SUMO-mediated Quality Control of Proteins Targeted to Mitochondria

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Eidesstattliche Erklärung

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dedicated to my mother

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SUMMARY

Posttranslational modifications by ubiquitin and ubiquitin-like proteins (UBLs) are of fundamental relevance for cellular function, regulation and development. Besides ubiquitin itself, the small ubiquitin-like modifier (SUMO) is one of the best-characterized UBLs. Protein modification by SUMO affects hundreds of cellular substrates and is crucial for the regulation of diverse physiological processes, including transcription, replication, chromosome segregation and DNA repair. Interestingly, SUMOylation has emerged as a predominantly nuclear modification but a number of cytosolic substrates have also been identified. By contrast, in the compartmentalized environment of the cell, not all proteins are accessible to SUMO enzymes. For instance, many organellar proteins such as luminal ER and intramitochondrial proteins are hidden from SUMOylation upon sorting. Particularly mitochondrial proteins are, however, often imported in a posttranslational manner. It is therefore conceivable that such proteins become SUMOylated at cytosolic stages of biogenesis. Nevertheless, the question, whether proteins transported into mitochondria are indeed SUMO substrates *in vivo* has not been experimentally elucidated so far.

Using a mass spectrometry-based approach, our laboratory identified multiple potential SUMO substrates annotated as mitochondrial proteins. Following up on these initial discoveries, I provide here a first in-depth characterization of mitochondria-targeted proteins as SUMO substrates. By analyzing the SUMOylation of individual proteins in direct assays, I could confirm that multiple mitochondrial matrix proteins are indeed modified by SUMO *in vivo*. The modification of these substrates is mediated by the SUMO E3 ligases Siz1 and Siz2 and targets both consensus and non-consensus SUMOylation sites. Consistent with the current understanding of the SUMO system, SUMOylation of mitochondria-targeted proteins is independent of their mitochondrial targeting sequences, strongly suggesting that the modification occurs prior to import. SUMO conjugation to mitochondria-targeted substrates is regulated by cytosolic HSP70 chaperones of the *SSA* subfamily, which, remarkably, not only influence the levels of SUMOylated substrates but also alter their modification in terms of site selectivity. Moreover, SUMOylated mitochondrial precursor proteins strongly accumulate in proteasome mutants and substrates conjugated by an isopeptidase-resistant SUMO variant are efficiently degraded in a proteasome-dependent manner. Thus, this study not only identifies mitochondria-targeted proteins as a novel and unprecedented group of SUMO substrates but also reveals an intriguing function of SUMO in cellular protein quality control.

1 INTRODUCTION

1.1 Protein modification by ubiquitin family proteins

Posttranslational modifications (PTMs) regulate protein function, stability and localization and thus greatly expand the functional diversity of the proteome. PTMs typically refer to the covalent attachment of small functional groups (*e.g.* phosphate, acetyl or methyl groups), fatty acids, isoprenoids or sugars to substrate proteins. A special class of posttranslational modifiers is the family of ubiquitin and ubiquitin-like proteins (UBLs) (Kerscher et al., 2006; van der Veen and Ploegh, 2012). Ubiquitin family proteins possess a highly conserved three-dimensional structure, the ubiquitin or β-grasp fold, and besides ubiquitin include the small ubiquitin-like modifier (SUMO), Rub1 (Nedd8), Atg8, Atg12, Urm1, Hub1, ISG15, UFM1, FAT10 and FUB1 (Kerscher et al., 2006). Most UBLs function as covalent modifiers and are typically attached to lysine residues of substrate proteins. One remarkable exception is the lipid modifier Atg8, which is covalently conjugated to the phospholipid phosphatidylethanolamine (Ichimura et al., 2000). Different from canonical UBLs is the protein Hub1, which acts by non-covalent binding to specific interaction partners (Ammon et al., 2014; Luders et al., 2003; Mishra et al., 2011).

1.1.1 Ubiquitin conjugation and deconjugation

Ubiquitin is synthesized *de novo* in the form of inactive precursor proteins. These ubiquitin precursors include linear polymers, in which ubiquitin units are arranged in a head-to-tail orientation (product of the *UBI4* gene in *S. cerevisiae*) (Ozkaynak et al., 1987) and single ubiquitin molecules fused to the N-terminus of ribosomal proteins (products of the *UBI1-3* genes in *S. cerevisiae*) (Finley et al., 1989). Consequently, the generation of free ubiquitin requires dedicated C-terminal hydrolases that catalyze the proteolytic processing of ubiquitin precursors. Notably, this type of maturation not only produces single ubiquitin molecules but also exposes the C-terminal double-glycine motif required for conjugation.

Ubiquitin conjugation to substrate proteins (referred to as ubiquitylation) involves a series of three enzymatic reactions (Hershko and Ciechanover, 1998) (Figure 1). First, an ATP-driven ubiquitin-activating enzyme (E1) forms a high-energy thioester bond between its active-site cysteine residue and the C-terminal carboxy group of ubiquitin. Second, ubiquitin is transferred to the catalytic cysteine of a ubiquitin-conjugating enzyme (E2) by a transesterification reaction. Third, ubiquitin is covalently attached to substrate proteins. Ubiquitylation typically involves the formation of an isopeptide bond between the C-terminal carboxy group of ubiquitin

and the ε-amino group of a lysine residue in the substrate protein. However, it has been reported that in some cases ubiquitin is conjugated to the N-terminus or alternative amino acid residues of substrate proteins (cysteine, serine and threonine) (Breitschopf et al., 1998; Shimizu et al., 2010).

Ubiquitin transfer to substrate proteins is catalyzed by ubiquitin ligases (E3), which are categorized into two classes (Figure 1). HECT ubiquitin E3 ligases contain a catalytic cysteine residue and form an E3-ubiquitin thioester intermediate before ubiquitin is attached to the substrate (Scheffner et al., 1995). RING and RING-like E3 ligases rather function as adaptor or scaffold proteins that bridge the ubiquitin-charged E2 and the substrate protein, thereby promoting the direct transfer of ubiquitin from the E2 to the substrate (Deshaies and Joazeiro, 2009).

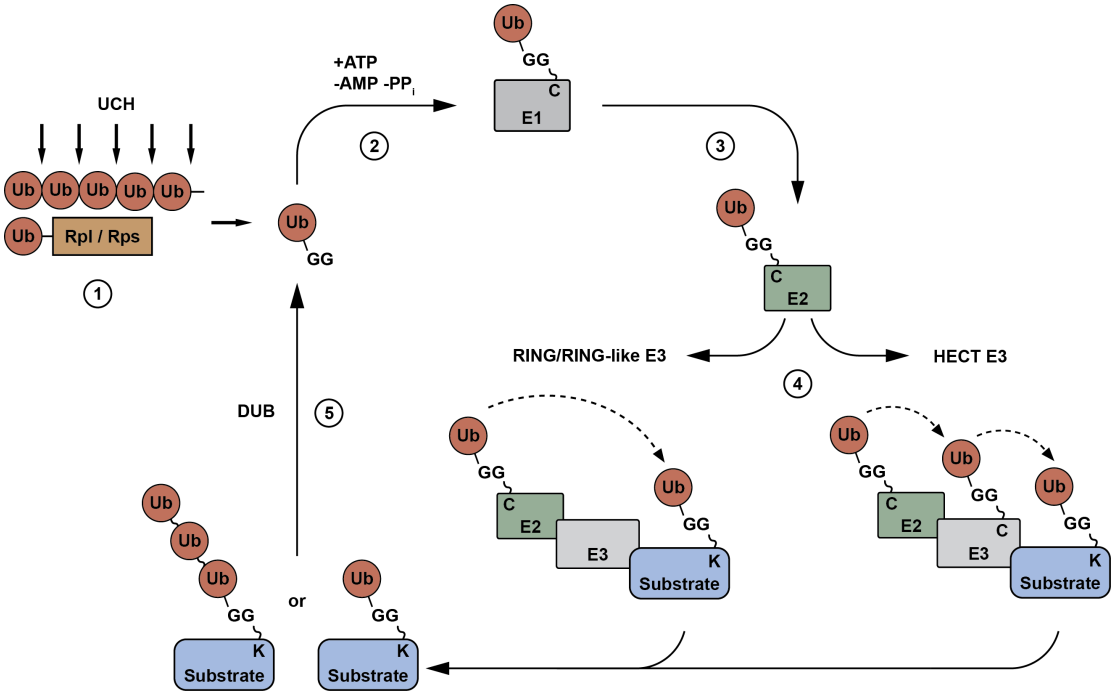


Figure 1. Ubiquitin conjugation and deconjugation. Ubiquitin (Ub) conjugation to substrate proteins involves a cascade of multiple enzymatic reactions. First, ubiquitin is translated in form of inactive precursor proteins, which comprise linear ubiquitin polymers and single ubiquitin molecules fused to ribosomal proteins of the large (Rpl) and small (Rps) ribosomal subunits. Ubiquitin precursors are processed by dedicated ubiquitin C-terminal hydrolases (UCH), which generate free ubiquitin moieties exposing the crucial double-glycine (GG) motif at their C-termini (1). Ubiquitin is then activated by a ubiquitin-activating enzyme (E1), which utilizes the chemical energy of ATP to form a high-energy thioester bond between its catalytic cysteine (C) residue and the C-terminus of ubiquitin (2). Next, ubiquitin is transferred to the catalytic cysteine residue of a ubiquitin-conjugating enzyme (E2) (3). Ultimately, ubiquitin ligases (E3) catalyze the covalent attachment of ubiquitin to lysine (K) residues of substrate proteins (4). Ubiquitin E3 ligases are categorized according to their reaction mechanisms used for ubiquitin transfer. HECT E3 ligases contain an active-site cysteine and form a thioester intermediate with ubiquitin prior to substrate ubiquitylation. RING and RING-like E3 ligases rather function as adaptor proteins that bridge the substrate protein and the ubiquitin-charged E2 in a suited orientation. Ubiquitin E3 ligases can catalyze the modification of substrate proteins with single ubiquitin moieties or polyubiquitin chains. Ubiquitylation is reversible and diverse deubiquitylating enzymes (DUBs) mediate the deconjugation of ubiquitin from substrate proteins (5).

Besides the modification with a single ubiquitin moiety (monoubiquitylation), substrate proteins can be simultaneously modified at multiple attachment sites (multiubiquitylation). Moreover, in many cases, substrate proteins are modified by polyubiquitin chains (polyubiquitylation) (Kerscher et al., 2006). Polyubiquitin chains are assembled by sequential steps of ubiquitin conjugation to an internal lysine residue (K6, K11, K27, K29, K33, K48 or K63) of a previously attached ubiquitin molecule. This results in the formation of polyubiquitin chains with specific linkage types, topologies and functions (Komander and Rape, 2012; Yau and Rape, 2016).

Similar to other PTMs, ubiquitin conjugation to substrate proteins is a reversible process and can be counteracted by various deubiquitylating enzymes (DUBs) (Komander et al., 2009).

1.1.2 Ubiquitin-dependent protein degradation

Since its discovery in the mid-1970s (Goldstein et al., 1975), ubiquitin has been implicated in the regulation of virtually all physiological processes. A major function of ubiquitylation is, however, to mark proteins for degradation by the 26S proteasome. Selective protein degradation by this multi-subunit self-compartmentalizing protease (Baumeister et al., 1998) is essential in all eukaryotes and plays critical roles in multiple cellular pathways including cell cycle regulation, signal transduction, protein quality control, endocytosis and antigen presentation (Varshavsky, 1997).

Canonical marks that label proteins for degradation by the proteasome are K48-linked polyubiquitin chains (Chau et al., 1989). Consistent with this crucial function, K48-linkages are the most abundant linkage type *in vivo* and strongly accumulate upon proteasome inhibition (Kaiser et al., 2011; Kim et al., 2011; Peng et al., 2003; Xu et al., 2009). However, other linkage types, for instance in form of K11- (Jin et al., 2008; Matsumoto et al., 2010; Williamson et al., 2009) and K29-linked polyubiquitin chains (Johnson et al., 1995; Koegl et al., 1999), can also trigger proteasomal degradation. Moreover, although predominantly implicated in non-proteolytic pathways, K63-linked polyubiquitin chains have been reported to mediate protein degradation in some cases (Saeki et al., 2009).

