sequence of Uba2 is a bipartite cNLS or contains two overlapping monopartite NLSs could not be conclusively clarified.

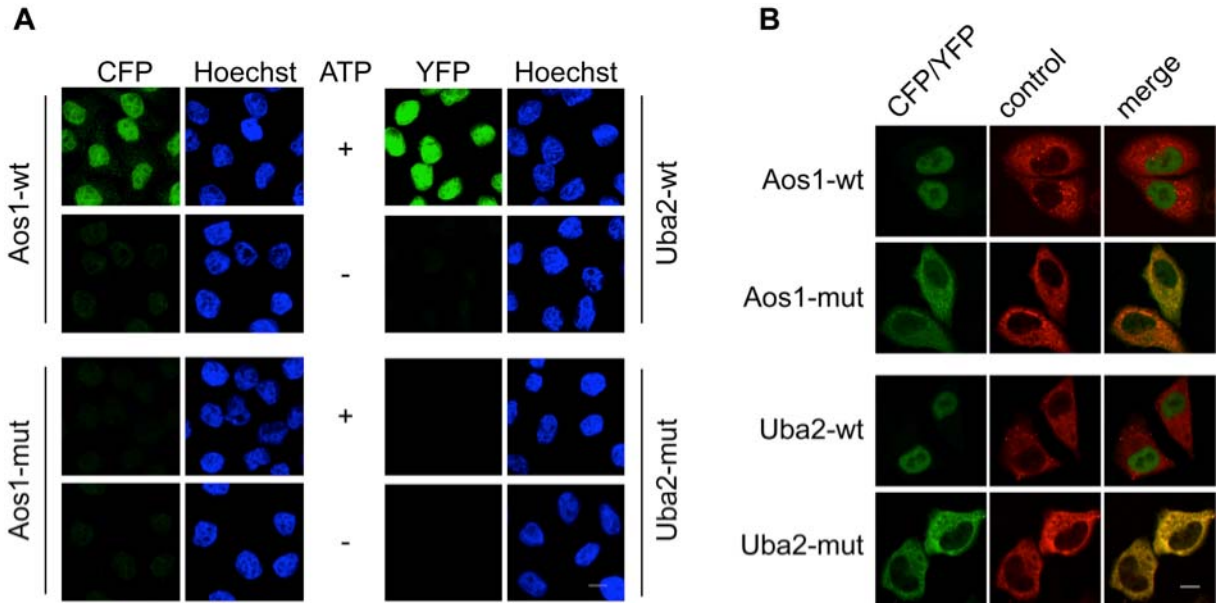


**Figure 13: Aos1 and Uba2 are independently imported in HeLa cells.** (A) *In vitro* import of recombinant purified CFP-Aos1 or Uba2-YFP by cytosolic extract in semipermeabilized HeLa cells. DNA was stained with Hoechst (blue) and nuclear accumulation of wild type proteins (CFP-Aos1-wt, Uba2-wt-YFP) and of the NLS mutants (CFP-Aos1-KR195,196$A_2$, Uba2-KR623,624$A_2$-YFP) was analyzed by fluorescence microscopy (green). Assays were performed in the presence of ATP-regenerating system (ATP +), whereas negativ control experiments contained ATP-depleting system (ATP -). Bar, 10 µm. (B) Intracellular localization CFP-Aos1 or Uba2-YFP after microinjection into the cytoplasm of HeLa cells. Prior to injection, recombinant wildtype proteins or the NLS mutants Aos1-KR195,196$A_2$ or Uba2-KR623,624$A_2$ (green) were mixed with TRITC-dextran which resided in the injected compartment (red). Intracellular localization was analyzed by fluorescence microscopy. Bar, 10 µm.

The finding that both E1 subunits contain distinct NLSs by which they can separately be imported into the nucleus could be confirmed by *in vitro* import assays and *in vivo* microinjection studies. Both recombinant wild type CFP-Aos1 and wild type Uba2-YFP accumulated in the nucleus of semi-permeabilized HeLa cells in *in vitro* import assays with cytosolic extract (Fig. 13A) and upon microinjection into the cytoplasm of living HeLa cells (Fig. 13B). Consistent with the transfection studies, the NLS mutants CFP-Aos1-KR195,196$A_2$ as well as Uba2-KR623,624$A_2$-YFP were not imported into the nucleus (Fig. 13A, B).

## 2.2. Importin β binds via importin α to both Aos1 and Uba2

The existence of independent NLSs in Aos1 and Uba2 immediately raised the question which transport receptors mediate the import of the two proteins. Since Aos1 contains a c-Myc-like import signal it may, like the c-Myc NLS peptide, interact with importin α and be imported via heterodimeric importin α/β (Conti et al. 2000).

Likewise is the classical bipartite NLS of Uba2 assumed to mediate interaction with importin α/β (Dingwall et al. 1982; Robbins et al. 1991).

In order to identify transport receptors capable of binding to the SUMO E1 subunits, I performed pull-down assays with immobilized Aos1 or Uba2 on CNBr-activated sepharose testing a variety of import receptors. Figure 14A shows that heterodimeric importin α/β indeed interacted with Aos1 wild type protein but not with the NLS mutant Aos1-KR195,196$A_2$ demonstrating that the interaction occurred via the identified nuclear localization signal in Aos1. Additionally, two other transport receptors, transportin and importin 13, were also capable of binding to Aos1. While binding of transportin required the NLS of Aos1, importin 13 bound to both wild type and NLS mutant Aos1. This result indicates that the interaction of importin 13 and

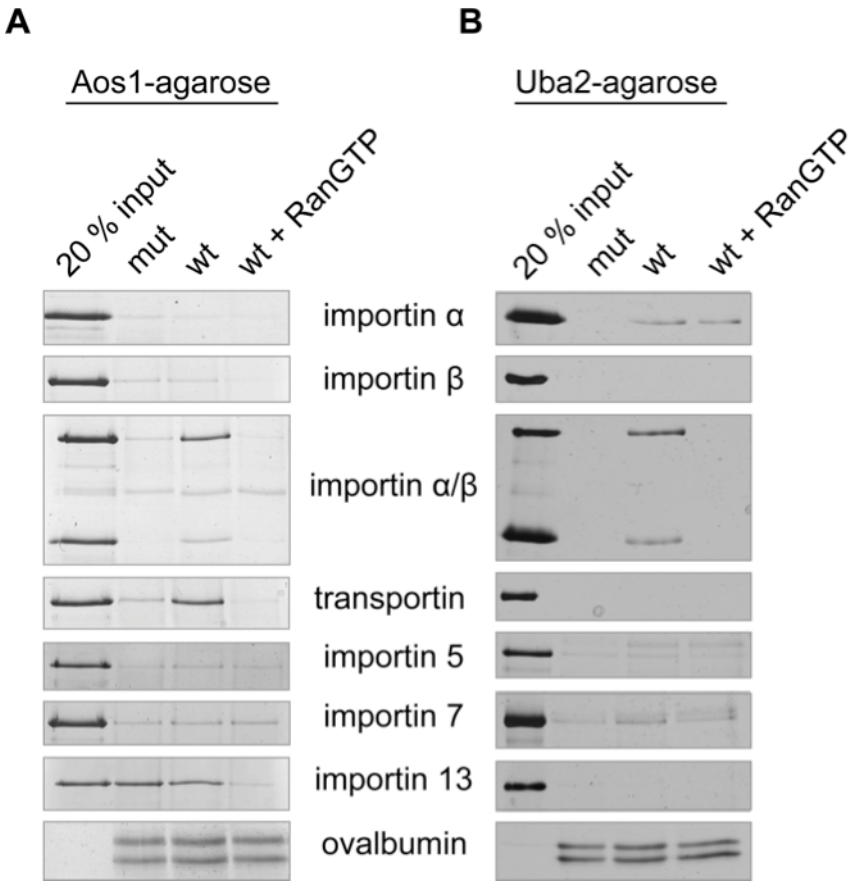


**Figure 14: Importin β binds to Aos1 and to Uba2 via the adaptor importin α.** Pulldown of recombinant import receptors importin α, β, α/β, transportin, importin 5, 7 or 13 with immobilized Aos1 (A) or Uba2 (B). Import receptors that bound to wild type beads (Aos1-wt, Uba2-wt) and to beads with NLS mutants (Aos1-KR195,196$A_2$, Uba2-KR623,624$A_2$) were eluted by SDS sample buffer and compared to 20 % of the input upon SDS PAGE and silverstaining. To control for specificity of binding, experiments were also performed in the presence of RanGTP that should interfere with formation of a typical receptor-cargo complex.

Aos1 involved another region of Aos1 than its identified NLS. However, the previous finding that the mutation of the Aos1 NLS lead to diminished nuclear import (Fig. 13, 14) suggests that importin 13, while interacting with Aos1 *in vitro*, is unlikely to be the main import receptor in cells (see also below Fig. 16).

When Uba2 was immobilized only monomeric importin α and heterodimeric importin α/β were able to bind (Fig. 14B) demonstrating that importin α mediates interaction with the receptor importin β. The interactions occurred via the mapped NLS of Uba2 since the receptor heterodimer bound to wild type Uba2 but not to the immobilized NLS mutant Uba2-KR623,624$A_2$ (Fig. 14B).

To control specificity of the interactions detected in pull-down assays, the effect of RanGTP addition was tested. RanGTP sensitivity is characteristic for bona fide import receptor/cargo complexes. As shown in Figure 14, interactions of Aos1 with importin α/β, transportin and importin 13, and interaction of Uba2 with importin α/β are indeed RanGTP sensitive. Due to the fact that importin α itself is not an import receptor but only an adaptor protein, the interaction of monomeric importin α with Uba2 was not inhibited by RanGTP.

## 2.3. Importin α/β mediates import of Aos1 and Uba2 *in vitro* and in cells

As importin α/β binds to both E1 subunits in a receptor/cargo-like manner, I performed *in vitro* import assays to test whether importin α/β can directly mediate import of Aos1 and Uba2 (Fig. 15). While importin α and importin β alone were not sufficient for nuclear accumulation of CFP-Aos1 and Uba2-YFP, the heterodimeric importin α/β complex efficiently mediated nuclear import of CFP-Aos1 and Uba2-YFP, but not of the NLS mutants. In control experiments, the addition of GTP-loaded RanQ69L, which binds to and blocks importin β, completely inhibited nuclear import. This indicates that the observed accumulation in the nucleus is due to active receptor-mediated nuclear import rather than passive diffusion through the nuclear pore complexes. Together, the experiments prove that the identified NLSs of Aos1 and Uba2 are indeed functional as nuclear import signals for import by dimeric importin α/β.

As living cells comprise more than the import receptors tested for *in vitro* interaction (Fig. 14) I wanted to find out whether transport by importin α/β is the main import

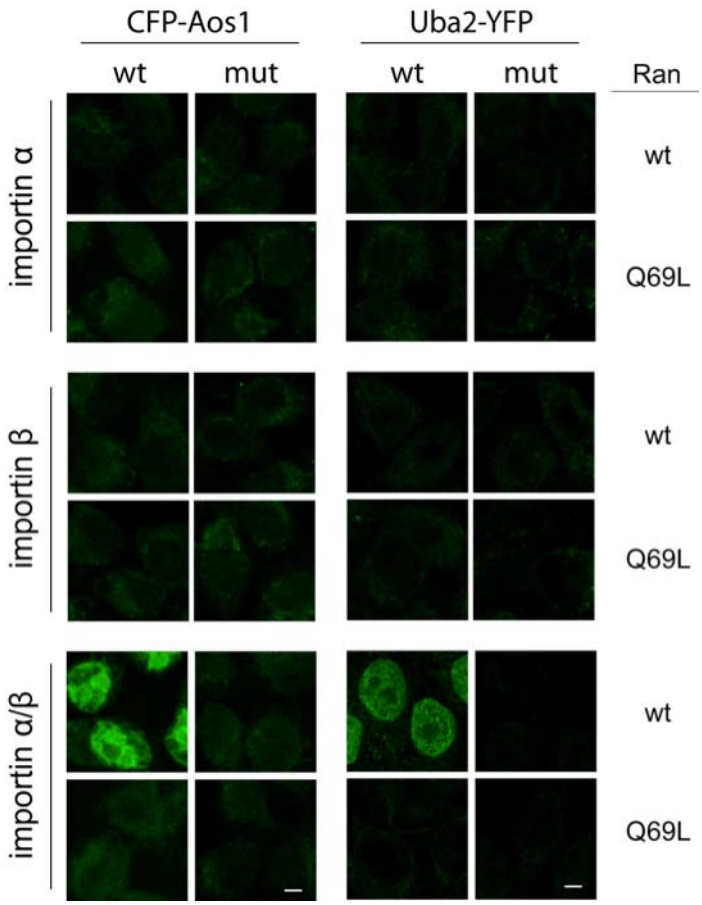


**Figure 15: Importin α/β mediates *in vitro* import of Aos1 and Uba2.** *In vitro* import of CFP-Aos1 or Uba2-YFP by importin α, β or α and β together in semipermeabilized HeLa cells. Nuclear accumulation of wild type proteins (Aos1-wt, Uba2-wt) and of the NLS mutants (Aos1-KR195,196$A_2$, Uba2-KR623,624$A_2$) was analyzed by fluorescence microscopy (green). Experiments were performed in the presence of wtRan or the mutant RanQ69L that can not hydrolyse GTP. Bar, 10 µm.

pathway or whether other cellular transport factors are even more potent in mediating nuclear import of Aos1 or Uba2. To address this question I microinjected fluorescent Aos1 and Uba2 into the cytoplasm of HeLa cells (Fig. 16) and compared the efficiency of nuclear import in the presence or absence of monoclonal α-importin β antibody, which was previously described to inhibit importin β-dependent nuclear import (Chi et al. 1995). Figure 16 shows that the import of both E1 subunits was completely abolished upon inhibition of importin β whereas nuclear import of the control M9-NLS, which is imported by transportin (Siomi et al. 1995; Nakielny et al. 1996), remained unaffected.

In combination with the *in vitro* finding that importin β can only bind to and import Aos1 or Uba2 in the presence of the adaptor importin α (Fig. 14, 15) these data demonstrate that nuclear import of monomeric Aos1 and Uba2 in cells is mainly mediated via the importin α/β pathway.

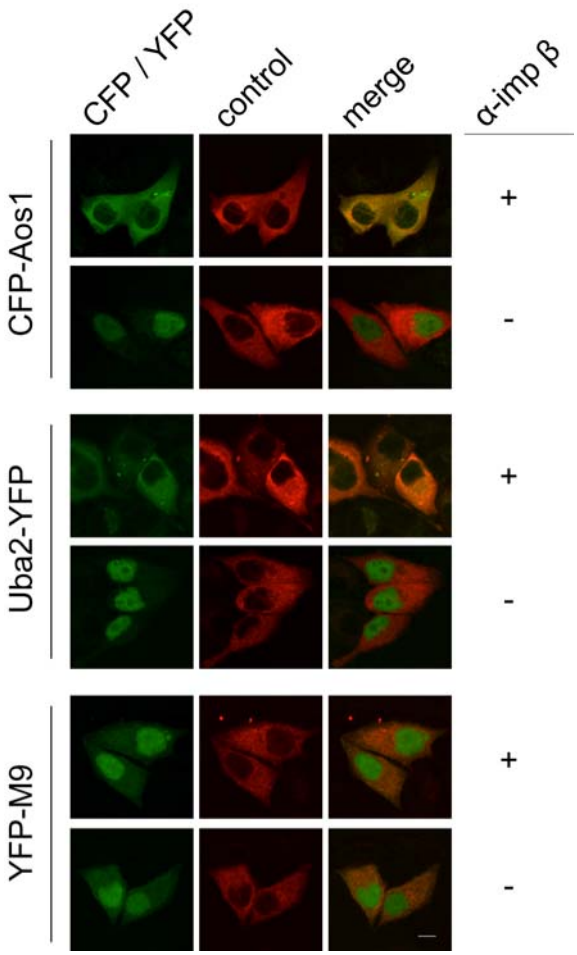


**Figure 16: Importin β mediates import of Aos1 and Uba2 in cells.** Mixtures of the fluorescent target protein (green), the injection control TRITC-dextran (red) and either monoclonal inhibitory α-importin β antibody 3E9 (α-imp β +) or transport buffer (α-imp β -) were microinjected into the cytoplasm of HeLa cells. Intracellular localization of CFP-Aos1, Uba2-YFP and YFP-M9 were analyzed by fluorescence microscopy. YFP-M9 is a cargo protein of the import receptor transportin (Siomi et al. 1995; Nakielny et al. 1996) and is used as control cargo whose nuclear import should be unaltered by inhibition of the importin β-transport pathway. Bar, 10 µm.

## 2.4. Pre-assembled E1 complex can be imported into the nucleus

While import of newly synthesized individual subunits may be sufficient to explain intranuclear localization of the SUMO E1 enzyme, preformed complexes need to reenter the nucleus after mitosis. To test whether whether assembled SUMO E1 complex can generally be imported into the nucleus, I first carried out *in vitro* import assays in the presence of HeLa cytosol. Figure 17A reveals that pre-formed wild type E1 complex consisting of CFP-Aos1-wt and Uba2-wt-YFP accumulated in the nuclei in an energy-dependent manner (rows 1 and 2).