Protein degradation by the 26S proteasome requires the specific recognition of ubiquitylated substrates by ubiquitin receptors. This can occur via two different mechanisms. First, ubiquitin modifications are recognized by receptor proteins that are stably associated with the proteasome. Examples are the ubiquitin-binding proteins Rpn10 (Deveraux et al., 1994; Elsasser et al., 2004; van Nocker et al., 1996) and Rpn13 (Husnjak et al., 2008), which act as canonical subunits of the 26S proteasome. Both Rpn10 and Rpn13 directly bind ubiquitylated proteins via a

ubiquitin-binding domain (UBD). Second, ubiquitin-modified proteins are delivered to the proteasome by so-called shuttling ubiquitin receptors. These receptors bind ubiquitylated substrates via a ubiquitin-associated (UBA) domain and reversibly interact with the proteasome via a ubiquitin-like (UBL) domain. Canonical members of the group of shuttling ubiquitin receptors include Rad23 (hHR23a/b in humans) (Elsasser et al., 2004; Rao and Sastry, 2002), Dsk2 (Ubiquilin 1/2 in humans) (Funakoshi et al., 2002; Rao and Sastry, 2002) and Ddi1 (Elsasser and Finley, 2005; Kaplun et al., 2005).

After delivery to the proteasome, ubiquitin-modified substrates are deubiquitylated by proteasome-associated DUBs (Leggett et al., 2002; Verma et al., 2002), unfolded and guided into the proteolytic cavity for degradation into smaller peptides (Elsasser and Finley, 2005).

Apart from the proteasome, clearance of ubiquitylated proteins is also mediated by autophagy, a cellular degradation system that delivers cytoplasmic material to the lysosome (in mammals) or the vacuole (in yeast) (Khaminets et al., 2016; Mizushima et al., 2011). Importantly, ubiquitin-selective autophagy requires specific adaptor proteins that link the ubiquitin system to the autophagy pathway (Khaminets et al., 2016; Kraft et al., 2010; Rogov et al., 2014). These adaptors bind ubiquitylated cargo via specific UBDs and known examples include the proteins p62 (SQSTM1) (Pankiv et al., 2007), NBR1 (Kirkin et al., 2009a; Kirkin et al., 2009b) and Tollip (Lu et al., 2014) in humans as well as Cue5 in yeast (Lu et al., 2014). Interestingly, although p62 and NBR1 preferentially interact with K63-linked polyubiquitin chains, all adaptors bind K48-linked polyubiquitin chains as well (Kirkin et al., 2009b; Lu et al., 2014). Thus, pathway choice between autophagic and proteasomal degradation might not solely rely on the topology of the polyubiquitin chains attached to a substrate.

A further common feature of autophagy adaptors is their ability to bind the autophagy factor Atg8 (LC3 and GABARAP in humans) on the autophagosomal membrane via so-called Atg8-interacting motifs (AIMs), which are also referred to as LC3-interacting regions (LIRs) (Kirkin et al., 2009a; Lu et al., 2014; Pankiv et al., 2007). Atg8 is generally required for autophagosome formation but also serves as docking module for adaptors during selective autophagy. Thus, by direct recognition of substrates via ubiquitin-binding domains and interacting with Atg8 via AIMs, ubiquitin-Atg8 adaptors specifically mediate the delivery of ubiquitylated cargo for autophagic degradation. Remarkably, substrates of ubiquitin-selective autophagy are diverse and include ubiquitylated aggregates, midbody rings, organelles and even bacteria (Khaminets et al., 2016).

1.1.3 SUMO conjugation and deconjugation

One of the best-studied UBLs besides ubiquitin is the small ubiquitin-like modifier (SUMO). Unicellular organisms and lower eukaryotes often express a single SUMO isoform (*e.g.* Smt3 in *S. cerevisiae*), whereas vertebrates and plants typically possess multiple genes encoding different SUMO isoforms (*e.g.* SUMO1-4 in *H. sapiens*) (Flotho and Melchior, 2013). All SUMO isoforms are synthesized as inactive precursors, which carry C-terminal peptide extensions of variable length (2-11 amino acid residues). Proteolytic maturation of these SUMO precursors is carried out by SUMO-specific enzymes that remove the amino acid residues C-terminal to the characteristic double-glycine motif of SUMO (Gareau and Lima, 2010).

Similar to ubiquitin, SUMO is usually attached to the ε-amino group of lysine residues in substrate proteins (referred to as SUMOylation) (Figure 2). Biochemically, SUMOylation highly resembles the activation and conjugation of ubiquitin. However, it requires a set of unique, SUMO-specific enzymes including a heterodimeric E1 (Aos1/Uba2 in *S. cerevisiae* and SAE1/SAE2 in *H. sapiens*) and the single E2 Ubc9 (Johnson, 2004). In many cases, SUMO-modified lysines are embedded within the core consensus motif ΨKX(D/E) in which Ψ is a hydrophobic amino acid (usually I, L or V) and X is any amino acid (Gareau and Lima, 2010). This motif can be directly bound by Ubc9 and does not essentially require an E3 enzyme for modification (Bernier-Villamor et al., 2002). However, in most cases, efficient SUMOylation is strongly dependent on E3 ligases *in vivo*. Many SUMO E3 ligases are members of the Siz/PIAS protein family and harbor a characteristic Siz-PIAS-RING (SP-RING) domain related to the RING domain of the corresponding class of ubiquitin E3 ligases (Gareau and Lima, 2010). The SP-RING family of SUMO E3 ligases comprises four proteins in *S. cerevisiae* (Siz1, Siz2, Mms21 and the meiosis-specific Zip3) and six proteins in *H. sapiens* (PIAS1, -2, -3, -4, ZMIZ1 and NSE2). Moreover, several other proteins with SUMO E3 ligase activity (RanBP2, Pc2, MUL1, TOPORS, HDAC4, -7, TRAF7, FUS, RSUME, MAPL) have been described in higher eukaryotes (Jentsch and Psakhye, 2013).

In many cases, SUMO substrates are modified by single SUMO moieties attached to one or multiple lysine residues (monoSUMOylation and multiSUMOylation). However, particular SUMO isoforms such as Smt3 in yeast (Bencsath et al., 2002) or SUMO2/3 in human cells (Tatham et al., 2001) can also form polySUMO chains (polySUMOylation). Formation of such polySUMO chains requires internal SUMOylation consensus sites, which are mainly localized within the N-terminus of SUMO (*e.g.* K11, K15 and K19 of Smt3). Other SUMO isoforms like human SUMO1 do not harbor N-terminal SUMOylation consensus sites and form

polySUMO chains with much lower efficiency *in vitro* (Tatham et al., 2001). However, hybrid chains of SUMO1 and SUMO2/3 have been reported to form via non-consensus sites (Cooper et al., 2005; Matic et al., 2008; Pedrioli et al., 2006).

Protein modification by SUMO is reversible and can be regulated by SUMO-specific isopeptidases (Figure 2). Notably, some of these enzymes not only act as isopeptidases but also possess a C-terminal hydrolase activity, which is required for the initial proteolytic maturation of SUMO precursors. All *bona fide* SUMO isopeptidases described to date are cysteine proteases and include the proteins Ulp1 and Ulp2 in yeast and six Ulp orthologs in *H. sapiens* (sentrin-specific proteases SENP1-3 and 5-7) (Hickey et al., 2012). Moreover, recent studies have reported the identification of three further SUMO isopeptidases in human cells, deSUMOylating isopeptidase 1 (DeSI-1), DeSI-2 (Shin et al., 2012) and ubiquitin-specific protease-like 1 (USPL1) (Schulz et al., 2012).

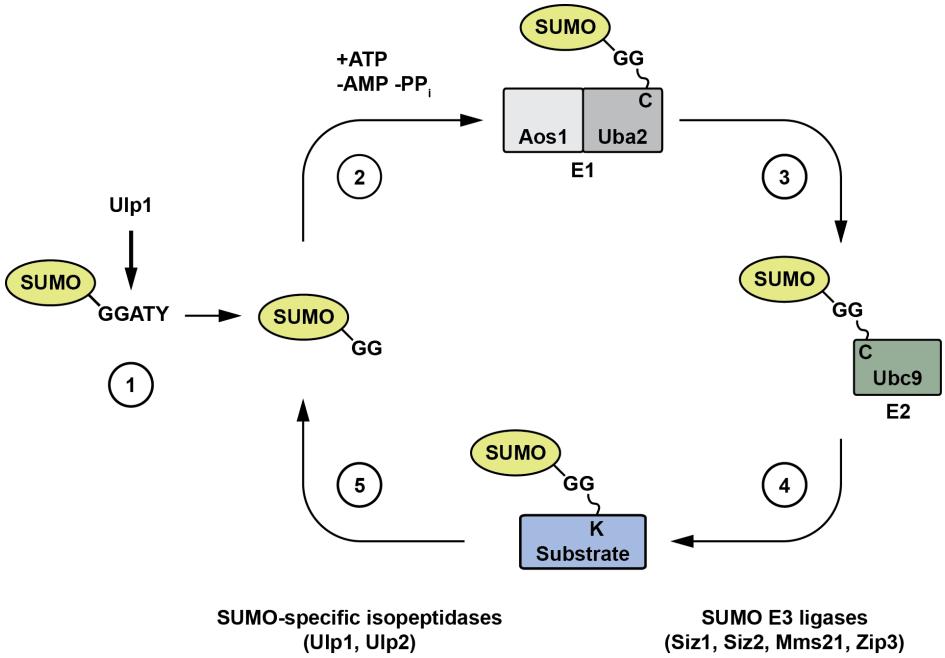


Figure 2. The SUMO conjugation and deconjugation system of *S. cerevisiae*. SUMO (Smt3 in *S. cerevisiae*) is translated as inactive precursor protein in which the C-terminal double glycine motif is followed by a peptide extension of several amino acids (ATY in *S. cerevisiae*). SUMO maturation and exposure of the C-terminal double glycine (GG) motif is mediated by SUMO-specific isopeptidases that possess a C-terminal hydrolase activity (Ulp1 in *S. cerevisiae*) (1). Mature SUMO is then activated by a dimeric SUMO-activating enzyme (E1) (Aos1/Uba2 in *S. cerevisiae*), which utilizes the chemical energy of ATP to form high-energy thioester bond between the active-site cysteine (C) of one subunit (Uba2) and the C-terminus of SUMO (2). Subsequently, SUMO is transferred to the catalytic cysteine (C) of the SUMO conjugating enzyme Ubc9 (3). Eventually, SUMO E3 ligases catalyze the covalent attachment of SUMO to the lysine (K) residue of a substrate protein (4). Protein modification by SUMO is reversible and the isopeptide bond between SUMO and a substrate protein can be hydrolyzed by SUMO-specific isopeptidases (Ulp1 and Ulp2 in *S. cerevisiae*) (5).

1.1.4 Molecular consequences of SUMOylation

Protein modification by SUMO affects a multitude of cellular substrates and regulates a large variety of physiological processes. Interestingly, SUMO substrates are typically modified to only a small percentage at steady state and only a very limited number of proteins appear to be quantitatively SUMOylated (Geiss-Friedlander and Melchior, 2007). However, low-level SUMOylation can sometimes entail strong effects, and, for instance, alter the localization, activity or stability of a modified protein.

Although the functions of protein SUMOylation are diverse and in many cases substrate-specific, the direct mechanistic consequences of SUMO modifications are mostly based on a few common molecular principles. First, SUMOylation can affect protein properties by competing with other lysine-directed PTMs for the same acceptor site. Competition between SUMOylation and ubiquitylation for the same amino acid residue has been reported for the protein IκB-α (Desterro et al., 1998). SUMO modification of IκB-α at Lys 21 blocks the ubiquitylation of this particular lysine residue and thereby interferes with the ubiquitin-dependent proteasomal degradation of the protein. A further example is a SUMO switch regulating the transcriptional activator myocyte-specific enhancer factor 2A (MEF2A). SUMOylation blocks the acetylation of MEF2A and thereby interferes with the transcriptional activation of the protein (Shalizi et al., 2006).