This finding allowed to test nuclear import of different combinations of wild type and mutant Aos1 and Uba2. Remarkably, a complex of mutant Aos1 and wild type Uba2 (CFP-Aos1-KR195,196$A_2$/Uba2-wt-YFP) also accumulated in the nuclei (rows 5 and 6). In contrast, E1 complexes with the import-defective NLS mutant of Uba2 (CFP-Aos1-wt/Uba2-KR623,624$A_2$-YFP and CFP-Aos1-KR195,196$A_2$/Uba2-KR623,624$A_2$-YFP) were not imported (Fig. 17A, rows 3,4,7 and 8). Together these findings demonstrate that the NLS of Uba2, but not of Aos1, is required and sufficient for

import of the assembled SUMO E1 complex. In addition to these *in vitro* findings, I investigated nuclear import of pre-assembled Aos1/Uba2 complex *in vivo* by microinjection in cells. As shown in Figure 17B, the complex containing wild type Uba2 localized to the nucleus (rows 1 and 3), whereas complex with Uba2-

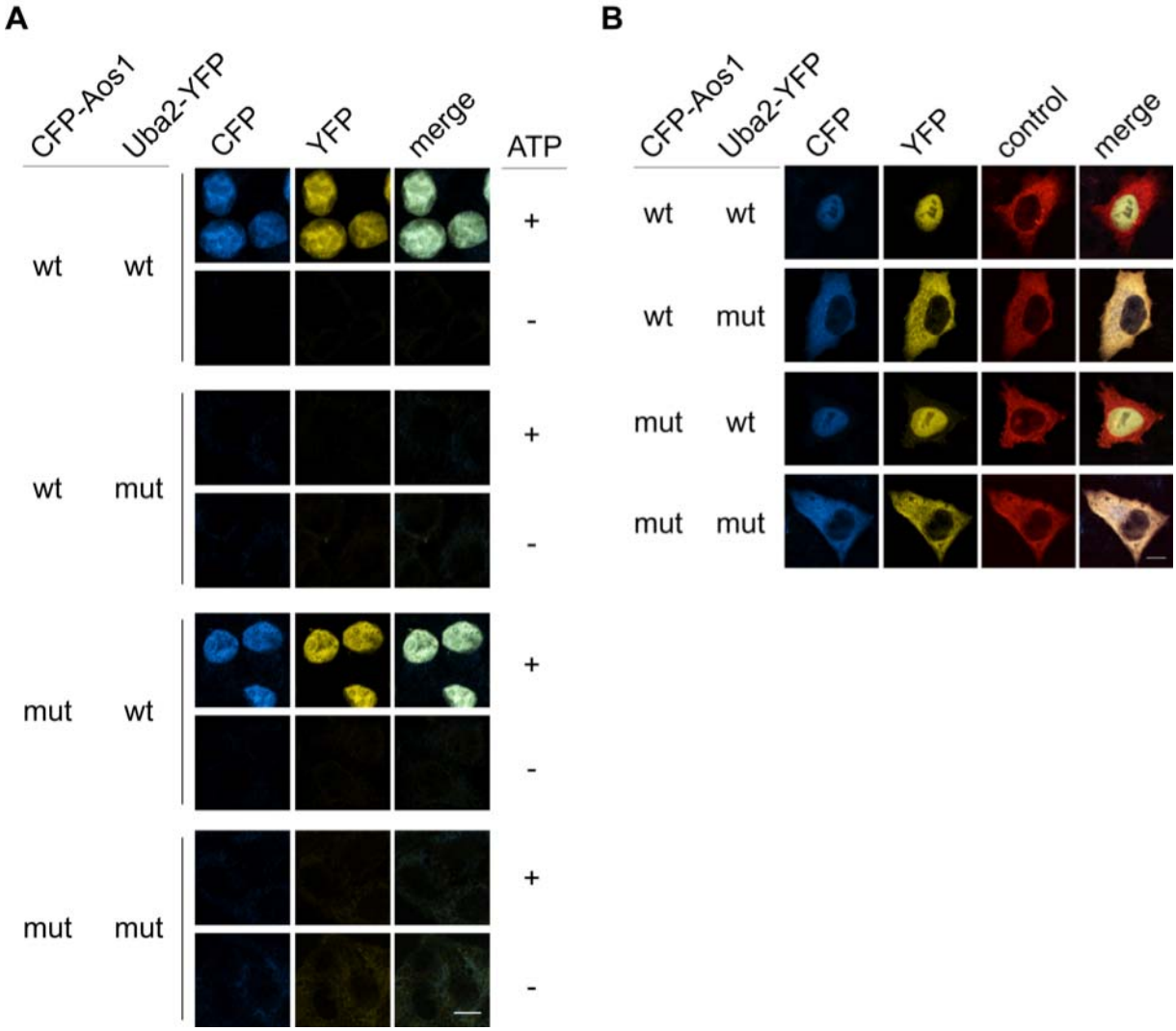


**Figure 17: The Uba2 NLS is required and sufficient for import of assembled E1 complex.** (A) *In vitro* import of different combinations of pre-assembled CFP-Aos1/Uba2-YFP complex. Prior to the experiment, complexes were reconstituted of wild type proteins (CFP-Aos1-wt, Uba2-wt-YFP) and/or NLS mutants (CFP-Aos1-KR195,196$A_2$, Uba2-KR623,624$A_2$-YFP). The assays were performed in semipermeabilized HeLa cells in the presence of cytosol and ATP-regenerating system (ATP +). Negativ control experiments contained ATP-depleting system (ATP -) instead. DNA was stained with Hoechst (blue) and nuclear accumulation of Aos1 (blue) and Uba2 (yellow) was analyzed by fluorescence microscopy. Bar, 10 µm. (B) Intracellular localization of CFP-Aos1 and Uba2-YFP after microinjection of pre-assembled complex into the cytoplasm of HeLa cells. E1 complexes composed of wild type proteins and/or NLS mutants were reconstituted prior to the experiment as elucidated in (A). TRITC-dextran (red) is contained in the injection mix and resided in the injected compartment, thereby serving as injection control. Intracellular localization of CFP-Aos1 (blue) and Uba2-YFP (yellow) was analyzed by fluorescence microscopy. Bar, 10 µm.

KR623,624$A_2$ remained in the cytoplasm (rows 2 and 4). These microinjection experiments document that the NLS of Uba2 is indeed required and sufficient for mediating nuclear import of pre-formed SUMO E1 complex in cells.

## 2.5. The NLS of Uba2 mediates E1 interaction with and import by importin α/β *in vitro*

Since I identified importin α/β as the main import receptor for both E1 subunits, I also analyzed its capability to interact with assembled E1 complex. For this purpose, I incubated different combinations of pre-formed E1 complex with a 5-fold molar excess of recombinant import receptors and subsequently analyzed the formation of complexes by analytical gel filtration (Fig. 18). Using a vast excess of import receptors allowed formation of receptor/cargo complexes containing more than one

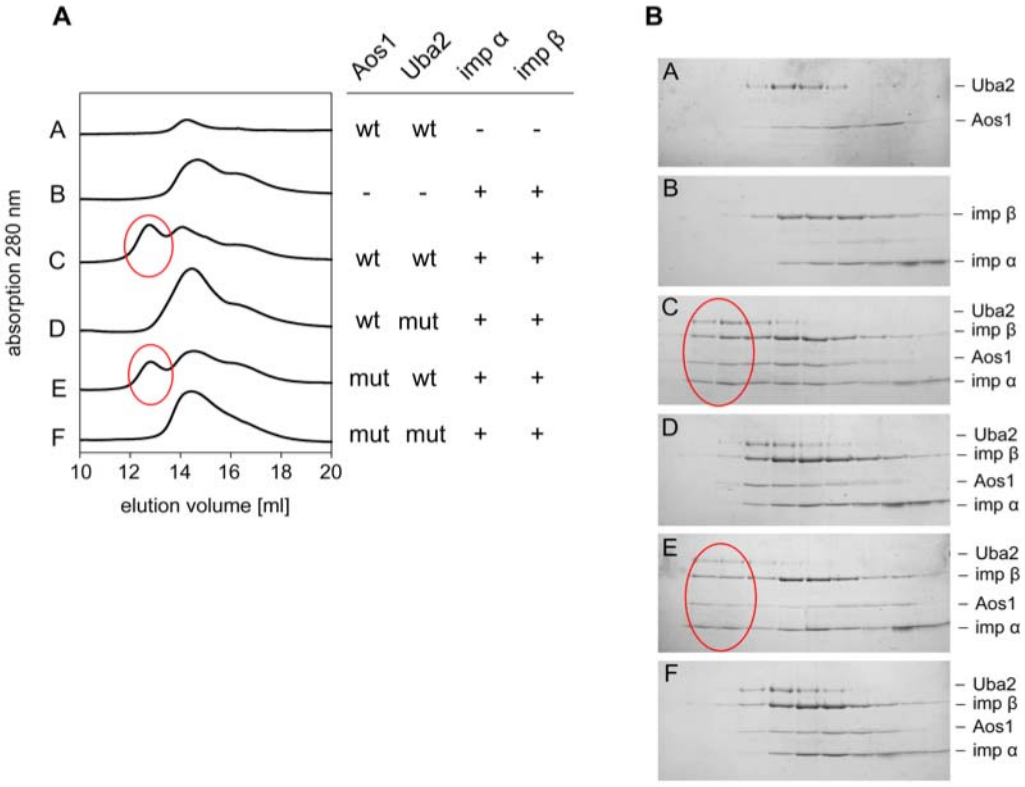


**Figure 18: Importin α/β binds to assembled E1 complex exclusively via the Uba2 NLS.** Analytical gelfiltration was performed after incubation of assembled CFP-Aos1/Uba2-YFP complexes with a 5fold molar excess of importins α and β. E1 complexes were reconstituted of Aos1-wt (wt) or Aos1-KR195,196$A_2$ (mut) and Uba2-wt (wt) or Uba2-KR623,624$A_2$ (mut). Elution profiles from the superose6-HR10/30 column were recorded by the Äkta purifier system (GE Healthcare) and processed with sigma plot 8.02 (Systat Software Inc.) (A). Fractions of 0.3 ml were collected and 50 µl of each fraction was analyzed by SDS PAGE and Coomassie-staining (B). E1/importin α/β complexes are highlighted in red.

receptor per cargo complex. Compared to the running behaviour of E1 complex alone (Fig. 18A, profile 1) or importins alone (profile 2), the elution-profile of Aos1-wt/Uba2-wt pre-incubated with importin ⟨/® showed an additional high-molecular weight peak eluting from the column at approximately 12.5 ml (profile 3). Subsequent SDS PAGE analysis revealed that this peak contained a complex composed of Aos1/Uba2/importin α/β in equimolar ratios (Fig. 18B, gel 3). A peak at the same elution volume was also detected upon incubation of importin α/β with Aos1-mut/Uba2-wt. This complex showed no difference compared to the wild type complex (profile and gel 5). In contrast, no complex formation was detected when the NLS in Uba2 was mutated (profiles and gels 4 and 6), demonstrating that the Uba2 NLS is absolutely required for formation of the tetrameric import complex Aos1/Uba2/importin α/β.

Next, I wanted to test whether the tetrameric Aos1/Uba2/importin α/β complex is functional in nuclear transport. Therefore, I tested the ability of recombinant importin α/β to mediate nuclear import of different pre-assembled E1 complexes in *in vitro*

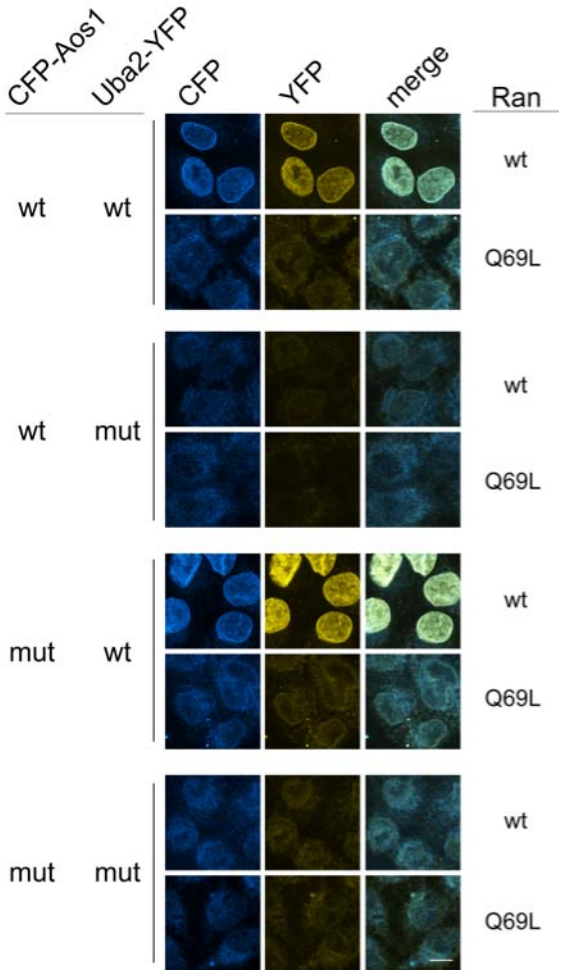


**Figure 19: *In vitro* nuclear import of Aos1/Uba2 complex is mediated by importin α/β via interaction with the Uba2 NLS.** *In vitro* import of pre-assembled CFP-Aos1/Uba2-YFP complexes by importin α, β or α and β together. E1 complexes are composed of CFP-Aos1-wt (wt) or CFP-Aos1-KR195,196$A_2$ (mut) and Uba2-wt-YFP (wt) or Uba2-KR623,624$A_2$-YFP (mut). The experiments were performed in the presence of wt Ran or RanQ69L. Nuclear accumulation of CFP-Aos1 (blue) and Uba2-YFP (yellow) was analyzed by fluorescence microscopy. Bar, 10 µm.

import assays (Fig. 19). SUMO E1 complex containing wild type Uba2 (rows 1, 2, 5 and 6) strongly accumulated in the nuclei in the presence of wild type Ran, whereas E1 complex with the NLS mutant Uba2-KR623,624$A_2$-YFP was not imported (rows 3, 4, 7 and 8). Consistent with the microinjection data, application of wild type or NLS mutant Aos1 did not influence the import behaviour of the pre-formed E1 complex (comparison of the rows 1 and 5 or 3 and 7). As expected, RanQ69L inhibited nuclear import.

Altogether, these results demonstrate that the NLS of Uba2 is required and sufficient for the interaction of pre-assembled E1 with importin α/β and resulting nuclear import of E1 complex.

## 2.6. The SUMO E1 complex in cells is mainly imported by importin α/β

The finding that the NLS of Uba2 is required for E1 import upon microinjection into living cells (Fig. 17B) in combination with the fact that the heterodimer importin α/β is required to import Uba2 alone (Fig. 14B, 15, 16) leads to the suggestion that the importin α/β-pathway is also the main cellular import pathway for assembled E1.

To test whether any cellular import receptor other than importin β is capable of mediating nuclear import of E1 complex, I analyzed the effect of inhibitory α-importin β antibody on import of micorinjected E1 comeplex (Fig. 20). Indeed, inhibition of importin β completely abolished nuclear import of E1 complex, which allows to conclude that the importin α/β-pathway is the main import-pathway for assembled

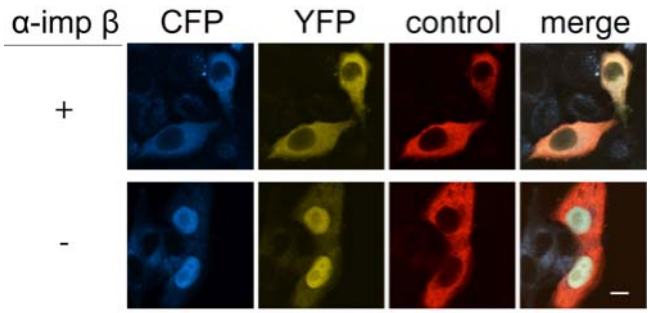


**Figure 20: Inhibition of importin β prevents nuclear import of assembled E1 complex in cells.** Mixtures of CFP-Aos1/Uba2-YFP complex (blue and yellow), the injection control TRITC-dextran (red) and either inhibitory α-importin β antibody (α-imp β +) or transport buffer (α-imp β -) were microinjected into the cytoplasm of HeLa cells. Intracellular localization of CFP-Aos1 (blue) and Uba2-YFP (yellow) were analyzed by confocal fluorescence microscopy. Bar, 10 µm.

Aos1/Uba2 complex in HeLa cells.

In conclusion, the results described here reveal that nuclear E1 complex can be generated in two ways (Fig. 21). Single subunits can be imported by importin α/β independently of each other via distinct NLSs mapped in Aos1 and Uba2 (left side); in addition, the pre-assembled Aos1/Uba2 complex can undergo nuclear import by importin α/β via interaction with the NLS in Uba2 (right side).