Second, SUMOylation can interfere with protein-protein interactions by shielding interaction surfaces present on a substrate protein. A well-studied example is the yeast DNA sliding clamp proliferating cell nuclear antigen (PCNA). PCNA is SUMOylated at Lys 127, which is located in close proximity to a surface area of PCNA that is recognized by PCNA-interacting proteins (PIP) via so-called PIP boxes (Moldovan et al., 2006). Accordingly, SUMO modification of PCNA at this particular lysine residue inhibits the binding of the PIP box-containing acetyltransferase Eco1 and thereby negatively regulates cohesion establishment during S phase.

Third, SUMOylation can recruit interaction partners to a modified substrate by providing an additional binding interface. Non-covalent binding of proteins to SUMO is typically mediated by short hydrophobic peptide stretches referred to as SUMO-interacting motifs (SIMs). SIMs harbor the core consensus sequence [V/I]-X-[V/I]-[V/I] and are sometimes flanked by acidic amino acid residues or phosphoacceptor sites (Hecker et al., 2006; Song et al., 2004; Stehmeier and Muller, 2009). Because SIM-containing proteins bind a specific surface patch on SUMO (*e.g.* comprising amino acid 35-55 in *S. cerevisiae* Smt3) with typically moderate affinities (Kerscher, 2007), SUMOylation often fosters interactions between proteins that already possess low

affinities for each other. A prominent example for the SUMO-dependent recruitment of a specific binding partner is once more PCNA. In addition to the modification at Lys 127, PCNA becomes SUMOylated at Lys 164 during S phase of the cell cycle (Hoege et al., 2002). The modification at this particular lysine residue facilitates recruitment of the SIM-containing helicase Srs2 to the replication fork, where Srs2 inhibits unwanted recombination events by disassembling Rad51 nucleoprotein filaments (Papouli et al., 2005; Pfander et al., 2005).

Notably, SUMO-SIM interactions can also occur in an intramolecular fashion. One example is the DNA repair protein thymine DNA glycosylase (TDG) (Steinacher and Schar, 2005). SUMOylation of TDG at Lys 330 leads to a conformational change, which is caused by an interaction of the attached SUMO moiety and a SIM of TDG itself. This structural rearrangement alters the DNA-binding properties of TDG and thereby releases this protein from chromatin.

A particularly interesting class of SIM-containing proteins are the so-called SUMO-targeted ubiquitin ligases (STUbLs), which represent a link between the SUMO and the ubiquitin system (Praefcke et al., 2012). STUbLs are specialized RING-type ubiquitin E3 ligases that harbor multiple SIMs and thereby are specifically recruited to polySUMOylated proteins. Prototypical members of this class of enzymes are Ris1 and the Slx5/Slx8 heterodimer in yeast (Uzunova et al., 2007) as well as RNF4 in vertebrates (Tatham et al., 2008). Moreover, by catalyzing substrate modifications with polyubiquitin chains, STUbLs regulate the proteasomal turnover of SUMO conjugates (Tatham et al., 2008; Uzunova et al., 2007).

An example for a non-proteolytic function of a STUbL is the ubiquitin E3 ligase Rad18 in *S. cerevisiae*. Rad18 binds SUMOylated PCNA in a SIM-dependent manner and mediates the non-proteolytic monoubiquitylation of different PCNA subunits (Parker and Ulrich, 2012). Similarly, RNF4 appears to possess non-proteolytic activities, which are involved in the DNA damage response in human cells (Yin et al., 2012).

1.1.5 Distinctive features of the ubiquitin and SUMO systems

Although protein modification by ubiquitin and SUMO is similar at multiple levels, both conjugation systems possess unique features. Characteristic for the ubiquitin system is its hierarchically organized and highly diversified enzymatic machinery. For instance, in the yeast *S. cerevisiae*, eleven E2s, 60-100 E3s and 20 DUBs have been discovered (Finley et al., 2012). By contrast, SUMOylation is controlled by a remarkably small number of enzymes, comprising a single E2 (Ubc9), four E3 ligases

(Siz1, Siz2, Mms21 and Zip3) and two SUMO-specific isopeptidases (Ulp1 and Ulp2) in *S. cerevisiae*.

PTMs often target individual proteins with high selectivity and enzyme diversification is of fundamental importance for substrate specificity in the ubiquitin pathway (Kerscher et al., 2006). Similar to ubiquitylation and despite the remarkable simplicity of its enzymatic apparatus, the SUMO system targets a plethora of cellular substrates as well. Thus, the question has been raised how substrate specificity in the SUMO system is achieved (Psakhye and Jentsch, 2012).

Among the mechanisms that ensure substrate specificity in the SUMO pathway, the targeting of SUMO E3 ligases to distinct cellular localizations and compartments is of particular relevance (Jentsch and Psakhye, 2013). Additionally, a recent study on the SUMOylation of proteins involved in homologous recombination revealed that the SUMO system frequently targets entire protein complexes rather than single substrates (protein group SUMOylation) (Psakhye and Jentsch, 2012). Thus, substrate selectivity can be achieved by the specific recruitment of SUMO enzymes to the vicinity of preassembled protein complexes. Moreover, it has been proposed that multiple SUMO modifications act synergistically to foster the stability of protein complexes by SUMO-SIM interactions. Hence, the intriguing concept of protein group SUMOylation also provides an explanation for the observation that removal of SUMO acceptor sites in single substrates often barely causes phenotypes. Accordingly, only the wholesale elimination of SUMOylation of an entire protein group has strong consequences and in case of the homologous recombination pathway significantly delays DNA repair (Psakhye and Jentsch, 2012).

1.1.6 The SUMO system in the context of cellular compartments

In contrast to other PTM pathways like phosphorylation, acetylation and ubiquitylation, which generally act throughout the cell, SUMOylation has emerged as a primarily nuclear modification (Kamitani et al., 1997). In fact, certain SUMO substrates require an intact nuclear localization signal (NLS) for efficient SUMOylation *in vivo* (Sternsdorf et al., 1999). Moreover, studies on an artificially designed reporter protein indicated that in some cases the combination of a ΨKX(D/E)-type consensus motif and an NLS is sufficient to trigger SUMOylation (Rodriguez et al., 2001).

The predominantly nuclear activities of the SUMO system have been further substantiated by several large-scale studies, which have identified hundreds of potential SUMO substrates in yeast and more than 1000 in human cells. The majority of these substrates indeed appears to be nuclear (Wohlschlegel et al., 2004) and a

recent evaluation of human SUMO proteomics studies has reported that in fact ~96 % of the top 200, ~93 % of the top 500 and ~86 % of the top 1000 most-frequently identified SUMO substrates are annotated as nuclear proteins (Hendriks and Vertegaal, 2016).

A prerequisite for the SUMOylation of substrate proteins in a given cellular compartment is the local presence of SUMO enzymes. Indeed, many components of the SUMO system predominantly reside in the nucleus (Johnson, 2004; Melchior et al., 2003; Seeler and Dejean, 2003) and dedicated nuclear import pathways have been reported for the SUMO E1 (Moutty et al., 2011) and E2 enzymes (Grunwald and Bono, 2011; Mingot et al., 2001). Moreover, distinct nuclear localizations have been described for several SUMO E3 ligases such as Pc2 (Kagey et al., 2003; Roscic et al., 2006), Mms21 (NSE2) (Potts and Yu, 2005; Zhao and Blobel, 2005) and members of the human PIAS protein family (Kotaja et al., 2002; Miyauchi et al., 2002; Sachdev et al., 2001). Likewise, in yeast, Siz2 is a predominantly nuclear protein and Siz1 is enriched in the nucleus during most phases of the cell cycle (Makhnevych et al., 2007; Takahashi and Kikuchi, 2005). Lastly, multiple SUMO isopeptidases primarily localize to the nucleus in both yeast and human cells (Hickey et al., 2012).

Despite its prevalence in the nucleus, the SUMO system is not entirely restricted to this compartment and a number of cytosolic SUMO substrates have been described (Figure 3). Consistently, enzymes of the SUMO conjugation system have been detected in the cytosol, albeit mostly in much smaller fractions than in nucleus (Bossis and Melchior, 2006; Donaghue et al., 2001; Lee et al., 1998; Makhnevych et al., 2007; Pichler et al., 2002; Takahashi et al., 2008; Takahashi and Kikuchi, 2005; Zhang et al., 2002). Well-studied examples of cytosolic SUMO substrates are the mammalian Ran GTPase-activating protein 1 (RanGAP1) at the cytoplasmic face of the nuclear pore complex (NPC) and the septins located at the bud neck in yeast cells. Septin SUMOylation requires a cytosolic pool of the SUMO E3 ligase Siz1, which is exported from the nucleus by the karyopherin Kap142/Msn5 prior to anaphase (Makhnevych et al., 2007). Notably, septins are deSUMOylated during cytokinesis by the SUMO protease Ulp1 (Makhnevych et al., 2007; Takahashi et al., 2000), demonstrating that SUMO isopeptidases exert distinct functions in the cytosol as well.

Apart from substrates with restricted localization, several soluble SUMO substrates have been identified in the cytosol. Examples range from yeast glycolytic enzymes such as Pgk1 (Psakhye and Jentsch, 2012) to intermediate filament proteins in *C. elegans* (Kaminsky et al., 2009). A particularly interesting case is the

mammalian proto-oncogene c-Myb, which is SUMOylated in the cytosol by the E3 ligase TRAF7. Compartment-specific SUMOylation of c-Myb inhibits its nuclear import and thereby causes the cytosolic sequestration of this protein (Morita et al., 2005).

Intriguingly, cytosolic SUMOylation also affects proteins at the cytosolic interfaces of the endoplasmic reticulum (ER), the mitochondrial outer membrane and the plasma membrane (Figure 3). First evidence for an implication of the SUMO system in the regulation of plasma membrane proteins came from studies on the glucose transporters GLUT1 and GLUT4 (Giorgino et al., 2000). Both GLUT1 and GLUT4 interact with the SUMO E2 enzyme Ubc9 via their cytosolic C-termini and it has been proposed that both transporters are modified by SUMO. Moreover, overexpression of Ubc9 severely altered the expression levels of the glucose transporters, leading to decreased abundance of GLUT1 and strongly increased abundance of GLUT4.

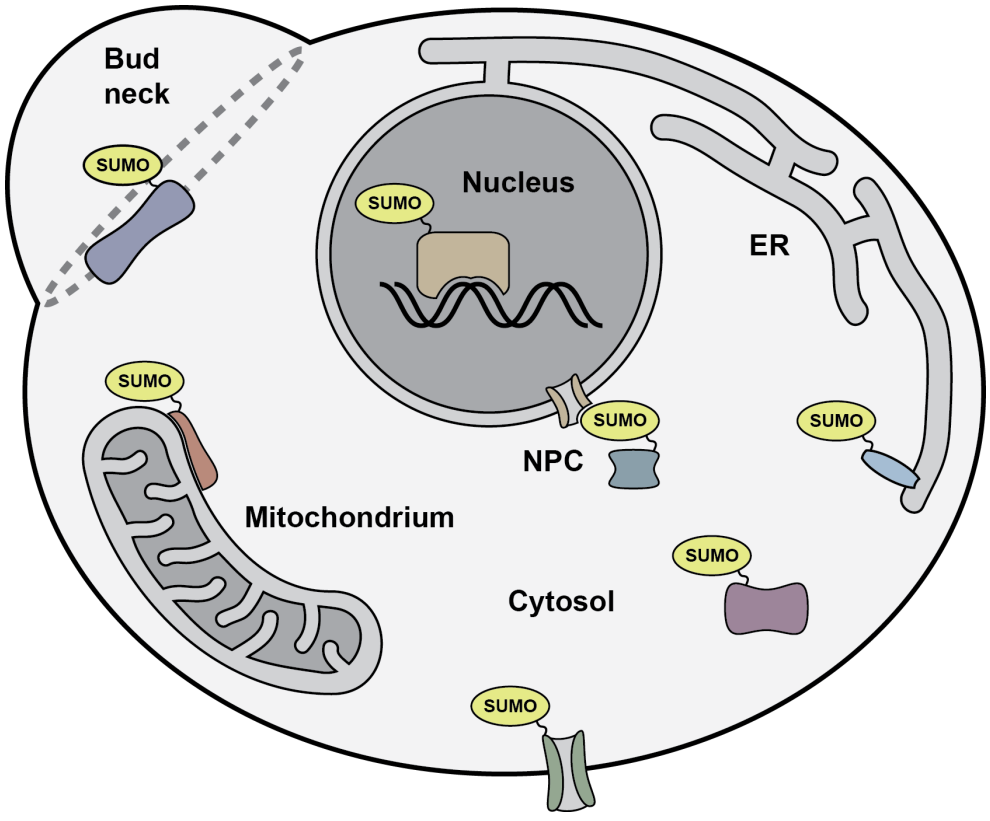


Figure 3. SUMO substrates in the context of cellular compartments. Protein modification by SUMO affects a multitude of cellular substrates. The majority of SUMO substrates localizes to the nucleus, where SUMOylation regulates essential processes such as replication, transcription and DNA repair. Apart from the nucleus, SUMO targets are also found in the cytosol and the cytosolic interfaces of the plasma membrane, the nuclear pore complex (NPC), the endoplasmic reticulum (ER) and mitochondria (see main text for details). A further group of cytosolic SUMO substrates are the septin proteins in yeast, which assemble at the bud neck during cytokinesis.