## 3. Analysis of the cytoplasmic E1 pool

Published work on the localization of the two SUMO E1 subunits Aos1 and Uba2

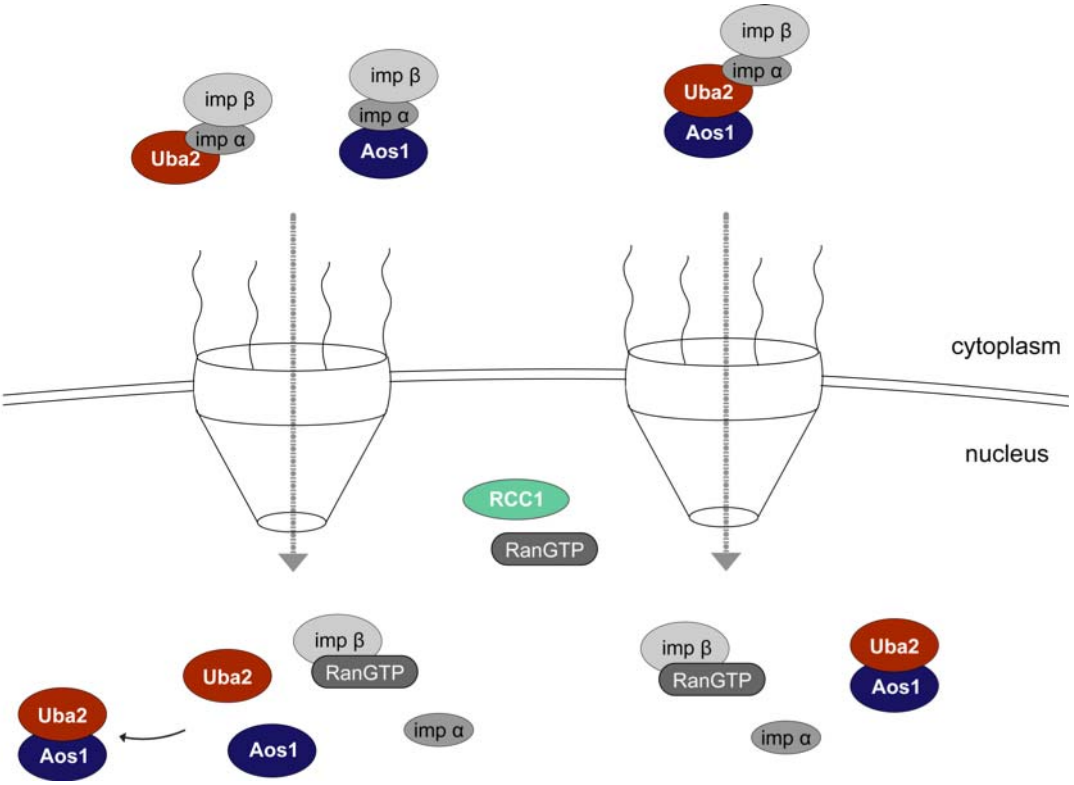


**Figure 21: Schematic model of the generation of active nuclear E1 complex.** (left) The single subunits Aos1 (blue) and Uba2 (red) are idependently imported by importin α/β via their distinct NLSs. After translocation through the nuclear pore the cargoes are released into the nucleus and can assemble to active SUMO E1 complex. (right) Already formed E1 complex interacts via the NLS of Uba2 with importin α/β, which mediate the translocation into the nucleus.

revealed that both proteins predominantly localize in the nuclear compartment (Dohmen et al. 1995; Azuma et al. 2001; Pichler et al. 2002). However, data from Dr.

Guillaume Bossis and Dr. Andrea Pichler showed the presence of both subunits also in cytosolic fractions and even pointed to a regulatory event specific to the cytosolic E1 pool (Pichler et al. 2002; Bossis et al. 2006). Furthermore, preliminary results obtained by Dr. K. Chmielarska in our lab suggested an enrichment of Aos1 at the Golgi apparatus (K. Chmielarska, PhD thesis). To obtain a more coherent picture of E1's localization I aimed to perform a more detailed analysis of the intracellular localization of the SUMO E1 enzyme in HeLa cells and hippocampal neurons.

## 3.1. Endogenous Aos1/Uba2 predominantly localizes to the nucleus of HeLa cells

Previous immunofluorescence studies using epifluorescence microscopes suggested that the main pool of SUMO E1 resides in the nuclei of cells (Dohmen et al. 1995; Azuma et al. 2001; Pichler et al. 2002). As epifluorescent microscopes detect the emitted light from the whole, or at least multiple layers of the specimen, the resulting image displays the sum of fluorescent intensities of all these layers. As the nucleus of a cell is thicker than the cytoplasm, a picture from a signal equally distributed between these compartments might therefore show increased signal intensity in the nucleus compared to the cytoplasm. Consequently, the epifluorescent images from the literature, which illustrated the nuclear enrichment of SUMO E1 in adherent cells, might lead to a partially wrong conclusion regarding the enzymes's distribution

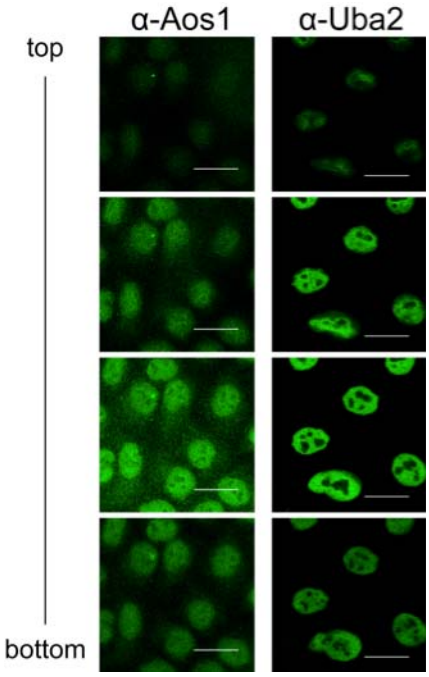


**Figure 22: Endogenous Aos1 and Uba2 predominantly localize to the nucleus.** Fixed HeLa cells were immunostained with affinity purified goat α-Aos1 or goat α-Uba2 antibody/donkey α-goat-Alexa488 (green). Images from different confocal planes through the cells were acquired by confocal laser scanning microscopy using a confocal LSM510meta (Zeiss). Bar, 20 μm.

between nucleus and cytoplasm. In order to analyze the intracellular distribution of endogenous Aos1 and Uba2 more accurately I verified the subcellular localization of the proteins by indirect immunofluorescence analyzed with confocal microscopy. Using this technique, only fluorescent light emitted very close to the focal plane in the specimen was detected, producing images with signal intensities that better reflect the intracellular concentrations of the fluorophores.

Images from different confocal planes ranging from the top to the bottom of the cells (Fig. 22) illustrate that endogenous SUMO E1 was significantly higher concentrated in the nucleus than in the cytoplasm. Aos1, but not Uba2, was to some extent also detected in the cytoplasm. This finding might be explained by an access of Aos1 over Uba2, which has previously been shown to occur during cell cycle progression (Azuma et al. 2001). A reason why endogenous Uba2 was not detected in the cytoplasm could be that high affinity epitopes of the protein are masked in the cytoplasm or that the amount of cytoplamic Uba2 is simply below the detection limit of the antibody. Consequently, this result confirms a predominant nuclear localization of both SUMO E1 subunits obtained by previous studies with epifluorescent microscopic analysis (Dohmen et al. 1995; Azuma et al. 2001; Pichler et al. 2002).

## 3.2. SUMO E1 is found in cytosolic fractions of HeLa cells

To address the question of the intracellular localization with an additinoal method that allows to detect small pools of Aos1/Uba2, I performed differential centrifugation of HeLa cells to obtain fractions of different cellular compartments. Equal protein amounts of each fraction were analyzed by SDS PAGE and immunoblotting to verify the presence of specific proteins (Fig. 23). Detection of the marker proteins RCC1 (nucleus), LDH (cytosol) and GM130 (Golgi apparatus) in the predicted fractions illustrated the successful separation of the different cellular compartments. In line with previous data from Dr. Guillaume Bossis and Dr. Andrea Pichler both SUMO E1 subunits Aos1 and Uba2 were clearly present in the nuclear and cytosolic fractions (Fig. 11, lane 1, 2, SN) (Pichler et al. 2002; Bossis et al. 2006). Interestingly, in contrast to the cytosolic marker protein LDH, Aos1 was also found in fraction 3 and Uba2 was found in fractions 3 – 6, demonstrating that Aos1 and Uba2 are also present in other compartments besides nucleus and cytosol. The detection of both subunits in fraction 3, which contains proteins of the Golgi apparatus (apparent from the Golgi marker GM130), is in line with preliminary data from Dr. Katarzyna

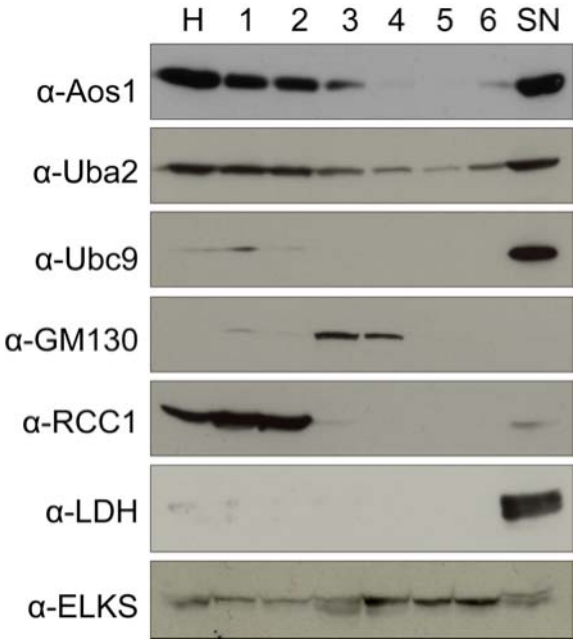


**Figure 23: Aos1 and Uba2 are present in the cytososlic fraction of HeLa cells.** Fractions of lysed HeLa suspension cells were obtained by differential centrifugation. Protein equivalents of the homogenate (H), fractions containing whole cells and intact nuclei (1), nuclear debris (2), mitochondria, lysosomes and golgi (3), lysosomes, golgi and peroxisomes (4), endoplasmic reticulum (5), polysomes (6) and of the cytosolic fraction (SN) were analyzed by immunoblotting with α-Aos1, α-Uba2, α-Ubc9, α-GM130, α-RCC1, α-LDH and α-ELKS for the presence of these proteins.

Chmielarska indicating the localization of Aos1 and Uba2 at the Golgi apparatus (K. Chmielarska, PhD thesis).

While analysis of protein equivalents, shown in Figure 23, reveals the presence of E2 enzyme mainly in the cytosol but not in the homogenate, analysis of cell equivalents show that the SUMO E2 is also present in the nuclear fraction as well as in the homogenate (data not shown). The analysis of cell equivalents (not shown) reveals that the amounts of Aos1, Uba2, and Ubc9 in the nuclear fraction was lower than the amounts detected in the cytosolic supernatant. This finding coincides with previous fractionation experiments performed by Dr. Andrea Pichler (Pichler et al. 2002) and contrasts the result of immunofluorescence analysis (Fig. 22), which revealed significantly higher amounts in the nucleus. A possible explanation for the observed discrepancy may be the fractionation procedure: The incubation on ice or the hypotonic swelling may trigger active export into the cytosol, or the lysis procedure slightly damages the nuclei and thereby causes leaking out of the nuclei.

Taken together, the immunofluorescence analysis of the distribution of Aos1 and Uba2 illustrates that the endogenous E1 subunits are predominantely localized in the nucleus of cells. While a pool of Aos1 was also found in the cytoplasm, Uba2 was only found in the nucleus. However, fractionation experiments verify the presence of both E1 subunits in the cytosol of cells. Consequently, the question how much SUMO E1 enzyme is located in the cytoplasm remains open.

## 3.3. Distribution of E1 in neuronal cells is analogous to HeLa cells

To expand the analysis of the intracellular localization of Aos1/Uba2, I turned to neuronal cells, where SUMOylation of synapse proteins like the transmembrane kainate-receptor subunit GluR6 and the metatropic glutamate receptor mGluR8 has been found to play a role in the regulation of synaptic function (Tang et al. 2005; Martin et al. 2007; reviewed in Coussen et al. 2007; Martin et al. 2007). These previous data raise the question whether the intracellular distiribution of the SUMO E1 enzyme in neuronal cells differs from the localization in HeLa cells.

To address this question, I analyzed the intracellular distribution of Aos1 and Uba2 in subcellular fractions of hippocampal neurons from mice (Fig. 24). Samples of the fractionation of isolated cells were kindly provided by the laboratory of Prof. Dr. Nils Brose (Max-Planck Institute for Experimental Medicine, Göttingen). Enrichment of the marker protein NMDAR1 comparing the homogenate (H) and the fraction of synaptic plasma membranes (SPM) served as an established indicator to control for successful SPM purification. Aos1 and Uba2 were detected in soluble fractions, mainly in the supernatant after nuclear sedimentation (S1) and in the cytosolic fraction (S3). Significantly, both subunits were also found in the cytosolic

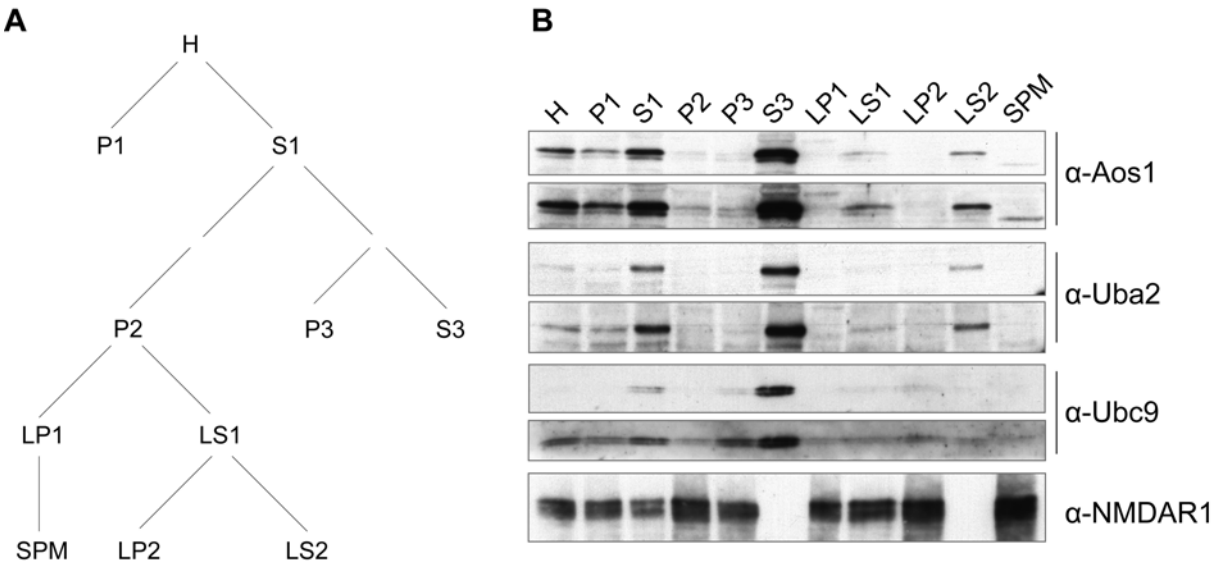


**Figure 24: Aos1 and Uba2 are present in the cytosolic synaptosomal fraction of murine hippocampal neurons.** Samples of fractionated hippocampal neurons from mice were kindly provided by the lab of Prof. Dr. Nils Brose (Max-Planck Institute for Experimental Medicine, Göttingen). (A) Schematic outline of the fractionation procedure according to (Jones et al. 1974). (B) Protein equivalents of the homogenate (H), nuclear pellet (P1), supernatant after nuclear sedimentation (S1), crude synaptosomal pellet (P2), light membrane pellet (P3), cytosolic fraction (S3), heavy membrane pellet (LP1), intermediate fraction LS1 (LS1), crude synaptic vesicles (LP2), cytosolic synaptosomal fraction (LS2) and synaptic plasma membranes (SPM) were analyzed by immunoblotting with α-Aos1, α-Uba2, α-Ubc9, α-NMDAR1.

synaptosomal fraction (LS2), revealing that some E1 indeed localizes to the cytoplasm of neuronal synaptic cells. The fact that the detected signal of Aos1 and Uba2 in the LS2 fraction was relatively low compared to S3 is not problematic, since the fractions were differentially purified from the fraction S1 and can therefore not be directly compared (Fig. 12A). However, the purification of LS2 from the crude synaptosomal pellet (P2) over LS1 goes along with a clear enrichment of Aos1 and Uba2, indicating the significance of the detected signal. For Ubc9 a similar enrichment in S1 and S3 was observed, but the protein was not found in fractions derived from the crude synaptosomal pellet P2.