Following this initial discovery, later studies provided detailed insights into the SUMO regulation of plasma membrane proteins. The first SUMO substrate to be described was the K^+ leak channel K2P1. It has been reported that K2P1 is SUMOylated at Lys 274, resulting in the inactivation of the channel (Rajan et al., 2005). However, a subsequent study questioned whether K2P1 is indeed regulated by SUMOylation (Feliciangeli et al., 2007), indicating that this issue requires further clarification. Nevertheless, SUMO-mediated regulation of channel activity has been confirmed for other substrates, for instance the voltage-gated potassium channel Kv1.5 (Benson et al., 2007). Kv1.5 is SUMOylated at two lysine residues located within cytosolic domains of the channel and the modification is involved in channel inactivation. Additional SUMO substrates at the plasma membrane include metabotropic and ionotropic glutamate receptors (Martin et al., 2007a; Tang et al., 2005; Wilkinson et al., 2008). Notably, it has been reported that SUMOylation of the ionotropic kainate receptor subunit GluR6 is crucial for the endocytosis of the receptor, thereby providing a link between the SUMO system and receptor-mediated endocytosis (Martin et al., 2007a).

SUMOylation also targets proteins at the cytosolic interface of cellular organelles. Such substrates include the ER-associated protein-tyrosine phosphatase-1B (Dadke et al., 2007) and the dynamin-related GTPase Drp1, which translocates from the cytosol to the outer mitochondrial membrane to regulate mitochondrial fission (Harder et al., 2004). Notably, Drp1 SUMOylation is dependent on the mitochondria-anchored protein ligase (MAPL), the first SUMO E3 ligase that has been reported to be associated with mitochondria (Braschi et al., 2009). Moreover, SUMOylation of Drp1 is negatively regulated by SENP5 (Zunino et al., 2007), thus highlighting a further example for the function of a SUMO-specific isopeptidase in the cytosol.

Taken together, current knowledge about the SUMO system indicates that SUMO modification targets diverse substrates, which are accessible to nuclear and cytosolic SUMO enzymes. However, SUMOylation appears to be absent within particular organelles such as the ER and mitochondria and the question whether proteins transported into these organelles are SUMO substrates *in vivo* has not been elucidated so far.

1.2 Biogenesis of mitochondrial proteins

Mitochondria are double-membrane organelles involved in multiple cellular pathways. Besides their prominent role in ATP production, mitochondria are crucial for the biosynthesis of lipids, amino acids and heme and at least one mitochondrial function, the formation of iron-sulfur clusters, is strictly essential for cell viability in all organisms (Lill and Muhlenhoff, 2008; Neupert and Herrmann, 2007). Moreover, in vertebrates, mitochondria have been implicated in the regulation of innate and adaptive immunity and are of fundamental importance for the execution of apoptosis (Wang and Youle, 2009; Weinberg et al., 2015).

Proteomic studies have suggested that mitochondria contain about 1000 proteins in yeast and 1500 proteins in human cells (Pagliarini et al., 2008; Perocchi et al., 2006; Sickmann et al., 2003). However, only a small number of these proteins are encoded in the mitochondrial genome and translated by mitochondrial ribosomes. The vast majority of mitochondrial proteins are encoded in the nuclear genome and synthesized as precursor proteins on cytosolic ribosomes. Subsequent sorting of these proteins into their functional environment often involves proteolytic processing, equipment with cofactors and assembly into larger functional protein complexes. Moreover, since mitochondria are made up of two membranes, proteins can be targeted to one out of four submitochondrial destinations: the outer membrane (OM), the intermembrane space (IMS), the inner membrane (IM) or the matrix.

1.2.1 Mitochondrial targeting signals

Protein import into mitochondria requires targeting sequences that harbor the information to which membrane or subcompartment a particular protein is sorted. Prototypical mitochondrial targeting signals are cleavable presequences, which are characteristically located at the N-termini of precursor proteins (Neupert, 1997; Neupert and Herrmann, 2007). They usually direct proteins to the mitochondrial matrix in an N to C direction and therefore are referred to as matrix targeting signals (MTS). MTSs typically comprise 10-80 amino acid residues and form amphipathic α-helices with one hydrophobic and one positively charged surface. After import into the mitochondrial matrix, most N-terminal targeting signals are proteolytically removed by the dimeric mitochondrial processing peptidase (MPP). Notably, the N-terminal localization of the MTS appears to be critical for its function and transplantation of a MTS to internal regions of proteins does not facilitate mitochondrial targeting. However, artificial fusion of an MTS to the C-terminus of a protein can mediate mitochondrial targeting but leads to a C- to N-terminal

translocation direction (Folsch et al., 1998). Remarkably, it has been reported that in one case, the yeast DNA helicase Hmi1, the MTS is naturally located at the C-terminus of the protein (Lee et al., 1999).

In addition to an MTS, a number of mitochondrial IM and IMS proteins contain further hydrophobic sorting signals C-terminal to the MTS, which are often followed by a cluster of charged amino acid residues (Rojo et al., 1998). For IM proteins, these hydrophobic sorting signals serve as transmembrane domains, which arrest translocation within the inner mitochondrial membrane and facilitate lateral sorting into the lipid phase (stop-transfer pathway) (Gartner et al., 1995; Glaser et al., 1990; Glick et al., 1992; Miller and Cumsky, 1993). Moreover, various IMS proteins contain so-called bipartite presequences, which are proteolytically processed after embedment into the inner membrane. Thereby the mature proteins are released into the intermembrane space (Gakh et al., 2002; Glick et al., 1992).

Besides the classical N-terminal presequences, a variety of less-defined internal mitochondrial targeting and sorting signals have been described. Such targeting signals are found in diverse mitochondrial proteins, including all proteins of the outer mitochondrial membrane, many intermembrane space and inner membrane proteins as well as a small number of matrix proteins (Chacinska et al., 2009).

1.2.2 Mitochondrial protein sorting

The majority of mitochondrial proteins are imported via the translocase of the outer membrane (TOM complex). Central component of this complex is the general import pore formed by the β-barrel protein Tom40 (Ahting et al., 2001; Model et al., 2008). Additional subunits are the receptor proteins Tom20, Tom70 and Tom22. Whereas Tom20 serves as major recognition site for preproteins with N-terminal targeting signals (Abe et al., 2000; Ramage et al., 1993; Saitoh et al., 2007; Sollner et al., 1989), Tom70 mainly binds proteins with multiple internal targeting signals such as carrier proteins (Chan et al., 2006; Sollner et al., 1990; Wu and Sha, 2006). A central receptor subunit, Tom22, promotes the general integrity of the TOM complex and transfers incoming proteins from Tom20 and Tom70 to the translocation pore (van Wilpe et al., 1999).

After passage through the TOM complex, mitochondrial proteins can follow different routes to reach their submitochondrial destination (Figure 4). The embedment of proteins into the outer mitochondrial membrane often requires the sorting and assembly machinery (SAM), which mediates the maturation of β-barrel (Paschen et al., 2003; Wiedemann et al., 2003) and a subset of α-helical proteins (Stojanovski et al., 2007).

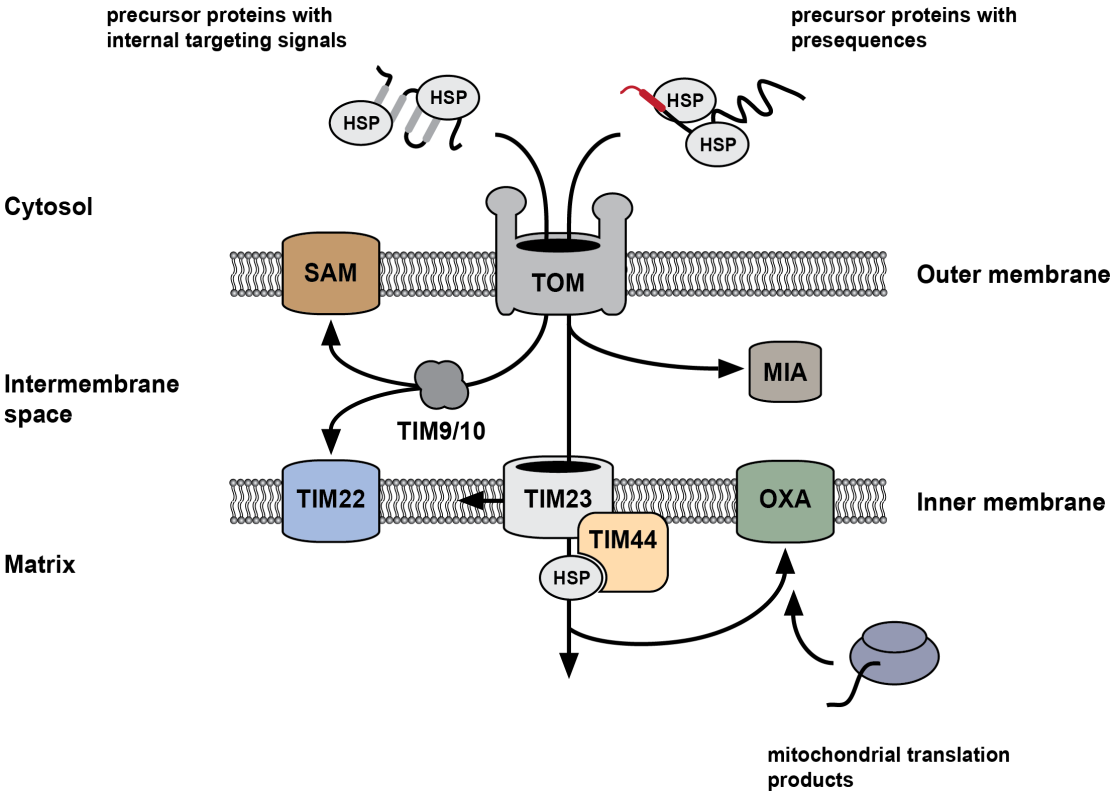


Figure 4. Mitochondrial protein sorting pathways. The vast majority of mitochondrial proteins are encoded in the nucleus and synthesized as precursor proteins on cytosolic ribosomes. In the cytosol, mitochondrial precursor proteins associate with molecular chaperones such as heat shock proteins (HSP), which maintain their import-competence and facilitate import into the organelle. Many precursor proteins harbor cleavable N-terminal targeting sequences (presequences), however, proteins with internal targeting signals have also been described. Almost all mitochondrial proteins are imported via a general entry gate formed by the translocase of the outer membrane (TOM complex). Specific components of the TOM complex also serve as receptors that bind mitochondrial preproteins at the cytosolic interface of the outer membrane. Following entry through the TOM complex, mitochondrial proteins follow different sorting pathways to one of the mitochondrial membranes or subcompartments. These include the outer mitochondrial membrane, the intermembrane space, the inner mitochondrial membrane and the mitochondrial matrix. Roughly 1 % of the mitochondrial proteome is encoded by the endogenous genome and synthesized as mitochondrial translation products. Depicted are various protein complexes involved in mitochondrial protein sorting: MIA, mitochondrial intermembrane space assembly; OXA, insertase/export machinery of the inner membrane; SAM, sorting and assembly machinery; TIM9/10, small TIM proteins that function as intermembrane space chaperones; TIM22 complex, carrier translocase of the inner membrane; TIM23 complex, presequence translocase of the inner membrane; TIM44, membrane anchor for mitochondrial HSP70 (mtHSP70); TIM44 and mtHSP70 are components of the presequence translocase-associated motor (PAM) complex.