To gain further insights into the intracellular localization of the SUMO E1 in different neuronal cells, I performed immunohistochemical analyses of endogenous Uba2 in tissue slides from normal mouse brain (Fig. 25). For this purpose commercially available tissue slides were deparaffinized, rehydrated and stained using $\alpha$-Uba2 antibody and Hoechst. The resulting images presented in Figure 13 exemplary show DNA, Uba2 and background fluorescence from four different regions of the brain. The samples displayed strong autofluorescence in the green and in the red channel, whereby the signal from the Uba2-staining was overlaid with background fluorescence. Comparing the background signal of the red channel with the green channel, the images from the regions 1 and 2 did not show any Uba2 signal above fluorescent background, whereas in the regions 3 and 4 specific Uba2 signal was detected. This Uba2-specific signal co-localized with Hoechst-stained DNA, in line with the predominantly nuclear Uba2 localization in cultured fibroblasts. However, no conclusions about the absence or presence of a smaller cytoplasmic pool of Uba2 could be drawn due to the high background fluorescence. Consequently, this experiment does not allow further analysis of the distribution of SUMO E1 in neuronal cells. Whether the finding that Uba2 was not detectable at all in some regions suggests cell type-specific expression of Uba2 or wheter it was due to technical problems requires further experiments.

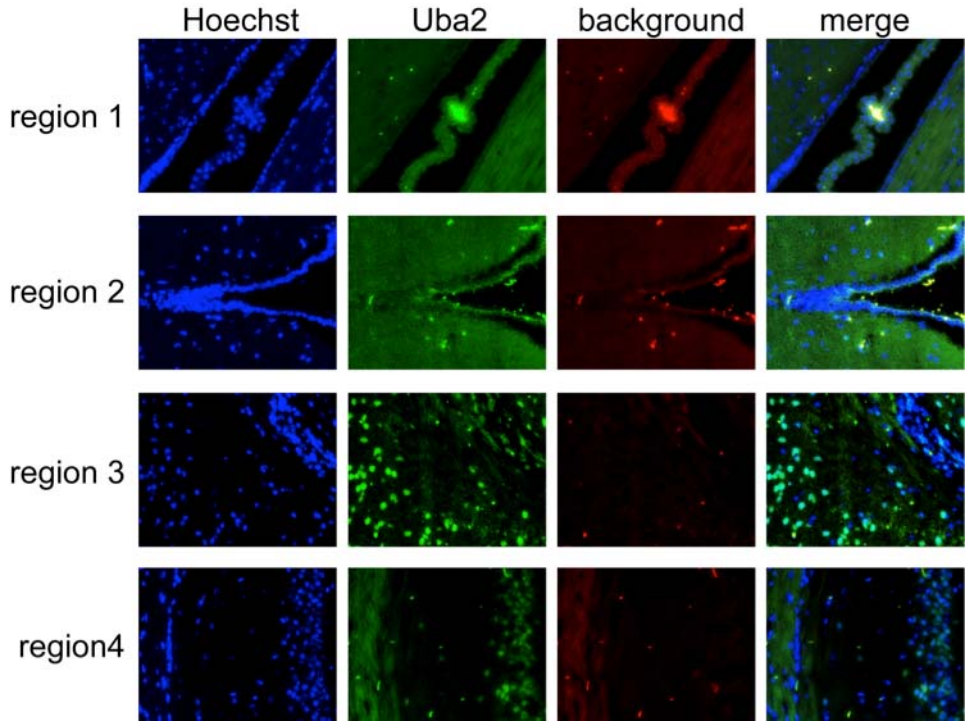


**Figure 25: Detection of endogenous Uba2 in slides of murine brain.** Tissue slides from murine brain were immunohistochemically stained with goat α-Uba2/donkey α-goat-Alexa488 (green), DNA was stained with Hoechst (blue) and autofluorescence background of the samples was detected in the red chanel (red). Images were acquired by fluorescence microscopy using a CellObserver (Zeiss).

The results obtained by analysis of neuronal cells reveal the presence of E1 in cytosolic synaptosomal fractions purified from murine brain but point to predominantly nuclear localization of the enzyme, as described for HeLa cells. As already mentioned, a number of identified SUMO targets are transmembrane proteins localized and modified at the synapses of neuronal cells (Tang et al. 2005; Martin et al. 2007; reviewed in Coussen et al. 2007; Martin et al. 2007). Whether SUMOylation of these targets requires the localization of a small E1 pool at the synapses and whether this localization of the E1 is due to passive diffusion or to an active transport mechanism are very interesting questions for future studies.

## 3.4. No indication for active export of the SUMO E1

The existence of a small cytoplasmic pool of Aos1/Uba2 immediately raises the question how this pool is generated. As both E1 subunits are predominantly localized in the nucleus, active shuttling between the nucleus and the cytoplasm could be one mechanism underlying the generation of the small cytoplasmic E1 pool. For this

reason I tested whether the E1 enzyme can be actively exported from the nucleus into the cytoplasm.

*Aos1 and Uba2 do not interact with CRM1 in vitro*

CRM1 is the major receptor for the export of proteins out of the nucleus (Fornerod et al. 1997; Stade et al. 1997; reviewed in Hutten et al. 2007). In most instances, the export complex is formed via interaction of CRM1 with a leucine-rich nuclear export signal (NES) within cargo proteins in the presence of RanGTP (Fischer et al. 1995). The fact that most proteins are exported via CRM1 in combination with the finding that potential NES consensus sequences L-$X_{2-3}$-(L,I,V,M,F)-$X_{2-3}$-L-X-(L,I,V) are present in Aos1 and in Uba2 (Fig. 26A) make CRM1 a promising candidate for mediating potential nuclear export of the SUMO E1. However, analysis of the Aos1/Uba2 structure revealed, that the consensus NESs are located at the interaction surfaces of both subunits, for which reason interaction CRM1 with assembled E1 complex is rather unlikely whereas the NESs are accessible in the monomeric subunits (Fig. 26B).

To test whether Aos1, Uba2 or assembled E1 complex binds to the export receptor CRM1 I performed pull-down experiments with immobilized CRM1. However,

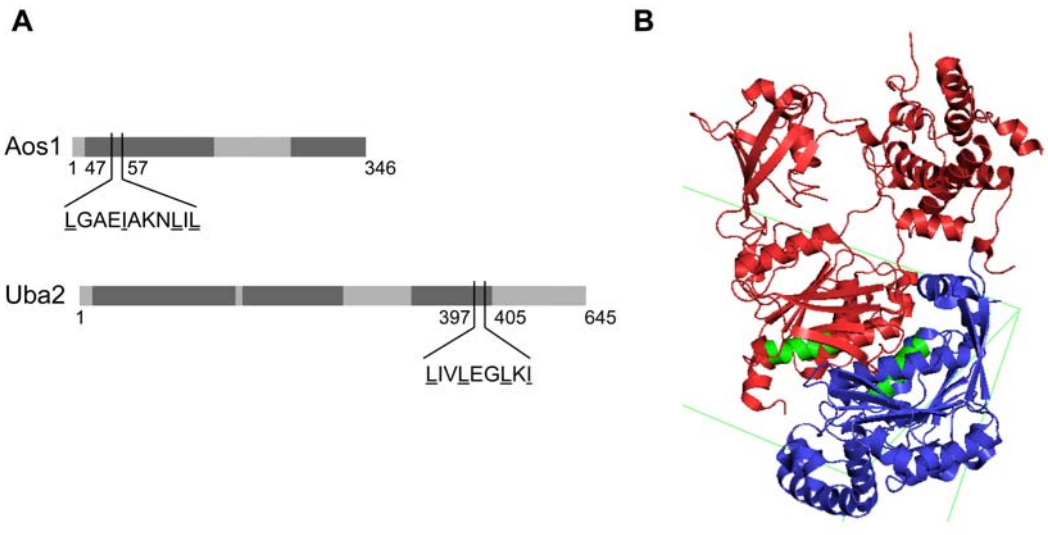


**Figure 26: Aos1 and Uba2 contain a potential consensus NES.** (A) Schematic illustration of the position and the sequence of the potential consensus NES in Aos1 and Uba2. (B) Illustration of the spatial localization of the potential NESs of Aos1 and Uba2 in a cartoon of the crystal structure of heterodimeric Aos1/Uba2 complex solved by Lois et al. (Lois et al. 2005). Aos1 is coloured in blue, Uba2 is shown in red and the potential consensus NESs are highlighted in green. The raw data were obtained from the Protein Data Bank (PDB) and images were generated with PyMOL v0.99 (DeLano Scientific LLC).

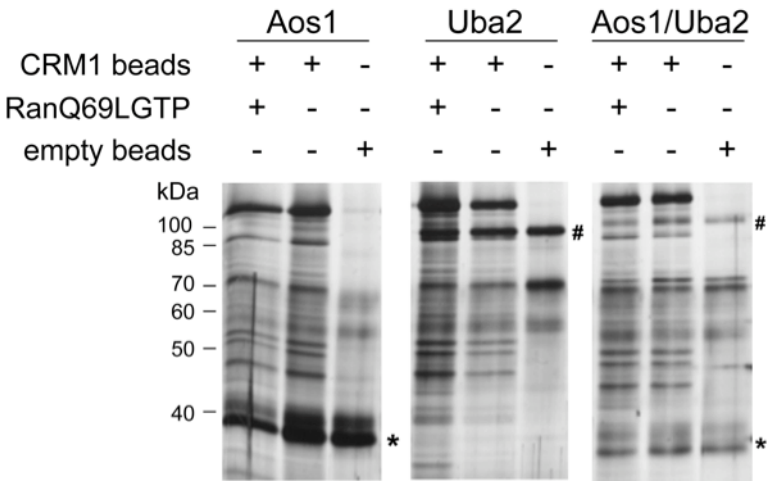


**Figure 27: No interaction of Aos1 or Uba2 with immobilized CRM1 in pulldown assays.** Aos1 (*), Uba2 (#) or assembled E1 complex (*,#) were subjected to CRM1 binding by incubation with beads containing immobilized CRM1. The assays were performed in the presence of RanQ69L whereas the negative control experiments did not contain Ran. Unspecific binding to beads was verified with empty beads. Bead bound proteins were analyzed by SDS PAGE and visualized by silver-staining.

comparable amounts of Aos1, Uba2 and assembled E1 comples bound to CRM1 beads and to empty beads (Fig. 27). This strong background binding to the empty beads indicates that the conditions of the pull-down experiments were not appropriate for the analysis of any specific interaction of the potential cargoes with CRM1.

Therefore, I turned to RanGAP assays, which are well-established experiments for analysis of CRM1-cargo interactions. The assay is based on the fact that RanGTP is protected from RanGAP-stimulated GTP-hydrolysis when RanGTP is present in a trimeric CRM1/RanGTP/cargo export complex, while in the absence of cargo RanGTP is hydrolyzed as assessed by the production of free phosphate. This method is applicable to verify even weak or transient interactions between CRM1 and NES bearing cargo proteins (Paraskeva et al. 1999; Engelsma et al. 2004). The positive control experiment in the presence of the NES peptide (NS2 protein of minute virus of mice, CVDEMTKKFGTLTIHDTEK; Askjaer et al. 1999) caused a significant decrease of GTP-hydrolysis by RanGAP (Fig. 28, reactions 4 and 5). The amount of free $\gamma$–phosphate decreased to 40 % compared to the reactions without any export signal containing cargo (reactions 2, 3 and 5), indicating that the NES peptide allowed the assembly of export complexes and thereby protected RanGTP from hydrolysis. In contrast, GTP-hydrolysis was unaltered upon addition of Aos1,

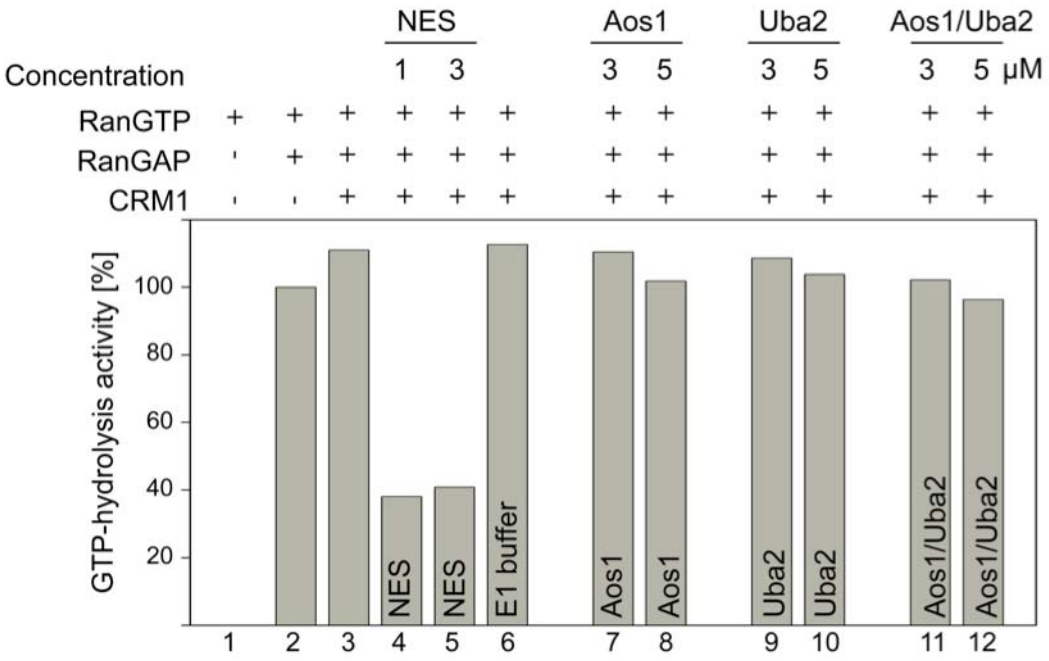


**Figure 28: Aos1, Uba2 or assembled E1 do not interact with CRM1 in RanGAP assays.** Aos1, Uba2 or assembled Aos1/Uba2 complex (3 or 5 µM final) were incubated with CRM1 (1 µM), RanGAP (60 nM), GTP (200 nM) and a limiting amount of [$\gamma$-$^{32}$P]RanGTP (30 nM). The reaction was stopped, [$\gamma$-$^{32}$P]RanGTP was removed by centrifugation with charcoal and the released [$\gamma$-$^{32}$P] was measured by scintillation counting. Background counts from a reaction without RanGAP were subtracted and GTP hydrolysis was expressed as the percentage of the value of radioactivity measured in a reaction with RanGAP and without CRM1. Positive control reactions contained nuclear export signal (NES) (1 or 3 µM) instead of SUMO E1.

Uba2 or assembled E1 complex (reactions 7 to 12), revealing that neither the single E1 subunits nor pre-formed E1 complex interact with the export receptor CRM1.

While CRM1 is the best-characterized export receptor, it certainly is not the only one. Other export receptors, as well as the transport receptors transportin and importin 13, primarily known for their import activity, have been shown to also mediate nuclear export of selected cargoes (Gallouzi et al. 2001; Mingot et al. 2001; Shamsher et al. 2002). Interestingly, transportin and importin 13 have both been shown to interact with Aos1 in *in vitro* pull-down assays, raising the question whether one of these transport receptors can mediate export of SUMO E1. However, their interaction with SUMO E1 subunits was inhibited in the presence of RanGTP (see Fig. 18) necessitating a more complex mechanism.

*No evidence for shuttling of CFP-Aos1 in HeLa cells*

To test in cells whether SUMO E1 is in general actively exported from the nucleus I analyzed the shuttling behaviour of Aos1 in interspecies heterokaryon assays. For

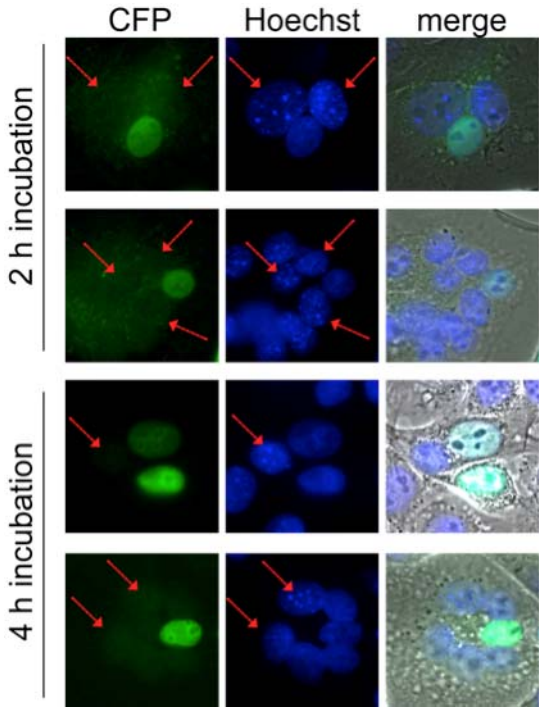


**Figure 29: No shuttling of Aos1 in heterokaryon assays.** Heterokaryate cells were obtained by fusion of untransfected mouse 3T3 cells with transiently transfected HeLa cells over expressing CFP-Aos1. After 2 or 4 hours of incubation DNA was stained with Hoechst (blue) and intracellular distribution of CFP-Aos1 (green) was analyzed by fluorescence microscopy. Nuclei from mouse 3T3 cells show characteristic bright heterochromatin in the Hoechst channel which are abscent from human HeLa nuclei.

these assays CFP-Aos1-expressing human cells and untransfected mouse cells were fused to heterokaryon cells containing at least one human and one murine nucleus. *De novo* protein biosynthesis was inhibited by the addition of cycloheximide, and the intracellular distribution of CFP-Aos1 was analyzed (Fig. 29). Since CFP-Aos1 was not newly synthesized, the accumulation of fluorescent signal in the blue spotted mouse nuclei (marked by arrows) would point towards shuttling of the CFP-Aos1 reporter protein: In this case, Aos1 would have been actively exported from a tranfected HeLa nucleus and subsequently re-imported into a mouse nucleus. Figure 29 demonstrates that the CFP signal did not accumulate in the mouse nuclei within 4 hours. Hence, shuttling of CFP-Aos1 was not detected.

However, interspecies heterokaryon assays only allow detection of shuttling if the levels of export are relatively high. In contrast, fluorescence microscopy techniques based on photobleaching are more sensitive methods that allowing the detection of even low levels of nucleocytoplasmic shuttling.

For this reason, I tested shuttling of CFP-Aos1 in multinuclear cells by using combined FRAP and FLIP (for details see chapter *METHODS – Combined FRAP and FLIP in multinuclear cells*). HeLa cells expressing the reporter protein CFP-Aos1 were fused to multinuclear cells. Then, the fluorescent signal in one of the nuclei was bleached (Fig. 30A, ROI-1, red) and images from different time points after bleaching were collected for quantification. Some selective images are shown in Fig. 30A. For

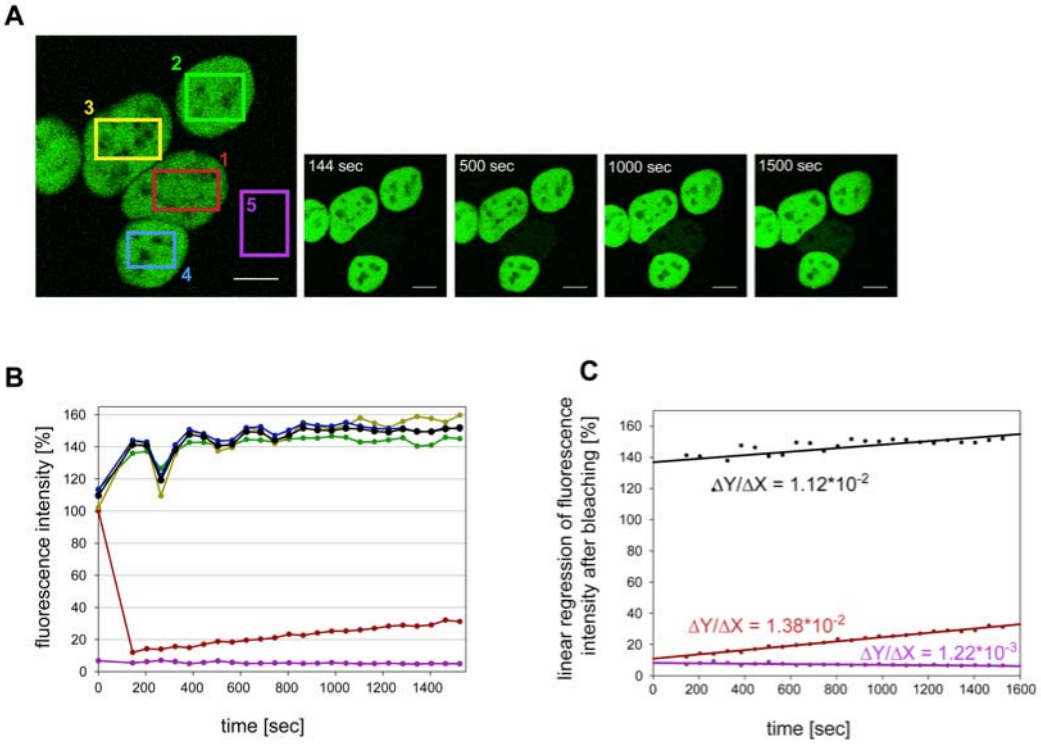


**Figure 30: Combined FRAP and FLIP analysis shows no shuttling of Aos1.** HeLa cells were transiently transfected with pcDNA3.1(-)-CFP-Aos1 and fused to homopolycaryons. CFP signal in one nucleus was completely bleached by multiple iteration of scanning with 100 % intensity of 458 nm from the argon laser using a CLSM510 (Zeiss). Images were aquired at different time points and signal intensities in 5 different ROIs (regions of interest) were tracked. (A) Selective images of different time points before and after bleaching. ROIs used for subsequent calculations are marked as follows: bleached nucleus ROI 1 (red), unbleached neighbouring nuclei ROIs 2 – 4 (green, yellow, blue) and cytoplasmic background ROI 5 (pink). (B) Graphs of the fluorescent signals of the ROIs 1 – 5 displayed in (A), are presented in the according colours red, green, yellow, blue and pink. The average signal from ROIs 2 – 4 are represented by the black graph. (C) Linear regression curves of the fluorescent signals in ROI 1 (red), ROIs 2 – 4 (black) and ROI 5 (pink) after bleaching. The calculated slopes represent the speed of signal accumulation. Data were processed with sigma plot 8.02 (Systat Software Inc.). Bar, 10 µm.

data processing, the fluorescent signal in the bleached nucleus (Fig. 30A, ROI-1, red), the surrounding unbleached nuclei (ROIs 2-4, green, yellow and blue) and in the unbleached cytoplasmic background (ROI-5, purple) were tracked within defined regions of interest (ROIs). Subsequently, fluorescent intensities in the ROIs were calculated in percentage of the intensity of ROI 1 before bleaching (Fig. 30B). The diagrams in Fig. 30B show that after bleaching of the marked nucleus (red graph) no strong increase in the bleached (FRAP) or decrease in an unbleached nucleus (FLIP) (green, yellow, blue graphs) was detected within the range of 25 minutes. Pictures taken at later time points, after re-adjusting the focus, showed similar signal distribution (data not shown). Linear post-bleaching regression curves were calculated of the signal in the bleached nucleus (Fig. 30C, red graph), of the average

signal from the unbleached nuclei (black graph) and from the background fluorescence (purple graph). To compare the speed of changes in the fluorescent signal, the slopes of the linear regressions corresponding to the speed were calculated. The resulting data of this analysis reveal that the fluorescent signal in bleached and unbleached nuclei increased with similar speed, whereas the background signal changed approximately 10 times slower. This result shows that no significant FRAP or FLIP occured, which would have become apparent by a faster increase in the bleached nucleus (ROI 1, red graph) compared to the unbleached nucleus (ROIs 2-4, black graph) in a static low background.

Since it has been shown that the level of SUMOylation of target proteins can be altered by exposing cells to different stresses (Mao et al. 2000; Sacher et al. 2006; reviewed in Tempe et al. 2008), cytoplasmic E1 may be generated by re-localization induced by a certain stress stimulus. However, analyses of the intracellular localization of endogenous Aos1 and Uba2 after incubation at 0.04 % $CO_2$, on ice or in the presence of 1 – 10 mM $H_2O_2$ did not point towards nuclear export of the E1 upon exposure to the tested stresses (data not shown).

Taken together, in of the performed experiments neither active export of Aos1 nor binding of Aos1, Uba2 or E1 complex to the export receptor CRM1 was detected, suggesting that cytoplasmic pool of SUMO E1 is not generated by constant export out of the nucleus. Hence, other mechanisms such as the inhibition of import or cytoplasmic retention may account for the generation of cytoplasmic E1 (for details see chapter *DISCUSSION*).

## 5. Analysis of the effects of mislocalized SUMO E1 in yeast

As described in the introduction, SUMOylation takes place both in the nuclear and cytoplasmic compartment. However, SUMO E1 enzyme is barely detectable in the cytoplasm and does not appear to shuttle between these compartments. One explanation for this apparent discrepancy is that the E1 enzyme is expressed in large excess, such that the low levels in the cytoplasm are not rate limiting. Alternatively

E1 may not be needed in a specific compartment, which is conceivable if the E2 enzyme can shuttle after beeing loaded with SUMO.

To test whether nuclear localization of E1 is required, I analyzed the effects of mislocalized SUMO E1 in yeast cells, which have the same natural E1 distribution as human cells (Dohmen et al. 1995; Azuma et al. 2001; Pichler et al. 2002). Since Aos1/Uba2 is essential in *S. cerevisiae* (Dohmen et al. 1995; del Olmo et al. 1997), this system allowed the generation of *UBA2* shuffle strains. Shuffle strains are yeast strains in which the endogenous copy of an essential gene is deleted and replaced with a marker, while cells are kept alive by an exogenous copy of this gene. Selective loss of this rescue gene and parallel expression of a plasmid-encoded variant protein subsequently allows phenotypical analysis of the variant. By means of a *UBA2* shuffle strain I aimed to analyze the cellular consequences of cytoplasmically localized Uba2 without the background of endogenous Uba2.

## 5.1. Human Uba2 can not substitute for deletion of yeast Uba2 in *UBA2* shuffle strains

By introduction of a shuffle plasmid containing the *UBA2* gene and disruption of the genomic *UBA2* gene I generated a yeast *UBA2* shuffle strain. The cells can grow on normal medium due to expression of Uba2 encoded by the shuffle plasmid, whereas 5-FOA containing medium selects for cells that have lost the shuffle plasmid. Since *UBA2* is an essential gene (Dohmen et al. 1995; del Olmo et al. 1997), the created shuffle strains could not survive without the shuffle plasmid and would consequently not grow on the selctive 5-FOA medium. However, cells with an additional 5-FOA insensitive plasmid with the ORF of Uba2 would still be able to grow in the presence of 5-FOA.

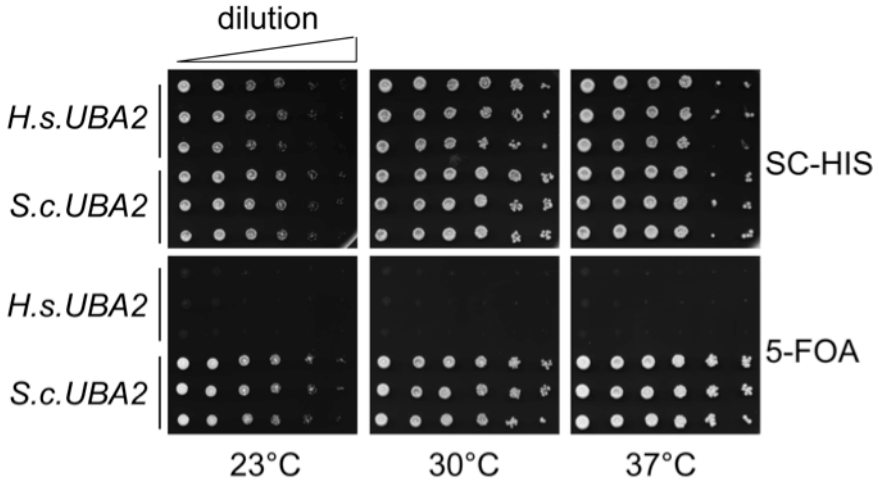


**Figure 31: Expression of *S.c.*Uba2 but not *H.s.*Uba2 rescues the deletion of endogenous Uba2 in yeast.** Endogenous Uba2 in yeast was replaced by expressing exogenous Uba2 in the shuffle strain ESM356-1/pRS316-*UBA2/uba2Δ::nat.* Exogenous Uba2 from *S.cerevisiae* or *H.sapiens* was overexpressed under ADH promoter from p413-expression constructs. Logarithmic growing cells with indicated genotypes were serially diluted and spotted onto plates that were incubated for 2 days at indicated temperatures. Growth on SC-HIS plates verifies equal dilutions and rescue for deletion of endogenous Uba2 was analyzed without the background of genomic Uba2 on 5-FOA plates.

To test the *UBA2* shuffle strain for functionality and to test whether human Uba2 can complement yeast Uba2, either human or yeast Uba2 was mildly overexpressed under ADH-promoter from a p413-construct that was transformed into the yeast strains (Fig. 31). Equal growth in the presence of the *UBA2* shuffle plasmid on SC-HIS plates demonstrated that an equal cell number of the two strains was spotted. Analysis of growth in the absence of *UBA2* shuffle plasmid on 5-FOA plates showed that the cells only survived when yeast but not when human Uba2 was overexpressed from the p413-construct. These experiments showed that the shuffle strain is functional and revealed human Uba2 does not rescue disruption of the essential *UBA2* gene in *S. cerevisiae*. Consequently, all following rescue experiments concerning the phenotypic analysis of Uba2 mutants had to be performed with variants of yeast Uba2.

## 5.2. Predominantly nuclear localization of Uba2 is not essential for the viabilty of yeast

To address the question whether nuclear localization of SUMO E1 is important I tested mislocalized NLS mutants of Uba2 for complementation of endogenous Uba2

in the generated yeast shuffle strains. For this purpose, *UBA2* shuffle strains expressing wild type yeast Uba2 (Uba2-wt) were compared to strains expressing the NLS mutant of yeast Uba2 (Uba2-ΔNLS) or the NLS mutant Uba2 with an additional NES (Uba2-ΔNLS-NES) to reinforce cytoplasmic localization.

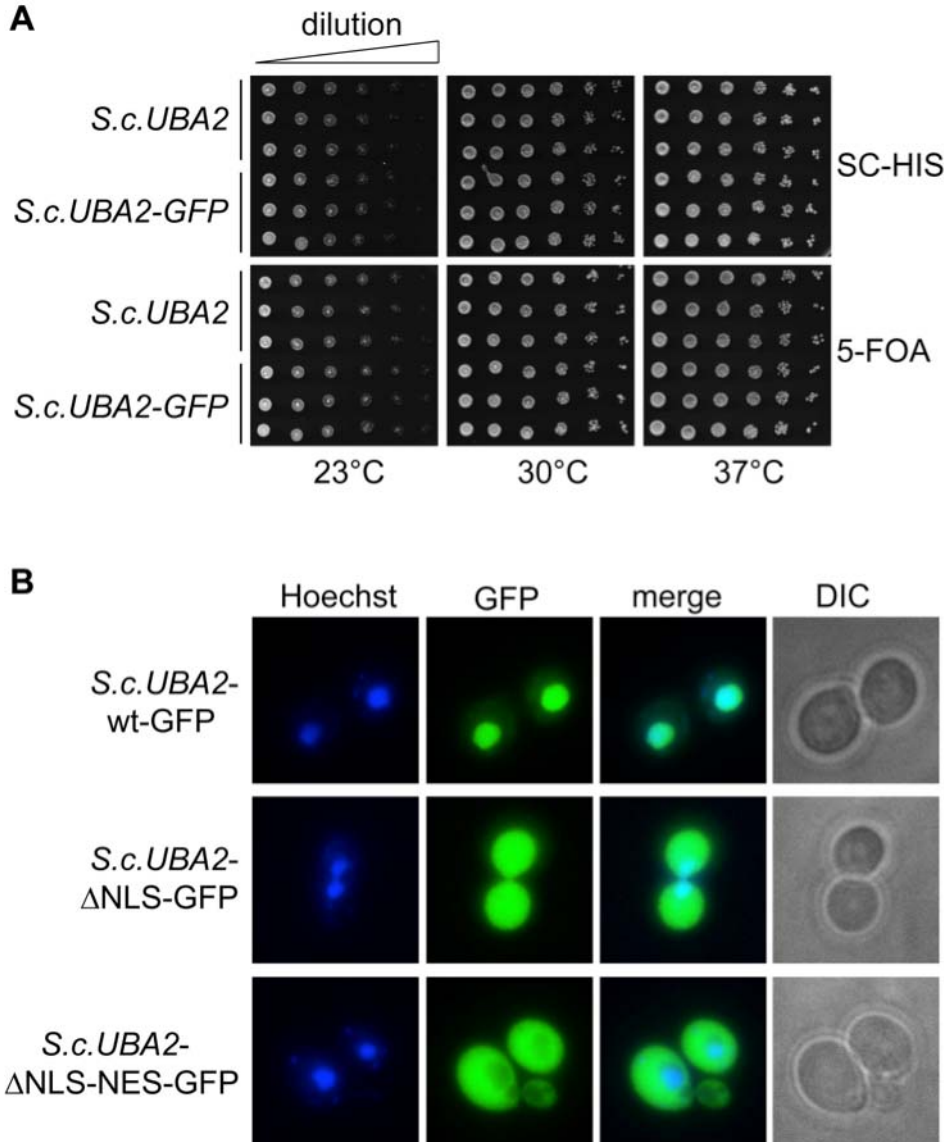


**Figure 32: Localization of *S.c.*Uba2-GFP in *UBA2* shuffle strains.** (A) Influence of a C-terminal GFP-tag on the functionality of Uba2 was analyzed by comparing the rescue ability of *S.c.*Uba2 and *S.c.*Uba2-GFP in the *UBA2* shuffle strain by growth on 5-FOA plates. (B) Variants of Uba2 were expressed in the shuffle strain without background of endogenous Uba2 after selection on 5-FOA plates. Abbreviations accord to the following variants: wild type (wt), KRTK619-622AATA (ΔNLS) and ΔNLS with an additional NES in the C-terminus of Uba2 (ΔNLS-NES). Cell cultures were harvested, DNA was stained with Hoechst (blue) and intracellular distribution of Uba2-GFP (green) was analyzed by fluorescence microscopy.