Proteins solely carrying N-terminal signal sequences are usually transported into the mitochondrial matrix. This sorting pathway involves the translocase of the inner membrane (TIM23 complex) and the presequence translocase-associated motor (PAM) complex (Chacinska et al., 2009; Neupert and Herrmann, 2007). In the matrix, N-terminal targeting sequences are typically removed by the mitochondrial processing peptidase (MPP). Moreover, emerging proteins are bound by mitochondrial HSP70 (mtHSP70), which is recruited to the inner mitochondrial

membrane by the protein Tim44. Both proteins are components of the PAM complex, which utilizes the energy of ATP to stimulate protein translocation into the matrix.

In addition to its role in the biogenesis of matrix proteins, the TIM23 complex is involved in the import of IM proteins via the stop-transfer pathway and also mediates the sorting of IMS proteins that contain bipartite presequences (see section 1.2.1). A special group of IMS proteins is sorted via the mitochondrial intermembrane space assembly (MIA) machinery. Central component of this pathway is the receptor protein Mia40 (Chacinska et al., 2004; Mesecke et al., 2005; Naoe et al., 2004), which binds cysteine-containing substrates emerging from the TOM complex via a hydrophobic interface and its redox-active cysteine-proline-cysteine (CPC) motif (Grumbt et al., 2007; Milenkovic et al., 2007; Milenkovic et al., 2009). Subsequently, Mia40 catalyzes the formation of disulfide bridges within precursor proteins and releases the oxidized and mature proteins into the intermembrane space (Muller et al., 2008; Terziyska et al., 2009).

Besides the TIM23-dependent pathway, an alternative route to the inner mitochondrial membrane is the so-called carrier pathway (Chacinska et al., 2009). Metabolite carrier proteins contain several internal targeting signals and in most cases are translated on cytosolic ribosomes. In the cytosol, carrier precursors are usually bound by molecular chaperones that protect them from aggregation and guide them to the Tom70 receptor at the outer mitochondrial membrane (see section 1.2.4). After translocation through the TOM complex, carrier proteins are recognized by a chaperone-like hexameric complex composed of the small TIM proteins Tim9 and Tim10 (Curran et al., 2002; Vasiljev et al., 2004). These factors facilitate the further transfer to the TOM22 complex, which eventually mediates the assembly of carrier proteins in the inner mitochondrial membrane.

Notably, the inner mitochondrial membrane also contains proteins encoded in the mitochondrial genome and synthesized on mitochondrial ribosomes. These proteins function as subunits of mitochondrial respiratory chain complexes and reach their mature state via the insertase/export machinery of the inner membrane (OXA complex). For some substrates, the OXA complex also participates in the so-called conservative sorting pathway (Hell et al., 1997; Hell et al., 1998). This pathway directs nucleus-encoded proteins from the cytosol into the matrix and from there into the inner membrane (Neupert and Herrmann, 2007).

1.2.3 Cotranslational and posttranslational protein import

A multitude of studies indicate that most mitochondrial proteins are translated on cytosolic ribosomes and posttranslationally imported into the organelle (Neupert,

1997; Neupert and Herrmann, 2007). First observations of posttranslational protein import into mitochondria were made by *in vivo* pulse and pulse-chase experiments using intact cells. It has been reported that fully translated precursors of mitochondrial proteins appear first in the cytosol and subsequently are converted into mature mitochondrial forms (Hallermayer et al., 1977). Importantly, the import kinetics vary for different precursor proteins and protein translocation into mitochondria continues even after the inhibition of translation by cycloheximide (Hallermayer et al., 1977). This strongly suggests that protein translocation into mitochondria is indeed not coupled to translation.

The findings that mitochondrial protein import can occur efficiently in a posttranslational manner *in vivo* were further substantiated by *in vitro* studies analyzing the import of proteins into isolated mitochondria. Using cell-free translation systems, protein synthesis and translocation into mitochondria can be entirely separated (Hartl et al., 1986). When precursor proteins are first translated and released into the postribosomal supernatant, mitochondrial import can be observed after the addition of isolated mitochondria to the supernatant.

Despite various observations that support a model of predominantly posttranslational protein import into mitochondria, cotranslational and posttranslational sorting modes are not mutually exclusive and appear to occur in parallel. In fact, it has been reported that cytoplasmic 80S ribosomes are associated with mitochondria (Kellems et al., 1974, 1975; Kellems and Butow, 1972, 1974) and that mitochondrial proteins are translated from both mitochondria-bound as well as free cytosolic polysomes (Suissa and Schatz, 1982). Moreover, distinct mRNAs encoding mitochondrial proteins appear to be selectively translated at the surface of the outer mitochondrial membrane. Targeting of mRNAs to mitochondria is mediated by diverse mechanisms (Fox, 2012) and for some proteins increases import efficiency (Margeot et al., 2002).

In general, since most mitochondrial proteins contain N-terminal targeting signals, it is conceivable that import initiates as soon as the N-terminus of a nascent polypeptide binds the import receptors at the outer mitochondrial membrane. Consistently, it has been reported that the enrichment of certain mRNAs at the mitochondrial surface requires translation and is dependent on the presequence-binding receptor Tom20 (Eliyahu et al., 2010).

In summary, compelling evidence indicates that mitochondrial protein import is not generally coupled to translation and occurs posttranslationally as well as cotranslationally *in vivo*. However, in any case, it appears to be a fast and efficient

process and cytosolic pools of probably most mitochondrial precursor proteins are barely detectable *in vivo* (Ades and Butow, 1980b).

1.2.4 A role for cytosolic factors in mitochondrial protein import

Protein transport into mitochondria requires the translocation of polypeptides through narrow import pores (Ahting et al., 2001; Schwartz and Matouschek, 1999; Truscott et al., 2001). Thus, preproteins adopt a largely unfolded state during import and usually traverse the mitochondrial import channels as linear chains (Rassow et al., 1990; Schwartz et al., 1999). In fact, it has been reported that the import of mitochondrial preproteins is impaired when their three-dimensional structure is stabilized (Eilers and Schatz, 1986; Rassow et al., 1989; Wienhues et al., 1991). Empowered by an electrochemical proton gradient and an ATP-driven import motor, mitochondria can actively unfold preproteins (Matouschek et al., 2000). However, to facilitate import, cytosolic mitochondrial precursor proteins are thought to generally adopt a more loosely folded state than their mature forms (Neupert, 1997). Remarkably, this might be partially attributed to the presence of N-terminal presequences, which can interfere with the folding of precursor proteins prior to import (Hoogenraad et al., 2002; Lain et al., 1995). Nevertheless, the import competence of mitochondrial preproteins is in many cases maintained by cytosolic factors, which stabilize unfolded conformations and prevent their aggregation in the cytosol. The existence of such factors was in fact suggested early on by the observation that rabbit reticulocyte lysates (Argan et al., 1983; Miura et al., 1983; Ohta and Schatz, 1984; Pfanner and Neupert, 1987; Randall and Shore, 1989; Sheffield et al., 1986) as well as yeast cytosolic extracts (Murakami et al., 1988; Ohta and Schatz, 1984) stimulate the uptake of polypeptides into isolated mitochondria *in vitro*.

1.2.4.1 HSP70 and HSP90

First evidence for a function of molecular chaperones in mitochondrial protein import came from studies on the *SSA* subfamily of HSP70s in yeast. Conditional depletion of this family of chaperones results in the accumulation of mitochondrial precursor proteins *in vivo* (Deshaies et al., 1988). In agreement with these findings, it has been reported that *SSA* family HSP70s also stimulate protein translocation into isolated mitochondria *in vitro* (Murakami et al., 1988).

Similar to yeast cells, cytosolic chaperones are also involved in mitochondrial protein import in mammals. *In vitro* import assays have revealed that the HSP70 isoform HSC70 delays the folding and inhibits the aggregation of purified

mitochondrial precursor proteins (Sheffield et al., 1990). Moreover, HSC70 stimulates the translocation of proteins into isolated rat liver mitochondria (Terada et al., 1995).

The activity of HSP70 proteins is regulated by a variety of co-chaperones that stimulate the ATPase activity of HSP70 or function as nucleotide exchange factors (NEFs). To date, no evidence for an involvement of NEFs in mitochondrial protein import has been provided. However, the yeast HSP40 protein Ydj1 (Atencio and Yaffe, 1992; Caplan et al., 1992) as well as its orthologs Dj2 and Dj3 in human cells (Kanazawa et al., 1997; Terada and Mori, 2000) have been linked to the biogenesis of mitochondrial proteins and are required for the import of at least a subset of proteins in cell-free assays.

Besides HSP70, mammalian cells employ the HSP90 chaperone system to stimulate protein import into mitochondria. Remarkably, HSP70 and HSP90 chaperones not only maintain the import competence of precursor proteins but also actively deliver preproteins to the Tom70 import receptor at the outer mitochondrial membrane (Young et al., 2003).

1.2.4.2 Mitochondrial import stimulation factor (MSF)

The mitochondrial import machinery of mammalian cells appears generally more complex than the corresponding system in yeast. Consistently, further cytosolic factors with active targeting functions have been identified. Best-characterized among these proteins is the mitochondrial import stimulation factor (MSF), which was purified from rat liver cytosol using a presequence peptide coupled to an affinity matrix (Hachiya et al., 1993). MSF belongs to the family of 14-3-3 proteins (Alam et al., 1994) and facilitates the import of multiple preproteins with different types of targeting signals (Hachiya et al., 1993). Thus, it has been suggested that MSF might generally bind mitochondrial precursor proteins independently of N-terminal presequences (Hachiya et al., 1993).

Import stimulation by MSF seems to be based on two different activities. First, MSF binds mitochondrial preproteins and maintains their import competence in a chaperone-like manner. Additionally, the chaperone-like functions of MSF comprise an ATP-dependent disaggregation activity, which enables it to resolubilize aggregated mitochondrial precursor proteins (Hachiya et al., 1993; Hachiya et al., 1994; Komiya et al., 1994). Second, MSF fulfills an active targeting function and guides proteins to the mitochondrial surface. MSF-dependent mitochondrial protein import seems to act in parallel to HSP70-dependent pathways and delivers client proteins to the mitochondrial import receptor Tom70. At the TOM complex, MSF is released in an ATP-dependent manner and the preproteins are transferred to the

import receptors Tom20 and Tom22. Subsequently, translocation through the outer mitochondrial membrane is initiated (Hachiya et al., 1995; Komiya et al., 1997; Komiya et al., 1996).

1.2.4.3 Targeting factor and presequence binding factor (PBF)

Apart from MSF, a number of cytosolic factors specifically implicated in protein transport into mitochondria have been identified. One example is a 28 kDa protein termed targeting factor, which stimulates mitochondrial import of several preproteins *in vitro*. Interestingly, targeting factor also increases the amount of preproteins bound to the mitochondrial outer membrane, suggesting that it actively delivers polypeptides to the TOM complex (Ono and Tuboi, 1988, 1990a, b).

A further import-stimulating protein termed presequence binding factor (PBF) has been isolated by its binding specificity towards the precursor of rat ornithine carbamoyltransferase (Murakami and Mori, 1990). PBF has been shown to maintain the import competence of certain mitochondrial precursor proteins in cell-free import assays and to stimulate the import of several polypeptides into isolated mitochondria (Murakami and Mori, 1990; Murakami et al., 1992).

1.3 Cellular mechanisms of protein quality control

Accurate folding into a distinct three-dimensional structure is a crucial prerequisite for the functions of cellular proteins. However, proteins are structurally dynamic macromolecules and misfolded proteins can arise from different sources such as errors during folding of *de novo* synthesized polypeptides or stress-induced unfolding of native proteins. In many cases, non-native proteins expose stretches of hydrophobic amino acids, which are normally buried inside the structure of an appropriately folded protein. Therefore, protein misfolding not only interferes with the functions of proteins but also causes the formation of insoluble and potentially cytotoxic aggregates. Consequently, cells have evolved a sophisticated network of surveillance mechanisms that monitor accurate protein folding and maintain the integrity of the proteome (proteostasis).