A comparison of the rescue by untagged and by C-terminally GFP-tagged yeast Uba2 (Fig. 32A) revealed that the GFP-tag, which was introduced for intracellular detection, did not alter viability of the strain. Then, fluorescence microscopic analyses of shuffle strains expressing GFP-tagged yeast Uba2 variants were performed to illustrate the intracellular localization of the variants. While the wild type protein was exclusively located in the nucleus, visualized by Hoechst staining of the DNA, the NLS mutant Uba2-ΔNLS mislocalized to the cytoplasm (Fig. 32B). However, the NLS mutant was additionally detected in the nucleus of the yeast cells. This partial nuclear localization of overexpressed Uba2-ΔNLS might be due to the fact that the NLS (according to the mapped NLS of the human homolog) was only partially mutated, and is conform with the localization of overexpressed human Uba2 NLS mutants in HeLa cells (Fig. 16). Since I aimed to analyse the phenotype of only cytoplasmically located Uba2, I created a variant that contains a mutated NLS and an additional nuclear export signal (NES). This Uba2 variant, Uba2-ΔNLS-NES, indeed mainly localized to the cytoplasm, which can be seen by the negative staining of the nucleus (Fig. 32B).

After ensuring cytoplasmic localization of the Uba2 variants, the variant strains were tested for rescue of the disruption of endogenous *UBA2* (Fig. 33). All strains showed comparable growth on SC-HIS plates verifying equal numbers of spotted cells. Unlike the negative control with human Uba2, the three strains expressing differently localizing yeast Uba2 variants grew similarly on 5-FOA, indicating that the intracellular distribution of Uba2 and thereby of SUMO E1 does not influence the viability of yeast under normal conditions.

Several examples from the literature document that the level of SUMO modification can change in response to stress (Saitoh et al. 2000; Kurepa et al. 2003; Zhou et al.

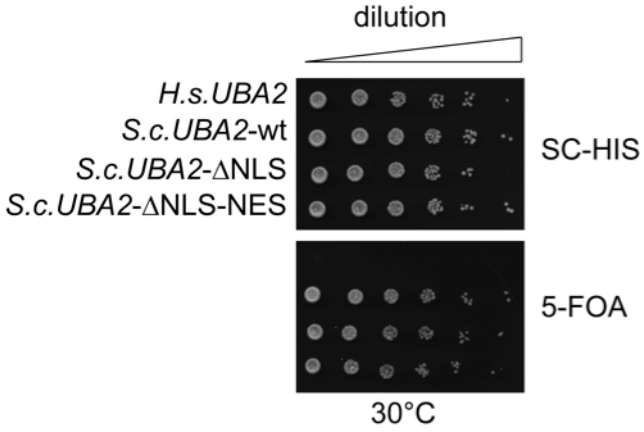


**Figure 33: Cytoplasmic *S.c.*Uba2 rescues the deletion of endogenous Uba2 in yeast.** Serial dilutions of logarithmic growing shuffle strains with indicated genotypes were spotted onto plates and incubated for 2 days at 30 °C. Rescue for deletion of endogenous Uba2 was analyzed without the background of genomic Uba2 on 5-FOA plates with cells expressing *H.s.*Uba2 serving as negative control.

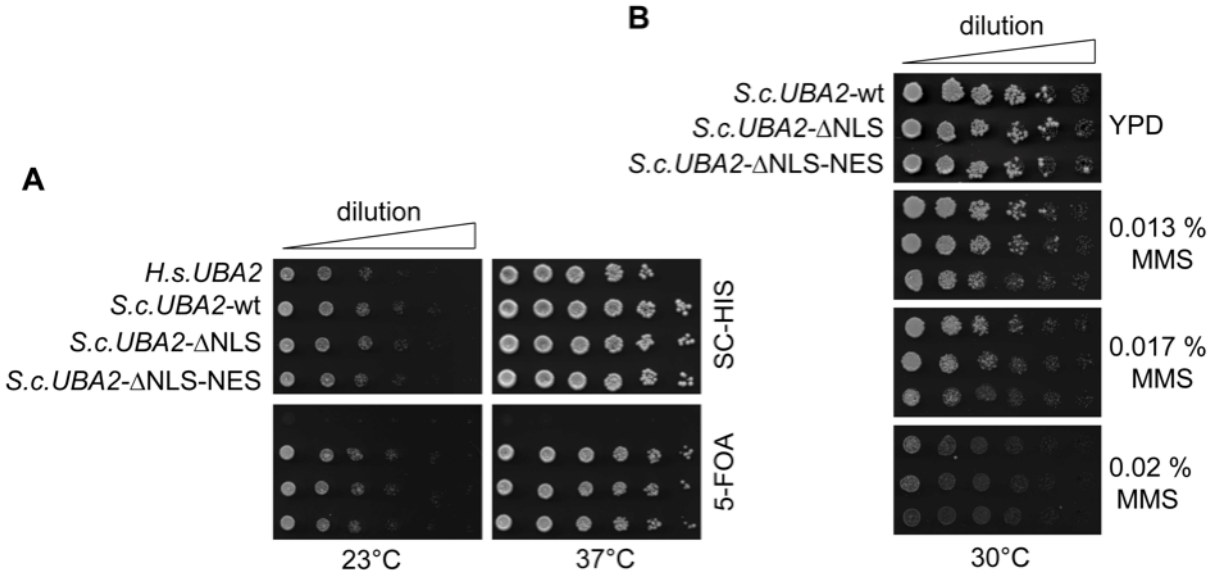


**Figure 34: Viability of yeasts with cytoplasmically localized Uba2 is not altered by suboptimal temperatures or increased DNA damage.** Serial dilutions of *UBA2* shuffle strains with indicated genotypes were spottet onto plates and incubated for 2 days. Growth tests under conditions of suboptimal temperatures were performed on 5-FOA plates at 23 or 37 °C (A). Growth under increased DNA damage stress was tested on YPD plates supplemented with different indicated amounts of the DNA damaging reagent MMS at 30 °C (B).

2004; Golebiowski et al. 2009; reviewed in Bossis et al. 2006; Tempe et al. 2008). A very recent study of rice SUMOylation showed the accumulation of high levels of SUMO conjugates under conditions of cold stress and salt stress (Chaikam et al. 2010), suggesting an interesting role of SUMOylation in handling stress conditions. Due to the role of SUMOylation in stress-response, I wanted to test whether the viability of yeast strains exposed to stress conditions is altered when the SUMO E1 is predominantly localized in the cytoplasm. Growth of the shuffle strain expressing partially or completely cytoplasmically localizing Uba2 (*S.c.UBA2*-ΔNLS, *S.c.UBA2*-ΔNLS-NES) was not altered compared to nuclear wild type Uba2 (*S.c.UBA2*-wt) when cells were subjected to conditions of suboptimal temperatures (Fig. 34A). Furthermore, the ability of the *UBA2* variants to rescue disruption of endogenous *UBA2* was tested under conditions of different intensities of DNA damage stress caused by growth on MMS-containing medium (Fig. 34B). Again, no drastic differences in viability of strains with nuclear versus cytoplasmic SUMO E1 were detected. Taken together, these results indicate that a predominantly nuclear localization of Uba2 and therefore of the SUMO E1 complex is not essential for the viability of *Saccharomyces cerevisiae*.

## 5.3. Cytoplasmic localization of Uba2 in yeast does not significantly alter the SUMOylation pattern

Since the E1 localization had no obvious effects on the viability of yeast, the question emerged whether SUMO modification is altered at all by mislocalization of E1. Published data, based on studies with a C-terminally truncated Uba2 fragment, indicate that the overall SUMOylation pattern in crude yeast cell lysate is slightly altered (Lois et al. 2005). However, I wanted to verify whether SUMOylation in the nucleus or the cytoplasm is altered specifically by mislocalization of the E1 enzyme. To address this question, I analyzed the Smt3 (yeast SUMO) conjugation pattern in nuclear and cytosolic fractions of yeast strains expressing nuclear (*S.c.UBA2*-wt) or cytoplasmic localized Uba2 (*S.c.UBA2*-ΔNLS, *S.c.UBA2*-ΔNLS-NES).

Figure 35 shows the analysis of protein equivalents of whole cell lysates (I), nuclear (N) and cytosolic fractions (C) of the indicated strains. The nuclear protein Nop1 (nucleolar protein 1) and the cytoplasmic protein PGK1 (phosphoglycerate kinase 1) were used as marker proteins to control for proper fractionation. Surprisingly, in 3 independent experiments, the overall level of Smt3 modified species in *S.c.UBA2*-wt strains were lower compared to the strains expressing the Uba2 mutants. However the reasons are still not understood. Nevertheless a longer exposure of the blot allowed to compare the Smt3 conjugation patterns.

The patterns of *S.c.UBA2*-ΔNLS and *S.c.UBA2*-ΔNLS-NES only showed marginal differences in the intensities of the two bands at 120 kDa and 110 kDa in the cytosolic fraction (marked with *****). The pattern of the wild type shuffle strain also showed low intensity of these two bands and furthermore completely lacked a 35 kDa band (marked with #) present in all fractions of the mutant strains. Besides these differences the overall ratio of Smt3-modified species in nuclear versus cytoplasmic fractions was similar in the tested strains, confirming findings from previous studies with C-terminally truncated Uba2 by Lois et al. (Lois et al. 2005).

Altogether, these results indicate that the localization of the SUMO E1 does not dramatically influence SUMOylation in the nucleus or in the cytoplasm. Since immunoblotting only allows detection of abundant SUMO targets, mass spectrometry analysis will be required for complete elucidation of effects caused on the level of individual targets.

How can it be that SUMOylation in the nucleus and in the cytoplasm is independent of the localization of the essential E1 enzyme? One attractive hypothesis is that the

SUMO E2 enzyme Ubc9 shuttles between both compartments in its free and in its thioester-charged form (for details see chapter *DISCUSSION*). This will be a topic for future investigations.

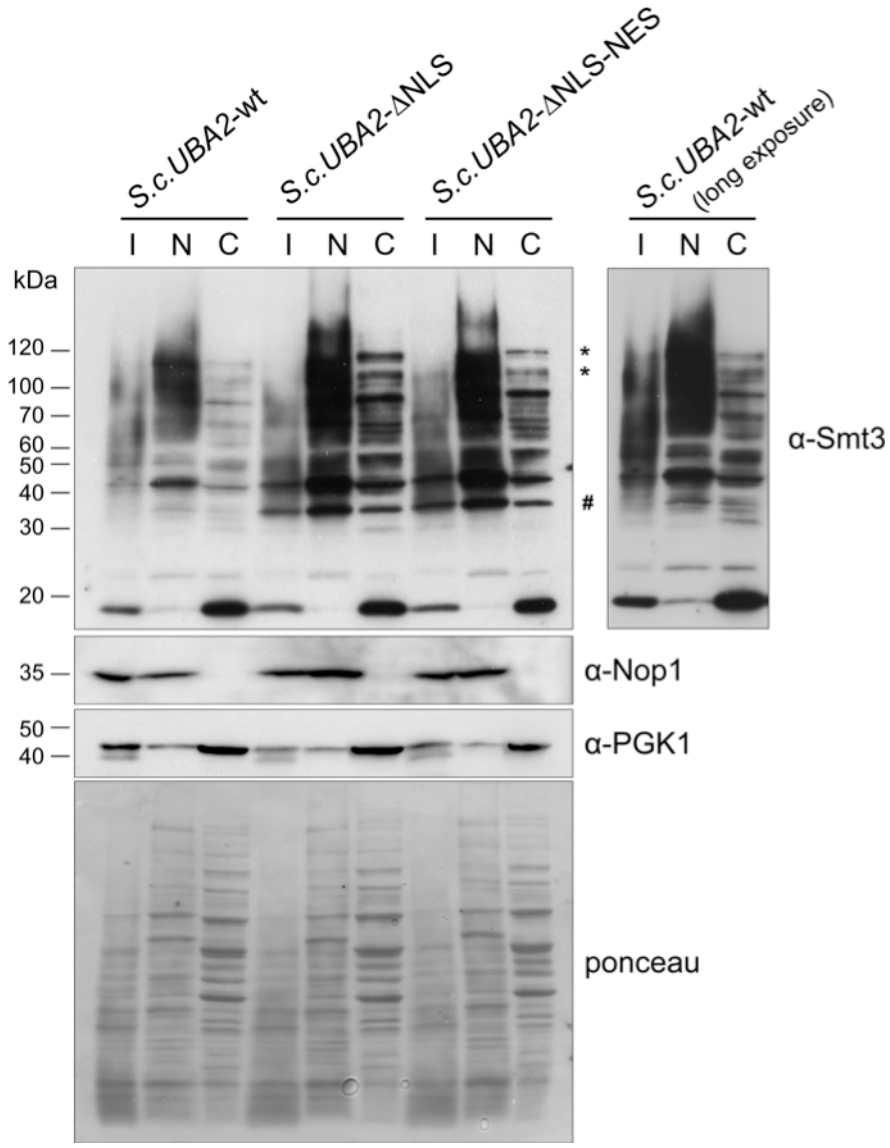


**Figure 35: No significant changes in SUMO-pattern of nuclear and cytosolic fractions upon cytoplasmic localization of Uba2 in yeast.** Cultures of *UBA2* shuffle strains with indicated genotypes were harvested and treated with zymosan. Resulting spheroblasts were homogenized and fractionated into nuclear and cytosolic fractions by centrifugation. Protein equivalents of input (I), nuclear (N) and cytosolic (C) fractions were subjected to SDS PAGE and analyzed by immunoblotting with α-Smt3, α-Nop1 or α-PGK1 antibody. A longer exposure of the wild type samples from the same blot allows better comparison.

# DISCUSSION

The heterodimeric Aos1/Uba2 complex is to date the only enzyme which is known to activate SUMO proteins. The work presented in this thesis aims to better understand the mechanisms that underlie the distinct intracellular localization of Aos1 and Uba2. Consistent with previous data I could demonstrate that the E1 complex predominantly localizes to the nucleus of cells whereas the cytoplasmic fraction of the enzyme is very small. The separation of the cellular SUMO E1 into a cytoplasmic and a nuclear pool served as a starting point to characterize the molecular mechanisms underlying the generation of these pools. In my studies I focused on the characterization of the nuclear import, which led to the identification of two ways of generating nuclear E1 complex. Additional investigations on potential nuclear export of the enzyme indicated that there is no frequent exchange between the two E1 pools. In combination with the fact that the cytoplasmic pool of Aos1 and Uba2 is very small, this led to the conclusion that the cytoplasmic SUMO activating activity is constantly low raising the question whether the E1's intracellular localization is at all important for SUMOylation in different cellular compartments.

In the following section some important details and interesting aspects arising from the presented results will be discussed in more detail.

## 1. Aos1 contains a c-Myc like NLS

Mutational analysis of the E1 subunit of Aos1 revealed that nuclear accumulation of Aos1 requires a cluster of three basic amino acids $^{195}$KRAK$^{198}$ (Fig. 12, upper lane). This essential cluster exactly matches the consensus sequence K-K/R-x-K/R of classical monopartite NLSs (Chelsky et al. 1989). Interestingly, further analysis of Aos1 revealed that the adjacent residues Leu-199 and Asp-200 also participate in the import of Aos1 (Fig. 12, upper lane). Hence, the identified NLS of Aos1 shares most residues of the conserved motif PxxKRxKLD of the unconventional NLS of the oncoprotein c-Myc ($^{320}$PAAKRVKLD$^{328}$) (Stone et al. 1987; Dang et al. 1988). While Pro-320 in the c-Myc NLS has been shown to be required for nuclear accumulation (Makkerh et al. 1996), the substitution of the corresponding Pro-192 in the sequence of Aos1 ($^{192}$PDTKRAK$^{198}$) did not alter Aos1's intracellular distribution. An explanation for the apparent discrepancy could be varying roles of the corresponding prolines in

the two motifs: The Pro-320 in c-Myc might be a necessary element to ensure structural flexibility required for the interaction with importin α, whereas in Aos1 this structural function might simply be carried out by other residues. In line with this hypothesis, crystallographic analysis by the laboratory of John Kuriyan revealed that the main interactions between the c-Myc NLS peptide ($^{319}$GPAAKRVKLDS$^{329}$) and importin α occur via the cluster $^{323}$KRVKL$^{327}$ (Conti et al. 2000). This motif binds in an extended conformation to the binding pockets of the large binding site of importin α forming the maximal number of hydrogen bonds possible and a number of salt bridges. In contrast, the hydrophobic N-terminal residues of c-Myc's NLS ($^{320}$PAA$^{322}$) are only anchored by a single hydrogen bond and are therefore likely to not mediate crucial interactions. Regarding Aos1, the crystal structure of SUMO E1 complex revealed that the identified NLS ($^{195}$KRAKLD$^{200}$) is located in a flexible loop (Lois et al. 2005). Due to insufficient electron density, the loop between Thr-179 and Glu-203 is missing in the solved structure of Aos1, indicating that the loop has a high structural flexibility. Since the loop already begins more than 10 amino acids N-terminal of the NLS, the flexibility may depend on structural elements other than Pro-192.