1.3.1 Recognition of non-native proteins by molecular chaperones

Central component of cellular protein quality control is a system of molecular chaperones, which recognize and bind non-native proteins. Many molecular chaperones were originally discovered as heat shock-induced proteins and the major families of these heat shock proteins (HSPs) are classified according to their molecular weight (HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs) (Hartl et al., 2011). Each class of molecular chaperones utilizes a distinct mode of client binding and usually possesses a specific set of substrate proteins. However, in many cases, non-native protein conformations are recognized by the interaction of HSPs with hydrophobic peptide segments, which are exposed by unfolded or misfolded proteins.

HSP70 proteins are part of a major ATP-dependent chaperone system that functions ubiquitously throughout the cell and interacts with a multitude of substrate proteins. HSP70 proteins are involved in a wide range of cellular processes including *de novo* folding of nascent polypeptides, refolding of misfolded or aggregated proteins, targeting of mitochondrial (see section 1.2.4) and secretory proteins and regulation of protein activity (Mayer and Bukau, 2005). On the molecular level, the functions of HSP70s rely on three different but related activities: promoting the solubility of unfolded proteins, assisting the folding process into the native state and resolubilization of aggregated protein species. All of these activities appear to be based on the property of HSP70 to bind short linear peptide segments, which are enriched in hydrophobic and basic amino acids (Rudiger et al., 1997; Zhu et al., 1996). Reversible binding to these hydrophobic stretches accounts for the solubility-promoting activity of HSP70 and might also provide time for the folding of substrate

proteins to proceed. Moreover, HSP70 has been implicated in active disaggregation processes (Diamant et al., 2000; Nillegoda et al., 2015; Rampelt et al., 2012), where it often cooperates with disaggregases of the HSP100 family (Glover and Lindquist, 1998; Goloubinoff et al., 1999).

Substrate interactions of HSP70s are regulated by repeated cycles of ATP binding, hydrolysis and ADP release during which HSP70 switches between a high-affinity ATP-bound state and a low-affinity ADP-bound state. Factors that stimulate the ATPase activity of HSP70 belong to the diverse class of J domain-containing co-chaperones (HSP40s). HSP40 proteins also interact directly with substrate proteins and recruit HSP70 to binding sites in close proximity, thereby contributing to the substrate specificity and functional diversity of the HSP70 chaperone system (Kampinga and Craig, 2010). Besides HSP40, the ATPase cycle of HSP70 is regulated by nucleotide exchange factors (NEFs) (Bracher and Verghese, 2015a, b). NEFs stimulate the release of ADP + P_i from HSP70 and allow a new round of ATP binding, which in turn triggers substrate release.

In addition to the HSP70 system, HSP90 and HSP60 represent further families of ATP-dependent chaperones, which function in protein folding and protein quality control (Kim et al., 2013; Taipale et al., 2010). Moreover, a number of ATP-independent small heat shock proteins (sHSPs) possess chaperone-like activities and participate in the HSP-dependent protective systems, which counteract the aggregation of cellular proteins (Garrido et al., 2012).

1.3.2 Functions of the ubiquitin-proteasome system in protein quality control

The maintenance of proteostasis by cellular protein quality control systems is critically balanced by the triage decision whether non-native proteins are refolded or degraded. Remarkably, for the clearance of proteins, which are refractory to refolding, cellular chaperone systems closely collaborate with the ubiquitin-proteasome system (UPS). This functional interconnection of HSPs, ubiquitin E3 ligases and the 26S proteasome ultimately mediates the degradation of the majority of soluble misfolded proteins in a cell (Figure 5).

A prototypical example for the collaboration of chaperones with the UPS is the mammalian ubiquitin E3 ligase carboxy terminus of HSC70-interacting protein (CHIP). CHIP interacts with HSP70 and HSP90 via its tetratricopeptide domain and catalyzes the ubiquitylation of HSP70 and HSP90 substrates via its U-box domain (Ballinger et al., 1999; Connell et al., 2001; Demand et al., 2001; Jiang et al., 2001; Murata et al., 2001). CHIP thereby mediates the chaperone-dependent proteasomal degradation of various proteins including the glucocorticoid receptor (Connell et al.,

2001), the receptor tyrosine kinase ErbB2 (Xu et al., 2002) and generally aggregation-prone chaperone substrates (Meacham et al., 2001; Petrucelli et al., 2004).

Interestingly, while CHIP appears to be a central factor of protein quality control in higher eukaryotes, links between chaperones and UPS-mediated protein degradation have also been identified in organisms, which lack a functional homolog of CHIP. For instance, in yeast, cytosolic misfolded proteins are degraded by a unique pathway that involves the parallel activities of the cytosolic ubiquitin E3 ligase Ubr1 and the nuclear E3 ligase San1 (Eisele and Wolf, 2008; Heck et al., 2010; Nillegoda et al., 2010; Prasad et al., 2010). It has been reported that substrates of this pathway are degraded in an HSP70-dependent manner (Park et al., 2007) and Ubr1-mediated ubiquitylation is indeed stimulated by HSP70 (Heck et al., 2010; Nillegoda et al., 2010).

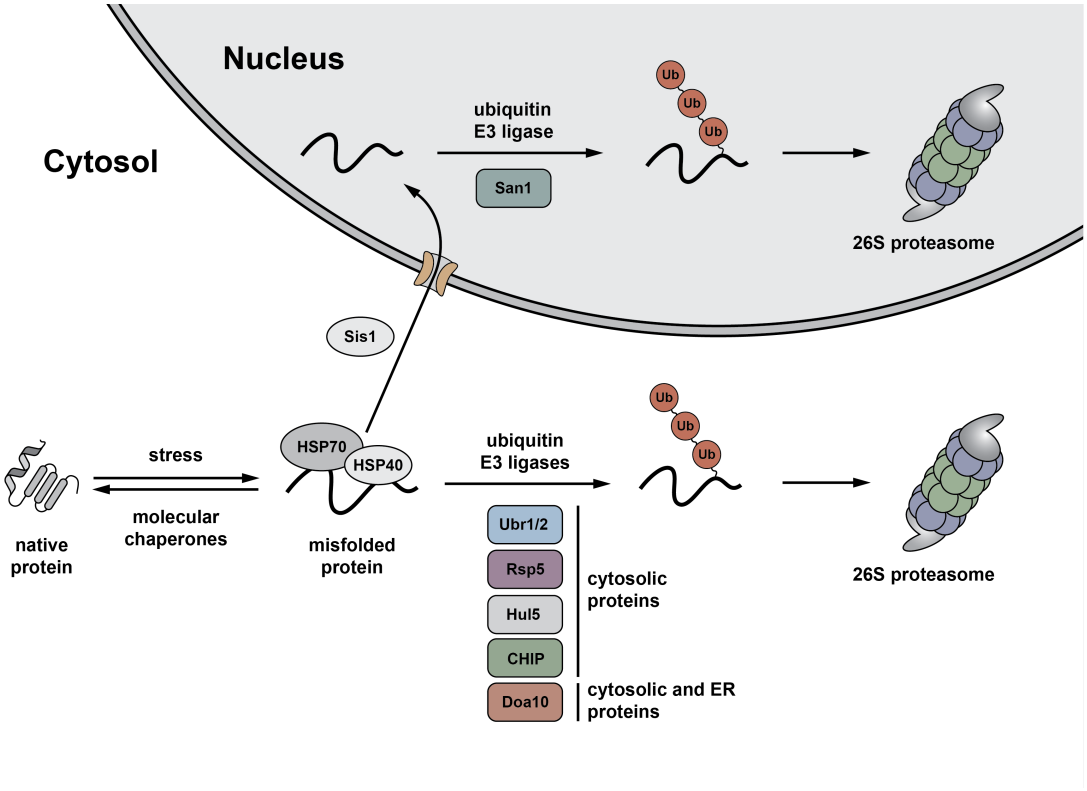


Figure 5. Pathways mediating the degradation of misfolded proteins by the ubiquitin-proteasome system. Various quality control components mediate the recognition, ubiquitylation and degradation of misfolded proteins. Particularly, molecular chaperones of the HSP70 and HSP40 families are involved in the recognition of non-native protein conformers. In concert with molecular chaperones, dedicated ubiquitin E3 ligases catalyze the ubiquitin (Ub) modification of misfolded proteins. Examples for ubiquitin E3 ligases involved in protein quality control are depicted (Ubr1/2, San1, Rsp5, Hul5 and Doa10 in *S. cerevisiae*; CHIP in vertebrates). Nuclear import of misfolded proteins for San1-mediated degradation requires specific HSP40 proteins (Sis1 in *S. cerevisiae*). The degradation of ubiquitylated substrates is ultimately mediated by the 26S proteasome.

Moreover, delivery of cytosolic substrates to the nuclear ubiquitin E3 ligase San1 requires HSP70 (Prasad et al., 2010) and the HSP40 protein Sis1 (Park et al., 2013), suggesting a dual role of chaperone factors in the degradation of misfolded cytosolic proteins. Remarkably, the transport of non-native proteins into the nucleus for degradation appears to be conserved among species and requires the Sis1 homolog DnaJB1 in mammalian cells (Park et al., 2013).

Protein misfolding can occur spontaneously in cells but is strongly induced by stress conditions such as heat shock. Under these circumstances, a variety of proteins are targeted for degradation by the proteasome. In yeast, heat shock-induced ubiquitylation exquisitely involves two ubiquitin E3 ligases, Hul5 (Fang et al., 2011) and Rsp5 (Nedd4 in humans) (Fang et al., 2014). Notably, heat shock-induced Rsp5-dependent ubiquitylation also requires the HSP40 protein Ydj1 (Fang et al., 2014), thus providing a further link between chaperones and the UPS in protein quality control.

Additional components of the ubiquitin system that have been implicated in protein quality control in yeast are the Ubr1 homolog Ubr2 (Nillegoda et al., 2010) and the ER-bound E3 ligase Doa10, which mediates the clearance of a number of soluble cytosolic and nuclear proteins (Metzger et al., 2008; Ravid et al., 2006; Swanson et al., 2001).

1.3.3 A role for SUMO in protein quality control

Despite the discovery of hundreds of potential SUMO substrates in yeast and mammalian cells, the molecular consequences and functions of SUMOylation have been revealed for only a subset of SUMO substrates. However, SUMO has been widely implicated as a “stress protein”. Similar to ubiquitylation, SUMOylation is strongly induced by diverse types of stress (Saitoh and Hinchey, 2000; Zhou et al., 2004), particularly by those that cause widespread protein misfolding such as heat shock (HS) (Golebiowski et al., 2009; Hendriks et al., 2014; Seifert et al., 2015) or proteasome inhibition (Castoralova et al., 2012; Hendriks et al., 2014; Tatham et al., 2011). Remarkably, although the exact function of HS-induced SUMOylation is still a matter of debate (Liebelt and Vertegaal, 2016; Niskanen et al., 2015; Seifert et al., 2015), it has been suggested that SUMO might exhibit chaperone-like activities that modulate the homeostasis of protein complexes at chromatin (Seifert et al., 2015).

Intriguing links between the SUMO system and proteostasis have also been revealed by the observation that SUMOylation targets multiple aggregation-prone proteins involved in neurodegenerative diseases (Krumova and Weishaupt, 2013; Liebelt and Vertegaal, 2016). It has been reported that SUMOylation modulates the

aggregation and stability of several disease-associated polyQ proteins including mutant Huntingtin (O'Rourke et al., 2013; Steffan et al., 2004), Ataxin-1 (Guo et al., 2014), Ataxin-7 (Janer et al., 2010) and the androgen receptor (Mukherjee et al., 2009). Moreover, SUMO has been shown to modify proteins such as Amyloid-β (Li et al., 2003; Zhang and Sarge, 2008) and α-Synuclein (Abeywardana and Pratt, 2015; Krumova et al., 2011), which are involved in Alzheimer's and Parkinson's disease. Remarkably, in many cases SUMOylation appears to increase the solubility of aggregation-prone proteins, thus reducing the extent of aggregate formation (Abeywardana and Pratt, 2015; Guo et al., 2014; Janer et al., 2010; Krumova et al., 2011; Mukherjee et al., 2009; Steffan et al., 2004; Zhang and Sarge, 2008). It has therefore been proposed that SUMO might function as a "protein solubility enhancer" (Krumova and Weishaupt, 2013). Additionally, it has been reported that SUMOylation promotes the clearance of multiple aggregation-prone substrates by the ubiquitin-proteasome system (Guo et al., 2014), indicating a functional cooperation of the SUMO and ubiquitin systems in protein quality control.

Taken together, SUMOylation appears to play a widespread role in the maintenance of proteostasis and might be an integral part of the cellular stress response interconnected to other protein quality control systems. However, the molecular mechanisms responsible for the functions of SUMO as a chaperone-like factor remain largely undefined.

2 AIM OF THIS STUDY

Since its discovery in the late 1990s (Mahajan et al., 1997; Matunis et al., 1996), substrates of the small ubiquitin-like modifier (SUMO) have been subject of extensive research. It has become evident that protein modification by SUMO affects a substantial part of the proteome and a multitude of nuclear and cytosolic proteins have been identified as SUMO substrates. However, current knowledge about the SUMO system strongly indicates that organellar proteins, for instance proteins residing inside mitochondria, are hidden from SUMO enzymes upon import (Flotho and Melchior, 2013). Accordingly, although a small number of mitochondrial proteins have been suggested as potential SUMO substrates in large-scale studies (Denison et al., 2005; Hannich et al., 2005; Panse et al., 2004; Wohlschlegel et al., 2004; Wykoff and O'Shea, 2005; Zhou et al., 2004), the question whether protein SUMOylation targets substrates prior to import into mitochondria has not been elucidated so far.

At the onset of this study, our laboratory had established a sensitive, mass spectrometry-based approach to study SUMOylated proteins in yeast (Psakhye and Jentsch, 2012, 2016). This experimental approach identified several potential SUMO substrates that were annotated as mitochondrial proteins. Struck by this remarkable finding, mitochondria-targeted proteins seemed to be an exceptionally fascinating group of novel SUMO substrates to study. Thus, the first objective of this study was to elucidate whether these proteins are indeed modified by SUMO *in vivo*. To this end, the SUMOylation of individual candidate proteins was analyzed in direct assays, thereby additionally allowing the identification of SUMO attachment sites and to study the involvement of SUMO E3 ligases. Based on this initial biochemical analysis, a second major aim of this study was to investigate the SUMOylation of mitochondria-targeted proteins in terms of regulation and function.

In the context of proteins transported into an organelle, it seemed particularly interesting to evaluate whether their SUMOylation was dependent on import. To this end, import-deficient mutant variants of mitochondria-targeted proteins were generated and subsequently analyzed in SUMOylation assays. A further objective was to screen for conditions and yeast mutants in which the SUMOylation of mitochondria-targeted substrates is increased. This analysis aimed to identify factors, which regulate the modification and to eventually reveal the molecular functions of mitochondria-targeted protein SUMOylation.

3 RESULTS

3.1 Discovery of mitochondria-targeted proteins as SUMO substrates

Systematic analyses of SUMO substrates have established an involvement of SUMOylation in multiple nuclear and cytosolic pathways (Geiss-Friedlander and Melchior, 2007). Notably, SUMOylation might also be involved in mitochondrial organization in yeast (Makhnevych et al., 2009) and has been implicated in the regulation of mitochondrial fission in mammalian cells (Braschi et al., 2009; Harder et al., 2004). However, convincing evidence for SUMOylation within inner mitochondrial compartments has not been provided to date and the question whether SUMOylation targets proteins “en route” to mitochondria has not been investigated so far.

3.1.1 A subset of mitochondrial matrix proteins are modified by SUMO *in vivo*

To gain deeper insights into the SUMO-modified proteome in yeast, our laboratory established a SILAC-based proteomics approach, which involves the purification of ^{His}SUMO conjugates from yeast cells followed by mass spectrometric (MS) analysis (Psakhye and Jentsch, 2012, 2016) (Figure 6A). This method relies on the usage of an N-terminal heptahistidine tag, which is compatible with the Ni-NTA-based purification of ^{His}SUMO-modified proteins under fully denaturing conditions. Thus transient SUMO modifications are preserved and the co-purification of interacting proteins is strongly reduced.

Intriguingly, among a number of more than 1000 potential SUMO substrates (Ivan Psakhye, Fabian den Brave and Stefan Jentsch; unpublished data), this approach revealed a set of 86 proteins that were annotated as proteins of inner mitochondrial subcompartments (Figure 6B). This group of potential SUMO substrates included a small number of intermembrane space proteins, whereas proteins of the inner mitochondrial membrane and the mitochondrial matrix were overrepresented. Importantly, only a minor fraction (less than 10 %) of potential SUMO substrates were annotated as proteins with dual localization (mitochondrial and cytosolic), suggesting the intriguing possibility that proteins targeted exclusively to mitochondria are indeed SUMOylated *in vivo*.

To confirm the SUMOylation of individual proteins in direct assays, several candidate proteins were fused to C-terminal 3HA epitopes and expressed from their endogenous and the *ADH1* promoter, respectively. Notably, the HA epitope tag was selected because it lacks lysine residues and therefore avoids the introduction of potential SUMO acceptor sites into a corresponding fusion protein.

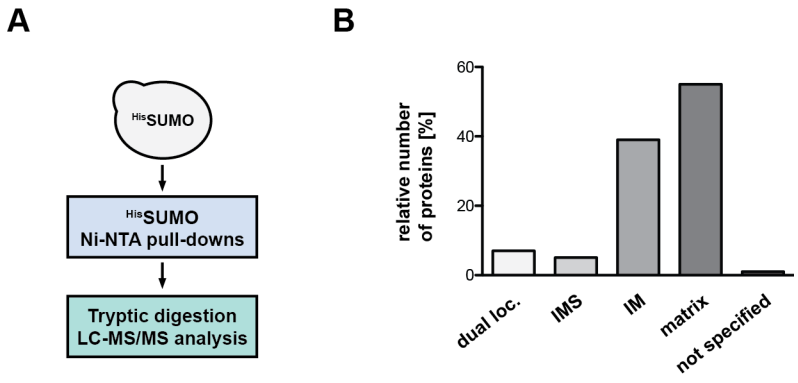


Figure 6. A mass spectrometry-based approach identifies mitochondria-targeted proteins as potential SUMO substrates. (A) Schematic representation of the experimental setup used to identify novel SUMO conjugates in the yeast *S. cerevisiae*. Yeast cells expressing N-terminally His-tagged SUMO from the *ADH1* promoter were employed to purify ^{His}SUMO conjugates by denaturing Ni-NTA pull-downs. The enriched SUMO substrates were then analyzed by tryptic digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS). **(B)** Localization and relative submitochondrial distribution of 86 mitochondrial proteins identified as potential SUMO substrates. Abbreviations indicate dual localization (dual loc.), mitochondrial intermembrane space (IMS) and mitochondrial inner membrane (IM).

To subsequently detect SUMOylated species of HA-tagged candidate proteins, SUMO conjugates were isolated from yeast cells co-expressing ^{His}SUMO from the *ADH1* promoter (Figure 7A). As controls, wild type yeast and cells solely expressing ^{His}SUMO or the HA-tagged candidate protein were included to ensure specificity of the approach. Moreover, to control for pull-down efficiency in these assays, the SUMOylation of endogenous 3-phosphoglycerate kinase (Pgk1) was analyzed.

Strikingly, western blot analysis of the enriched SUMO conjugates confirmed several substrates identified by MS analysis and demonstrated that a subset of structurally and functionally distinct mitochondrial matrix proteins are indeed modified by SUMO *in vivo*. These proteins include Ilv6 (Figure 7B), the regulatory subunit of acetolactate synthase involved in branched-chain amino acid biosynthesis (Cullin et al., 1996; Pang and Duggleby, 1999), Adh3 (Figure 7C), a mitochondrial alcohol dehydrogenase isoform (Lutstorf and Megnet, 1968; Sugar et al., 1970) and Mrpl23 (Figure 7D), a mitochondrial ribosomal protein (Kitakawa et al., 1997). Moreover, all confirmed substrates are nuclear-encoded proteins and contain N-terminal MTSs, which enable their import into the mitochondrial matrix.

SUMOylation of each of these substrates gave rise to a single slower-migrating protein form, which could be specifically detected in samples from cells expressing the respective HA-tagged protein in combination with ^{His}SUMO (Figure 7B-D). The apparent molecular weights of these slower-migrating species were

increased by roughly 20 kDa compared to the unmodified proteins, which is characteristic for the modification of substrate proteins with a single ^{His}SUMO moiety (Hoege et al., 2002; Psakhye and Jentsch, 2012). Notably, only a small fraction of each SUMO substrate was modified at steady state and SUMOylated protein species could not be detected in total cell extracts.

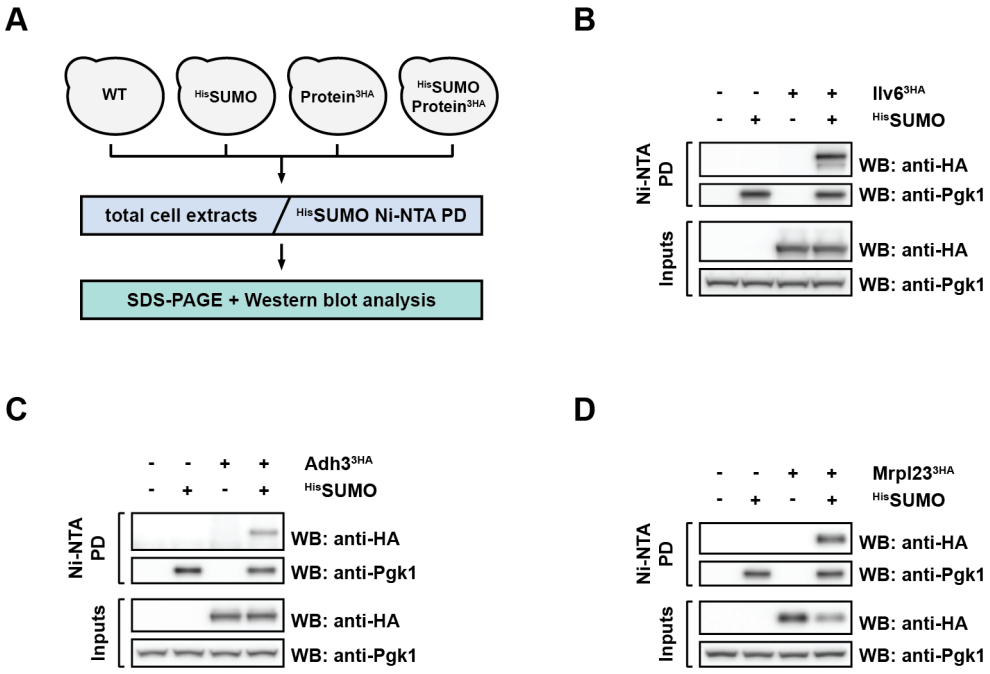


Figure 7. A number of mitochondrial matrix proteins are modified by SUMO *in vivo*. (A) Schematic depiction of the experimental design used to analyze the SUMOylation of individual proteins. Total cell extracts (Inputs) were prepared by TCA precipitation. SUMO conjugates were purified by denaturing Ni-NTA pull-downs (Ni-NTA PD) from cells expressing ^{His}SUMO from the *ADH1* promoter. Proteins were separated by SDS-PAGE on 12 % Bis-Tris gels and analyzed by western blotting using specific antibodies. **(B)** Identification of Ilv6 as SUMO substrate. Denaturing Ni-NTA pull-downs (Ni-NTA PD) were performed to isolate ^{His}SUMO conjugates from different yeast strains. Cells expressing ^{His}SUMO from the *ADH1* promoter or/and Ilv6 fused to a C-terminal 3HA epitope from the endogenous promoter are indicated. ^{His}SUMO conjugates and proteins from total cell extracts (Inputs) (prepared by TCA precipitation) were separated on 12 % Bis-Tris gels and analyzed by western blotting using HA epitope- and Pgk1-specific antibodies. Pgk1 SUMOylation was analyzed to control for pull-down efficiency. Levels of unmodified Pgk1 served as loading control. **(C)** Identification of Adh3 as SUMO substrate. Similar to (B) but with cells expressing C-terminally 3HA-tagged Adh3 from the endogenous promoter. **(D)** Identification of Mrpl23 as SUMO substrate. Similar to (B) but with cells harboring a plasmid that expresses C-terminally 3HA-tagged Mrpl23 from the *ADH1* promoter.