Altogether, the data from crystallographic analyses of the NLSs of c-Myc and of Aos1 support the suggestion that the prolines adjacent to the basic cores of the NLSs probably have different functions. It would be interesting to test whether an intermolecular exchange of the NLSs in c-Myc and Aos1 would result in an altered sensitivity of the nuclear import of the proteins towards mutation of the respective proline residues.

## 2. Nuclear E1 is generated in two ways

The characterization of the nuclear import of SUMO E1 resulted in a model schematically illustrated in Figure 21. According to this model active nuclear E1 can be generated in two ways: One possibility is the independent import of the single subunits via their distinct import signals by importin α/β and subsequent assembly of active Aos1/Uba2 complex inside the nucleus (Fig. 21, left). The second mechanism allows the import of pre-assembled E1 complex via the NLS of Uba2, which is also mediated by heterodimeric importin α/β (right).

Nuclear import of Aos1 and Uba2 is required in two scenarios, after *de novo* protein biosynthesis and after breakdown and reformation of the nucleus during mitosis. The model for E1 import allows for a solution for both situations: After *de novo* synthesis of the subunits in the cytoplasm Aos1 and Uba2 could be imported independently by their distinct NLSs followed by E1 complex formation. On the other hand, re-import of already assembled E1 complex after mitosis can occur via the NLS of Uba2 and thereby makes disassembly before import and reassembly after import unnecessary.

Biochemical and cell biological analyses revealed that the interaction of importin $\alpha/\beta$ with already formed E1 complex occurs exclusively via the NLS of Uba2, whereas the NLS of Aos1 does not mediate importin-E1 interactions. This observation may be explained by altered accessibilities of the NLSs in the single subunits compared to assembled E1 complex. The crystal structure of the Aos1/Uba2 complex, solved by the laboratory of Chris Lima allows comparing the spatial positions of the NLSs of Aos1 and of Uba2 (Fig. 36) (Lois et al. 2005):

In the case of Uba2 the whole C-terminal domain from amino acid 551 on has not been solved in the structure, which is why the closest residue Ala-550 must serve as a marker for the approximate position of this domain. Fig. 36A gives a rough idea of the potentially exposed position of the missing domain adjacent to the UbL-domain of Uba2. The relatively long distance of this domain from the interaction sites of Aos1

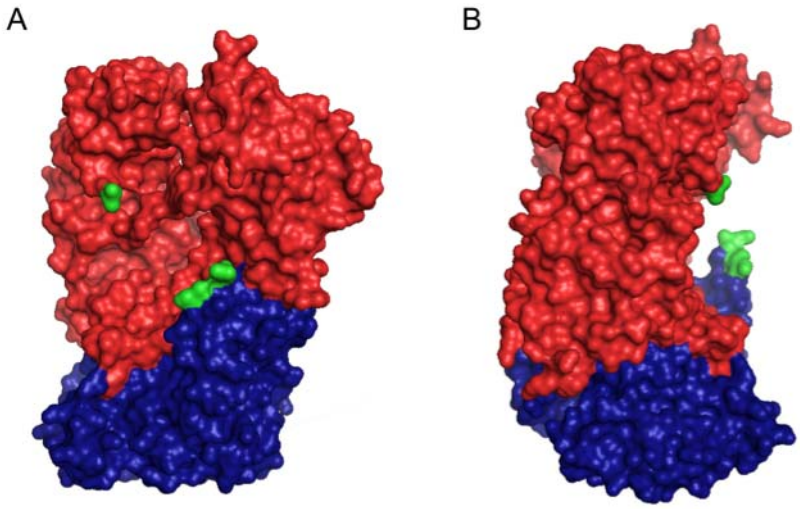


**Figure 36: Crystal structure of Aos1 and Uba2.** Illustration of the crystal structure of heterodimeric Aos1/Uba2 complex solved by Lois et al. (Lois et al. 2005). Aos1 is coloured in blue with Thr-179 and Glu-223, which flank the missing loop with the identified NLS, marked in green. Uba2 is shown in red and the C-terminal Ala-550 of the solved Uba2 fragment highlighted in green. (A) and (B) represent different views on the same complex. The raw data were obtained from the Protein Data Bank (PDB) and images were generated with PyMOL v0.99 (DeLano Scientific LLC).

and Uba2 suggests that the accessibility of Uba2's NLS is probably not altered by complex formation.

In the case of Aos1, the loop in which the NLS is located was also too flexible for detection. Therefore, the adjacent amino acids Thr-179 and Glu-223 indicate the position of the loop (Fig. 36B). The structure of the E1 complex shows that the NLS of Aos1 extends into a cavity-like topology formed by both subunits. In fact, this hidden position of the Aos1 NLS Aos1 is even more drastic than displayed since the missing domain of Uba2 is just above this region of Aos1. Due to this direct surrounding of the Aos1 NLS by Uba2, it is obvious, that formation of the E1 complex causes masking of the NLS of Aos1.

Consequently, the accessible position of the Uba2 NLS and the hidden localization of the Aos1 NLS in assembled E1 complex provide a likely explanation why only the Uba2 NLS is capable of mediating interaction of formed SUMO E1 complex with importin $\alpha/\beta$.

## 3. How is the cytoplasmic E1 pool generated?

Immunofluorescent analyses by epi- and confocal fluorescence microscopy indicate that Aos1 and Uba2 are almost exclusively located in the nuclei of cells (Fig. 22) (Dohmen et al. 1995; Azuma et al. 2001; Pichler et al. 2002; Pichler et al. 2004). However, the analysis of subcellular fractions of HeLa cells and hippocampal neurons performed within this work, in line with fractionation of RAW264.7 macrophages by Dr. Guillaume Bossis (Bossis et al. 2006), emphasize that a portion of Aos1 and Uba2 is located in the cytoplasm of cells. The existence of a cytoplasmic SUMO E1 pool immediately raises the question how this pool is generated.

Analogous to its nuclear import, Aos1 and Uba2 could be exported from the nucleus into the cytoplasm. Since data obtained within this work indicate that active export via the main export receptor CRM1 does most likely not occur, I also investigated the general behaviour of Aos1 regarding nucleocytoplasmic shuttling. The applied method of combined FRAP and FLIP in homopolykaryons is a state-of-art method for the analysis of a slow shuttling reporter construct. However, in addition to the lack of a positive control for the performed experiment, the experimental setup itself was not well suited to analyze E1 shuttling: The assumption that most cellular Aos1 and Uba2 exists in form of a stable heterodimeric complex suggests that potential shuttling

would occur as heterodimeric Aos1/Uba2 complex. For this reason the experiment should be set up such that potential shuttling of E1 complex can be determined. In the performed experiment this was not ensured. Since Aos1 alone is rather stable, the performed experiment with the overexpressed CFP-Aos1 reporter protein served to analyze single Aos1 rather than the Aos1/Uba2 complex. In contrast to monomeric Aos1, single Uba2 has been shown to be strongly degraded, whereas complex formation with Aos1 protects it from degradation (Boggio et al. 2007). Therefore, expression of Uba2 likely results in low levels of expressed Uba2, which would mostly exist in a complex with Aos1. Consequently, instead of CFP-Aos1, an Uba-YFP reporter construct or co-expression of both subunits would be more suited for FRAP/FLIP experiments to find out if the E1 complex shuttles between nucleus and cytoplasm.

Inhibition of nuclear import by posttranslational modifications displays an additional potential mechanism to generate cytoplasmic SUMO E1. Selective inhibition of nuclear import of target proteins could for example result from phosphorylation of residues adjacent to NLSs which have been shown to decrease the affinity for importin $\alpha$ (Kaffman et al. 1999; Harreman et al. 2004). Since the Uba2 NLS is required for nuclear import of SUMO E1 complex, masking of the Uba2 NLS would be sufficient to keep active E1 in the cytoplasmic compartment. And indeed, both basic clusters of the Uba2 NLS contain adjacent serine residues that could potentially be phosphorylated. In addition, SUMOylation itself presents another intriguing possibility to regulate the localization of the E1 enzyme. Interestingly, the lysines 623 and 630 of Uba2, which are located in (Lys-623) or adjacent (Lys-630) to the second cluster of the identified NLS, have been shown to function as a SUMO acceptor site *in vitro* (Hsiao et al. 2009). This finding immediately raises the question whether SUMOylation itself might influence the intracellular distribution of the E1 enzyme. Hence, it would be of special interest to investigate the posttranslational modification of Aos1 and Uba2, especially their cytoplasmic pools, in detail.

An alternative mechanism for potential cytoplasmic retention of E1 could be specific interactions with proteins that recruit E1 complex to the cytoplasm. Interaction of cytoplasmically localized proteins with Aos1 or Uba2 could, due to the high stability of assembled E1 complex, retain the whole SUMO E1 complex in the cytoplasm. Potential candidates could for example be the Rab6-interacting protein 2 (ELKS) that has previously been shown to interact with Aos1 (K. Chmielarska, PhD thesis) or the

partially cytoplasmic protein DJ-1 (PARK7), which interacted with Uba2 in a yeast two-hybrid screen by Eunsung Junn and his coworkers (Junn et al. 2005). However, co-immunoprecipitation experiments performed within this work failed to confirm the potential interaction of DJ-1 and Uba2 (data not shown). For the identification of new interaction partners of the SUMO E1 I set up yeast two-hybrid screens with full length Aos1 and full length Uba2 as baits. Unfortunately, the screens carried out by Dr. Manfred Kögl from the Genomics and Proteomics Core Facilities of the DKFZ in Heidelberg did not result in any potential interaction partner for Aos1 or Uba2 (data not shown).

Altogether, neither previous data nor analyses performed within this work give any indication which mechanism underlies the constitution of cytoplasmic SUMO E1. Consequently, the question how cytoplasmic E1 enzyme is generated remains open.

## 4. Is the E1's intracellular distribution subjected to regulation?

The regulation of SUMOylation on the level of the enzymatic machinery has to date, unlike regulation on the level of individual targets, hardly been characterized. Potential mechanisms could alter the activity, abundance or localization of SUMO enzymes and thereby regulate SUMOylation in total (reviewed in Bossis et al. 2006). Various cellular stresses like heat shock (Saitoh et al. 2000), osmotic stress (Kurepa et al. 2003), oxidative stress (Zhou et al. 2004; Bossis et al. 2006) or genotoxic stress (Mao et al. 2000; Mo et al. 2002; Park et al. 2005; Wang et al. 2005; Sacher et al. 2006) as well as hibernation (Lee et al. 2007) have already been shown to generally affect SUMOylation (reviewed in Tempe et al. 2008).

Regarding the E1 enzyme, altered abundance of Aos1/Uba2 by the CELO adenovirus (Boggio et al. 2005) and altered activity induced by oxidative stress (Bossis et al. 2006) have been reported to decrease overall SUMOylation. In addition, previous studies on the intracellular localization of the Uba2 homolog from *Drosophila melanogaster* revealed drastic changes in the localization of *D.m.*Uba2 in different developmental stages (Donaghue et al. 2001; Shih et al. 2002). Whereas Uba2 is initially distributed throughout the whole cell it has been shown to gradually enrich in the nucleus during embryogenesis. Considering the fact that most human Uba2 exists in form of assembled E1 complex (Azuma et al. 2001), this finding points towards an interesting and so far unidentified regulation of the intracellular

localization of Aos1/Uba2. Besides this developmental change of the intracellular distribution, additional mechanisms may exist that also affect the localization of Aos1/Uba2 in non-deviding cells.

Different localization of the SUMO E1 could for example be triggered by a specific stimulus like cellular stresses, some of which have been shown to inhibit classical nuclear import pathways (Stochaj et al. 2000; Kodiha et al. 2009). However, preliminary analysis of the effect of $H_2O_2$ suggests that oxidative stress does not influence the distribution of Aos1 or Uba2 (data not shown).

Alternatively, intracellular localization could also be a cell type-specific phenomenon. In line with a regulatory extranuclear role of SUMOylation in neuronal cells (Martin et al. 2007), Aos1 and Uba2 are found in the cytosolic synaptosomal fraction of hippocampal neurons (Fig. 24). Unfortunately, further analysis of the localization of Uba2 in tissue slides of murine brain did not allow determining precisely the intracellular localization of Uba2 (Fig. 25).

In conclusion, changes in the localization of Uba2 during embryogenesis of *Drosophila melanogaster* demonstrate that regulation of the distribution of Aos1/Uba2 exists. However, it remains to be elucidated whether the localization of E1 complex is subjected to additional regulations and which molecular mechanisms underly the regulated localization of the SUMO E1 observed in fruit fly.

## 5. Can the localization of Aos1 and Uba2 be seperately regulated?

In contrast to the monomeric ubiquitin E1 the SUMO E1 is a heterodimeric complex. This dimeric composition easily seduces to speculations about seperate regulations or even additional functions of the two subunits Aos1 and Uba2. Previous studies by the laboratory of Mary Dasso revealed differences in the levels of Aos1 during cell cycle, pointing towards a seperate regulation of the abundance of the two subunits (Azuma et al. 2001). Yet, differential regulation of the localizations or even additional functions of the single SUMO E1 subunits have to date not been reported. However, the identification of distinct functional NLSs in both Aos1 and Uba2 offers the possibility of seperate regulations of the intracellular localization of the subunits. A prerequisite for different localizations of the subunits would be the availability of the proteins as single subunits. Microinjection experiments with assembled E1 complex performed within this work documents a high stability of the Aos1/Uba2 complex (Fig.

17B). E1 composed of wild type Aos1 and NLS mutant Uba2 did not accumulate in the nucleus after microinjection in the cytoplasm of cells. Since dissociation of the E1 complex would have resulted in import of the wild type Aos1 subunit, this result indicates that the Aos1/Uba2 heterodimer is a stable complex in the environment of a living cell. Additionally, gel filtration analysis of cell extracts revealed that Aos1 and Uba2 co-migrate in a complex, demonstrating that the vast majority of both subunits exists in form of assembled E1 complex (Azuma et al. 2001). The conclusion that most cellular Aos1 and Uba2 exist in form of a stable heterodimeric E1 complex reasons that the accessibility of Aos1 and Uba2 as single subunits is very limited. Hence, interactions of single Aos1 or Uba2 with other proteins that could perhaps seperately regulate their localization or regulate other additional functions are rather unlikely. However, it remains possible that interactions with other proteins might induce the dissociation of the E1 complex and thereby enable so far unknown regulations or functions of the SUMO E1 subunits.

## 6. Is cytoplasmic E1 a prerequisite for cytoplasmic SUMOylation?

The amount of E1 in the cytoplasm is usually very small compared to the nuclear pool. In light of the fact that SUMOylation is an essential cellular process, the differences in the levels of E1 enzyme raised the fundamental question, whether the E1's intracellular distribution is important for cells. Possibly, the vast amount in the nucleus may be needed for nuclear SUMOylation or cells may only tolerate low amounts of E1 in the cytoplasm to avoid unspecific SUMOylation.

A previous study by the laboratory of Dohmen on the Uba2 homolog from *Saccharomyces cerevisiae* revealed that a fragment of *S.c.*Uba2$_{1\text{-}554}$ lacking 82 amino acids at the C-terminus, overexpressed under Gal1-promoter, is able to rescue the deletion of endogenous *UBA2*, even though the fragment mislocalized to the cytoplasm (Dohmen et al. 1995). A more sensitive phenotypic analysis of the *S.c.*Uba2$_{1\text{-}602}$ fragment under the control of the natural *UBA2* promoter was performed in the laboratory of Lima. Expression of the fragment that only lacked the last 34 residues of *S.c.*Uba2 (including both basic clusters according to the mapped NLS in human Uba2) fully rescued the deletion of endogenous *UBA2* gene but resulted in slightly altered Smt3 conjugation pattern of cell lysate (Lois et al. 2005). In contrast to these studies, Del Olmo and his co-workers observed that partial

disruption of the *UBA2* gene, resulting in *S.c.*Uba2$_{1-594}$ - a fragment lacking 42 C-terminal residues, leads to a 2fold slower growth and larger cells compared to wild type yeast strains (del Olmo et al. 1997).

In line with the previous findings by Dohmen and Lima, the data obtained with the NLS-mutant *S.c.*Uba2-ΔNLS strongly suggest that a predominant cytoplasmic localization of the E1 does not alter the viability of yeast under normal or stress conditions (Fig. 33, 34). However, even the variant *S.c.*Uba2-ΔNLS-NES, which contained a disrupted NLS and an additional NES, was partially detected in the nucleus of cells (Fig. 32). In line with this finding, the study of Dohmen demonstrated that the C-terminal deletion fragment of *S.c.*Uba2, which lacked both basic clusters corresponding to the mapped NLS in *H.s.*Uba2, localized to both nucleus and cytoplasm (Dohmen et al. 1995). Therefore it can be assumed, that a partial nuclear localization of Uba2 very likely also underlied the studies by Lima and Del Olmo, in which the intracellular localization of the Uba2 was not explicitly tested (del Olmo et al. 1997; Lois et al. 2005). Consequently, the residual nuclear amount of E1 enzyme may explain the consistent findings that yeast strains with predominantly cytoplasmic Uba2 were viable and had no significant changes in the Smt3-modification pattern. To study the effect of E1 solely present in the cytoplasm, I already began cloning of an Uba2 variant with a cytoplasmic membrane anchor. Such a variant would exclude the localization of any residual E1 in the nucleus and would therefore allow the phenotypic analysis of exclusively cytoplasmically localized E1.

If it proves true that the intracellular localization of SUMO E1 does indeed not significantly influence SUMOylation in the nucleus or in the cytoplasm, what does this mean for the mechanisms underlying SUMOylation in these two compartments?

In the case of exclusively nuclear localized E1 (scenario A), SUMO-charged E2 enzyme Ubc9 would have to be exported into the cytoplasm to provide sufficient SUMOylating activity for modification of cytoplasmic targets (Fig. 37). Subsequently, the empty Ubc9 would be imported into the nucleus for another round of loading with SUMO by the nuclear localized E1. In the artificially created situation of exclusively cytoplasmic localized E1 (scenario B; tested in yeast strains), Ubc9 would have to be loaded in the cytoplasm and be imported into the nucleus for ensuring sufficent SUMO modifying activity in the nuclear compartment. After transfer of SUMO to a nuclear target, the empty Ubc9 would be exported to the cytoplasm for another round of SUMO transfer. If it proves true that both scenarios A and B do not cause drastic

changes of SUMOylation in either compartment, it could be deduced that both uncharged Ubc9 as well as SUMO-charged Ubc9 can shuttle in and out of the nucleus. This attractive model has some precedence, as ubiquitin was reported to serve as an import signal for the class III ubiquitin E2 enzyme UbcM2 (Plafker et al. 2004). Nuclear import of UbcM2 and interaction with its import receptor importin 11 were shown to depend on the charging of UbcM2 with ubiquitin.

Interestingly, the transport receptor importin 13 that has been shown to mediate nuclear import of Ubc9 has also been reported to mediate nuclear export of the eukaryotic initiation factor eIF1A (Mingot et al. 2001). This unusual double function would make importin 13 a potential candidate to mediate shuttling of Ubc9. However, since the presence of RanGTP has been shown to interfere with Ubc9/importin 13 complex formation, an alternative mechanism at least for the export of empty Ubc9 would be likely. Furthermore it would be interesting to analyze whether and how the according transport receptor(s) can distinguish between the empty and SUMO-charged form of Ubc9.

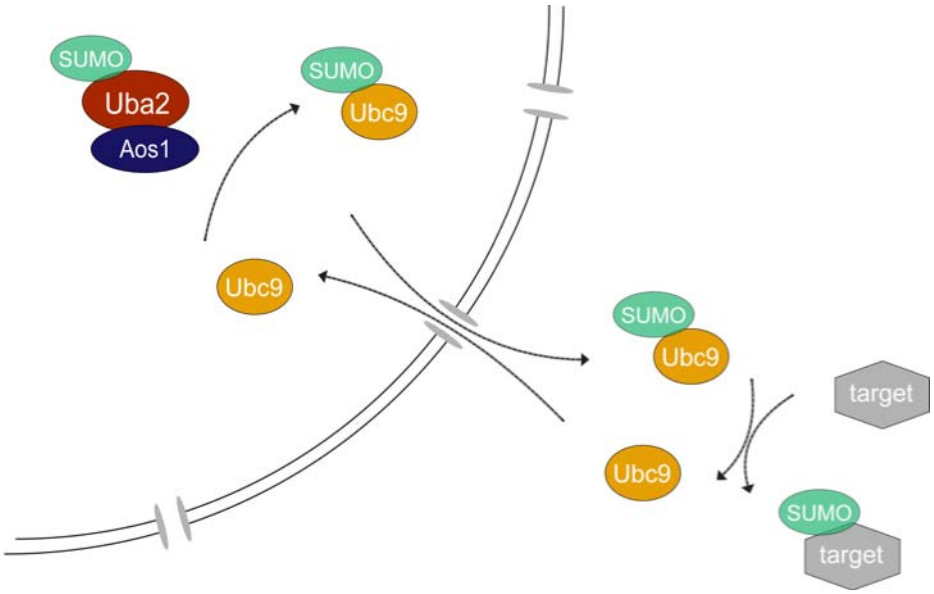


**Figure 37: Working model of the nucleocytoplasmic transport of Ubc9.** In the case of exclusively nuclear E1 the E2 Ubc9 is loaded with SUMO inside of the nucleus. Ubc9-SUMO is exported into the cytoplasm where it is required for SUMOylation of cytoplasmic targets. Subsequently, empty Ubc9 is imported to be again loaded with SUMO for another round of SUMOylation.

## 7. Perspectives

Generally, the SUMO E1 enzyme Aos1/Uba2 is unequally distributed between the nuclear and the cytoplasmic compartment. The presented work provides a detailed picture of the generation of the major nuclear pool. However, intensive studies will be needed to clarify the potential roles of active nuclear export, inhibition of nuclear import or cytoplasmic retention in the generation of the cytoplasmic pool of Aos1/Uba2.

Interestingly, preliminary results indicate that the intracellular localization of Aos1/Uba2 might not be of key importance for SUMOylation, raising the possibility that Ubc9 may play a critical role providing activated SUMO in different cellular compartments. If this proves true, it will be very interesting to further investigate the mechanisms underlying the intracellular localization of the E2 Ubc9. Here, it would be particularly interesting to study the mechanisms underlying SUMOylation of targets in the plasma membrane of synapses. The long distance from the nucleus, where most E1 and E2 is located, points towards an additional yet unknown mechanism that assures the local presence of sufficient SUMOylating activity. There, detailed analysis will be needed to elucidate whether cell type-specific or signal-triggered differences in the intracellular localization of the SUMO E1 and E2 enyzems exist.

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# SUPPLEMENTARY MATERIAL

**Supplemantary Figure 1: Sequence alignment of Aos1.** Amino acid sequences of Aos1 from *H. sapiens, M. musculus, D. rerio, X. laevis, D. melanogaster and S. cerevisiae* were aligned using ClustalW2. Conserved clusters of basic amino acids potentially participating in nuclear import are underlined and highlighted in bold.

```
H. sapiens          ---MVEKEEAGGG----ISEEEAAQYDRQIRLWGLEAQKRLRASRVLLVGLKGLGAEIAK 53
M. musculus         ---MVEKEEAGGGGGGGISEEEAAQYDRQIRLWGLEAQKRLRASRVLIVGMKGLGAEIAK 57
D. rerio            MIDTIEKEDT------IISEEEAAQYDRQIRLWGLDAQKRLRGSRVLLVGLRGLGAEVAK 54
X. laevis           ---MVEKEEA------VISEEEAAQYDRQIRLWGLEAQKRLRTSRVLLVGMRGLGAEVAK 51
D. melanogaster     ---MVVDMDTSET-AVELTEAENELYDRQIRLWGLESQKRLRTAKILIAGLCGLGAEITK 56
S. cerevisiae       ---MDMKVEK-------LSEDEIALYDRQIRLWGMTAQANMRSAKVLLINLGAIGSEITK 50
                          . :        ::* *   *********: :* .:* :::*: .: .:*:*::*

H. sapiens          NLILAGVKGLTMLDHEQVTPEDPGAQFLIRTGSVGRNRAEASLERAQNLNPMVDVKVDTE 113
M. musculus         NLILAGVKGLTMLDHEQVSPEDPGAQFLIQTGSVGRNRAEASLERAQNLNPMVDVKVDTE 117
D. rerio            NLILAGVKGLTLLDHEQVTEESRRAQFLIPVDADGQNHAQASLERAQFLNPMVEVKADTE 114
X. laevis           NLILAGVKALTLLDHEQVSSEDSRAQFLIPSGSLGQNRAEASLNRARNLNPMVSVEADTE 111
D. melanogaster     NIILSGVNSVKLLDDKDVTEEDFCSQFLVPRESLNTNRAEASLTRARALNPMVDISADRE 116
S. cerevisiae       SIVLSGIGHLTILDGHMVTEEDLGSQFFIGSEDVGQWKIDATKERIQDLNPRVELNFDKQ 110
                    .::*:*:  :.:** . *: *.  :**::     .  : :*:  * : *** *.:. * :

H. sapiens          DIEKKPESFFTQFDAVCLTCCSRDVIVKVDQICHKNSIKFFTGDVFGYHGYTFANLG-EH 172
M. musculus         DVEKKPESFFTKFDAVCLTCCSRDVIIKVDQICHRNSIKFFTGDVFGYHGYTFANLG-EH 176
D. rerio            PVESKPDDFFFQFDAVCLTRCSRDLMVRVDQLCASRNIKVFCGDVYGYNGYMFSDLGQEY 174
X. laevis           NINQKSDDFFTQFDVVCLTSCPSDLLVRVNHICHKHNIKFFTGDVYGYHGSMFADLG-EH 170
D. melanogaster     PLKEKTSEFFGQFDVVVVNGATNEELLRIDTICRDLGVKFIATDVWGTFGFYFASLQ-KH 175
S. cerevisiae       DLQEKDEEFFQQFDLVVATEMQIDEAIKINTLTRKLNIPLYVAGSNGLFAYVFIDLI--- 167
                     ::.* ..** :** *  .    :  :::: :    .: .   .  *  .  * .*

H. sapiens          EFVEEKTKVAKVSQGVEDGPDT<u>**KRAKLD**</u>SSE----------TTMV<u>**KKK**</u>VVFCPVKEALEV 222
M. musculus         EFVEEKTKVAKVSQGVEDGPEA<u>**KRAKLD**</u>SSE----------TTMV<u>**KKK**</u>VLFCPVKEALEV 226
D. rerio            HYVEEKPKVVKGSNEANDGPEA<u>**KKPKID**</u>PNE----------TTMV<u>**KK**</u>TISFCSLKEALEV 224
X. laevis           EFVEEKAKVTKAKPLVEDGPEA<u>**KKAKID**</u>PTE----------TILV<u>**KKK**</u>VQFCPLKDALEI 220
D. melanogaster     SYVEDVINHKVVAN-------SE<u>**KKKK**</u>YETV----------SIPTQRDVDYPGYSAWLDF 218
S. cerevisiae       EFISEDEKLQSVRPTTVGPISSNRSIIEVTTRKDEEDEKKTYERIKTKNCYRPLNEVLST 227
                     ::.:  :            :::     .              :    :   .  *.

H. sapiens          DWSSEKAKAALKRTTSDYFLLQVLLKFRTDKGRDPSSDTYEEDSELLLQIRNDVLDSLGI 282
M. musculus         DWSGEKAKAALKRTAPDYFLLQVLLKFRTDKGRDPTSESYKEDAELLLQIRNDVFDSLGI 286
D. rerio            DWTTEKAKSSLKRIPADYFLLQVLLKFRTDKGRDPQPDSFAEDSQLLLQIRDDVLETMGL 284
X. laevis           DWRSEKAKSALKKTPTDYFLLQVLMKFRTDKGRDPQPSSYQEDSELLLQICSDVLDSLGV 280
D. melanogaster     DVTEPSYLRKLKRNGPGVLLLSVLQKFRTTHKRDPSYKTREADLELLRGIRDELLPNS-- 276
S. cerevisiae       ATLKEKMTQRQLKR-VTSILPLTLSILQYDLNQKGKAISFEQMKRDAAVWCENLGVPATV 286
                         .      :     :*  .*  ::    :.    :     .      .::

H. sapiens          SPDLLPEDFVRYCFSEMAPVCAVVGGILAQEIVKALSQRDPPHNNFFFFDGMKGNGIVEC 342
M. musculus         SPDLLPDDFVRYCFSEMAPVCAVVGGILAQEIVKALSQRDPPHNNFFFFDGMKGSGIVEC 346
D. rerio            SSDLLPNTFVSYCFSEMSPVCAVVGGVLGQEIVKALSQRDAPHRNFFFFDGLKGSGVVDY 344
X. laevis           SPDLLPKDFASYCFSEMAPVCAVVGGVLGQEIVKALSLRDAPHNNFFFFDGKTSNGIVDC 340
D. melanogaster     ---ILGDEALGLIFAQISPAVAVVGGVVAQEVIKVVTKLEAPHRNLFVFDPETCAGYVEA 333
S. cerevisiae       VKDDYVQQFIKQKGIEFAPVAAIIGGAVAQDVINILGKRLSPLNNFIVFDGITLDMPLFE 346
                          .       :::*. *::** :.*:::: :    .* .*::.**  .    :

H. sapiens          LGPK 346
M. musculus         LGPQ 350
D. rerio            FSSK 348
X. laevis           LGSK 344
D. melanogaster     IGAK 337
S. cerevisiae       F--- 347
                    :
```

# ABBREVIATIONS

## General abbreviations

| | |
|---|---|
| aa | amino acids |
| Co-IP | co-immunoprecipitation |
| Da | dalton |
| DMEM | Dulbeccos's modified Eagles medium |
| *E.coli* | *Escherichia coli* |
| ECL | enhanced chemical luminescence |
| FCS | fetal calf serum |
| FLIP | fluorescence loss in photobleaching |
| FRAP | fluorescence recovery after photobleaching |
| FRET | fluorescence reconance energy transfer |
| *H.s.* | *Homo sapiens* |
| His- | hexahistidine tag |
| IF | immunofluorescence |
| LB | Luria-Bertani |
| MCS | multiple cloning site |
| NCS | newborn calf serum |
| NES | nuclear export signal |
| NLS | nuclear localization signal |
| NPC | nuclear pore complex |
| ORF | open reading frame |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| *S.c.* | *Saccharomyces cerevisiae* |
| *S.cerevisiae* | *Saccharomyces cerevisiae* |
| v/v | volume per volume |
| w/v | weight per volume |
| WB | western blot |
| wt | wildtype |
| ∆NLS | mutated, unfunctional NLS |

## Proteins

| | |
|---|---|
| BSA | bovine serum albumine |
| CFP | cyan fluorescent protein |
| E1 | activating enzyme |
| E2 | conjugating enzmye |
| E3 | ligase |
| GAP | GTPase acitvating protein |
| GFP | gree  fluorescent protein |
| imp | importin |
| S1 | SUMO1 |
| S2/3 | SUMO2/3 |
| SUMO | small ubiquitin related modifier |
| Ub | ubiquitin |
| YFP | yellow fluoresencent protein |

## Chemicals

| | |
|---|---|
| APS | ammonium persulfate |
| ATP | adenosine-5’-tripphosphate |
| DMSO | dimethyl sulfoxide |
| dNTP | 2’-desoxynucleoside-5’-triphosphate |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| GDP | guanosine diphosphate |
| GTP | guanosine-5’- triphosphate |
| HEPES | [4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid |
| IPTG | isopropyl-®-D-1-thiogalactopyranoside |
| LMB | leptomycin B |
| PBS | phosphate buffered saline |
| PMSF | phenylmethanesulphonylfluoride |
| SDS | sodium dodecyl sulfate |
| TEMED | tetramethylethylenediamine |
| Tris | tris(hydroxymethyl)aminomethane |

| | |
|---|---|
| TRITC | rhodamine isothiocyanate |
| Triton-X100 | 4-octylphenol polyethoxylate |
| Tween-20 | polyoxyethylene (20) sorbitan monolaurate |

## Physical units

| | |
|---|---|
| °C | degree celsius |
| g | gram |
| g | acceleration of gravity on earth |
| h | hour(s) |
| l | liter |
| m | meter |
| M | molar (mol/l) |
| min | minute(s) |
| OD | optical density |
| pH | potential hydrogen |
| rpm | rotations per minute |
| sec | second(s) |

## Prefixes

| | | |
|---|---|---|
| k | kilo- | $10^{3}$ |
| c | centi- | $10^{-2}$ |
| m | mili- | $10^{-3}$ |
| μ | micro- | $10^{-6}$ |
| n | nano- | $10^{-9}$ |
| p | pico- | $10^{-12}$ |

## Code for amino acids

| | | |
|---|---|---|
| A | Ala | alanine |
| C | Cys | cysteine |
| D | Asp | aspartate |
| E | Glu | glutamate |

| | | |
|---|---|---|
| F | Phe | phenylalanine |
| G | Gly | glycine |
| H | His | histidine |
| I | Iso | isoleucine |
| K | Lys | lysine |
| L | Leu | leucine |
| M | Met | methionine |
| N | Asn | asparagine |
| P | Pro | proline |
| Q | Gln | glutamine |
| R | Arg | arginine |
| S | Ser | serine |
| T | Thr | threonine |
| V | Val | valine |
| W | Trp | tryptophane |
| Y | Tyr | tyrosine |
| x | - | any |
| z | - | apolar residue |
| Ψ | - | bulky hydrophobic residue |

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## Publications

Werner A, **Moutty MC**, Möller U, Melchior F. *Performing in vitro sumoylation reactions using recombinant enzymes*; Methods Mol Biol. 2009; 497:187-99.

Edlich F, Maestre-Martínez M, Jarczowski F, Weiwad M, **Moutty MC**, Malesević M, Jahreis G, Fischer G, Lücke C. *A novel calmodulin-Ca2+ target recognition activates the Bcl-2 regulator FKBP38*; J Biol Chem. 2007; 282(50):36496-504.

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