3.1.2 SUMOylation of mitochondria-targeted proteins is mediated by specific SUMO E3 ligases

In most cases, the SUMOylation of substrate proteins *in vivo* is strongly dependent on SUMO E3 ligases. To test whether the SUMOylation of mitochondria-targeted proteins is stimulated by distinct SUMO E3 ligases, the levels of SUMO-modified HA-tagged Ilv6 were analyzed in cells lacking one of the known SUMO E3 ligases Siz1

(*Δsiz1*), Siz2 (*Δsiz2*), Zip3 (*Δzip3*) or expressing a ligase-defective mutant variant of the essential SUMO E3 enzyme Mms21 (*mms21-11*). Western blot analysis of SUMO conjugates isolated by ^{His}SUMO Ni-NTA pull-downs indicated that the SUMOylation of Ilv6 is mediated by Siz1 and to a minor extend by Siz2 (Figure 8A). By contrast, Ilv6 SUMOylation was unaltered upon deletion of Zip3, which is consistent with its meiosis-specific functions (Agarwal and Roeder, 2000; Cheng et al., 2006; Eichinger and Jentsch, 2010). Different from the other E3 ligase mutants, *mms21-11* cells displayed lower Ilv6 protein levels in total cells extracts. Moreover, SUMOylation of both Ilv6 and Pgk1 was impaired in samples from these cells. Since it appears unlikely that the cytosolic enzyme Pgk1 is SUMOylated by the strictly nuclear E3 ligase Mms21, this suggested an indirect effect that was probably related to the slow growth phenotype of this particular mutant (Figure 8B).

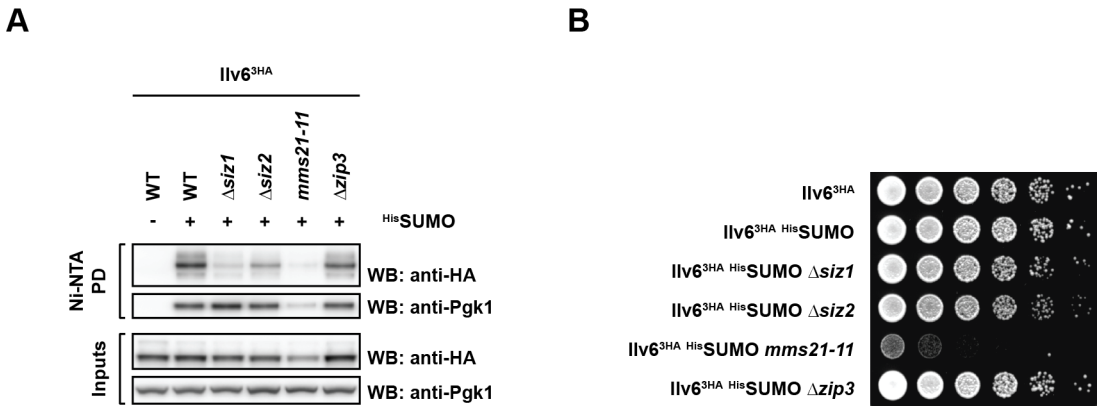


Figure 8. Ilv6 SUMOylation is catalyzed by the SUMO E3 ligases Siz1 and Siz2. (A) Ilv6 SUMOylation is specifically reduced in cells lacking Siz1 or Siz2. Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from wild type cells, mutants lacking one of the known SUMO E3 ligases Siz1 (*Δsiz1*), Siz2 (*Δsiz2*), Zip3 (*Δzip3*) or expressing a mutant variant of the SUMO E3 ligase Mms21 that lacks E3 ligase activity (*mms21-11*). All cells used in (A) express C-terminally 3HA-tagged Ilv6 from the endogenous promoter. Cells expressing ^{His}SUMO from the *ADH1* promoter are indicated. **(B)** Growth phenotypes of yeast strains used in (A). Five-fold serial dilutions of cells were spotted on YPD plates and incubated at 30°C for 2 days.

Indeed, Ilv6 SUMOylation was virtually absent in double deletion mutants lacking both Siz1 and Siz2 (*Δsiz1 Δsiz2*) (Figure 9A), demonstrating that these two SUMO E3 ligases of the conserved Siz/PIAS protein family mediate the modification of Ilv6. Strikingly, with highly similar contributions, Siz1 and Siz2 also catalyzed the SUMOylation of Adh3 (Figure 9B) and Mrpl23 (Figure 9C), demonstrating that all mitochondria-targeted SUMO substrates identified in this study require an identical combination of SUMO E3 ligases for modification.

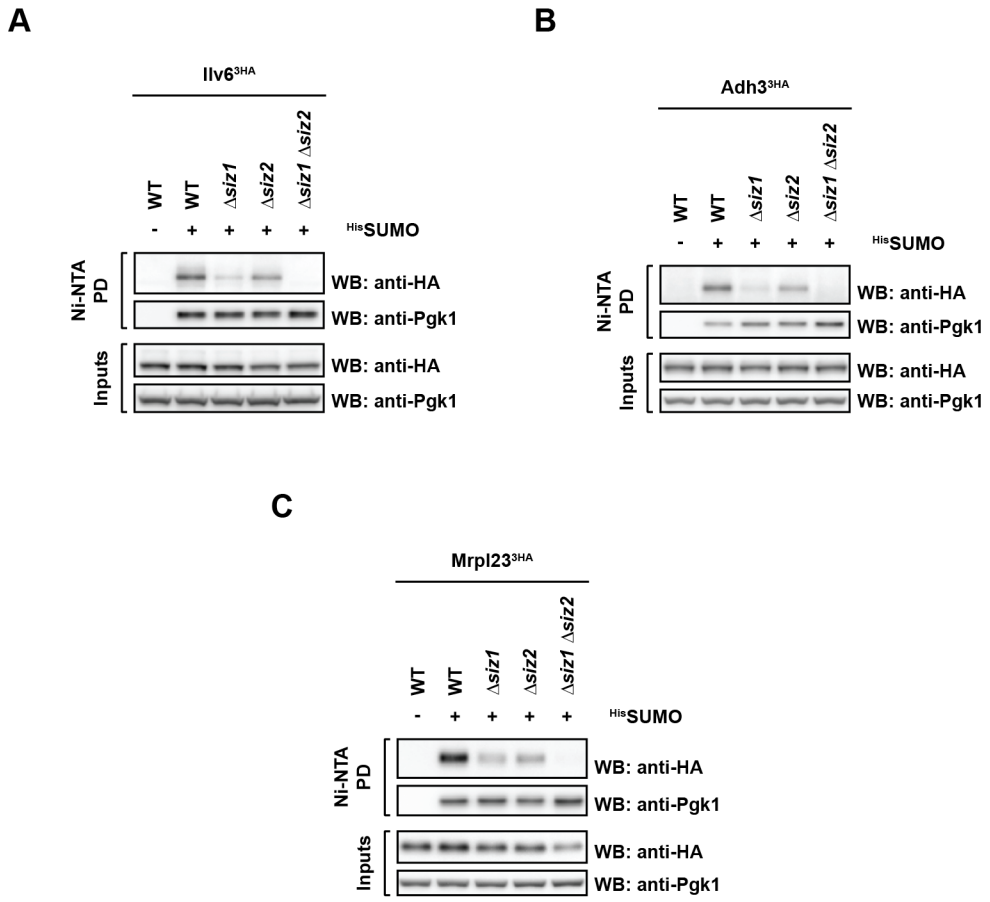


Figure 9. SUMOylation of mitochondria-targeted proteins requires the combined activity of the SUMO E3 ligases Siz1 and Siz2. (A) Ilv6 SUMOylation is virtually absent in cells lacking the SUMO E3 ligases Siz1 and Siz2. Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from wild type cells, mutants lacking Siz1 (*Δsiz1*), Siz2 (*Δsiz2*) and the double deletion mutant (*Δsiz1 Δsiz2)*. **(B)** SUMOylation of Adh3 is almost undetectable in cells lacking the SUMO E3 ligases Siz1 and Siz2. Similar to (A) but with cells expressing C-terminally 3HA-tagged Adh3 from the endogenous promoter. **(C)** SUMOylation of Mrpl23 almost undetectable in cells lacking the SUMO E3 ligases Siz1 and Siz2. Similar to (A) but with cells harboring a plasmid that expresses C-terminally 3HA-tagged Mrpl23 from the *ADH1* promoter.

3.1.3 Identification of SUMO acceptor sites of mitochondria-targeted proteins

SUMO substrates are often modified at one or multiple specific lysine residues. To identify the SUMO acceptor sites of Ilv6, all lysine residues of the protein were individually replaced by arginine. The corresponding *KR* mutant variants were tagged with C-terminal 3HA epitopes and expressed from the endogenous promoter in yeast. Among these variants, a mutant at Lys 260 (*K260R*) strongly reduced the levels of Ilv6^{3HA}-SUMO conjugates (Figure 10A), indicating that this particular lysine residue is the major SUMO attachment site of Ilv6. Sequential replacement of three additional lysine residues by arginine (*K218R*, *K284R* and *K296R*) further decreased the SUMOylation of Ilv6 in a stepwise manner. Accordingly, a mutant variant lacking

all four lysine residues (*ilv6^{3HA}-K218R, K260R, K284R, K296R* termed *ilv6^{3HA}-4KR*) was almost completely refractory to SUMOylation (Figure 10A and B).

Interestingly, analysis of Ilv6 SUMOylation using different *KR* mutant variants also revealed that modification of individual lysine residues of Ilv6 gives rise to differentially migrating protein species (Figure 10A). This suggests that the relative electrophoretic mobility of Ilv6-SUMO conjugates is dependent on the position of the modified lysine residues. Similar findings have been made for the SUMO substrates PCNA (Hoege et al., 2002) and Rad52 (Sacher et al., 2006), further confirming that the gel migration behavior of SUMO-protein conjugates not only depends on their size but also on the positions of the branched peptides.

Ilv6 contains two lysine residues (K158 and K218) embedded within a ΨKX(D/E)-type SUMOylation consensus motif. However, only one of these lysine residues (K218) was detectably SUMOylated in wild type cells and the modification of Lys 218 occurred with much lower efficiency than the SUMOylation of the non-consensus Lys 260. Thus, the SUMOylation of Ilv6 is not restricted to SUMOylation consensus sites and occurs with even stronger preference at alternative lysine residues.

In case of Adh3, computational analysis using the GPS-SUMO software (Zhao et al., 2014) identified two potential SUMO attachment sites at Lys 305 and Lys 375. Individual and simultaneous replacement of these two lysine residues by arginine revealed a prominent modification of Adh3 at Lys 305, which is embedded within a SUMOylation consensus motif (Figure 10C and D).

Remarkably, all SUMO attachment sites identified in Ilv6 and Adh3 appeared to exclusively localize to C-terminal segments of these proteins (Figure 10B and D). Similarly, SUMOylation of C-terminal acceptor sites could be observed for Mrpl23, where the simultaneous mutation of the two most C-terminal lysine residues (K155 and K163) to arginine reduced the levels of Mrpl23^{3HA}-SUMO conjugates by around 50 % (Figure 10E and F). Additionally, SUMO conjugates of the resulting mutant variant (*mrpl23^{3HA}-K155R, K163R*) showed an altered and more dispersed electrophoretic mobility compared to conjugates of the wild type protein. This suggests that Lys 155 and Lys 163 indeed serve as primary SUMO attachment sites of Mrpl23 and that alternative lysine residues are modified when Lys 155 and Lys 163 have been experimentally removed. Notably, neither of these two lysine residues is embedded within a ΨKX(D/E)-type sequence motif, confirming that mitochondria-targeted SUMO substrates are modified at both consensus and non-consensus SUMOylation sites.

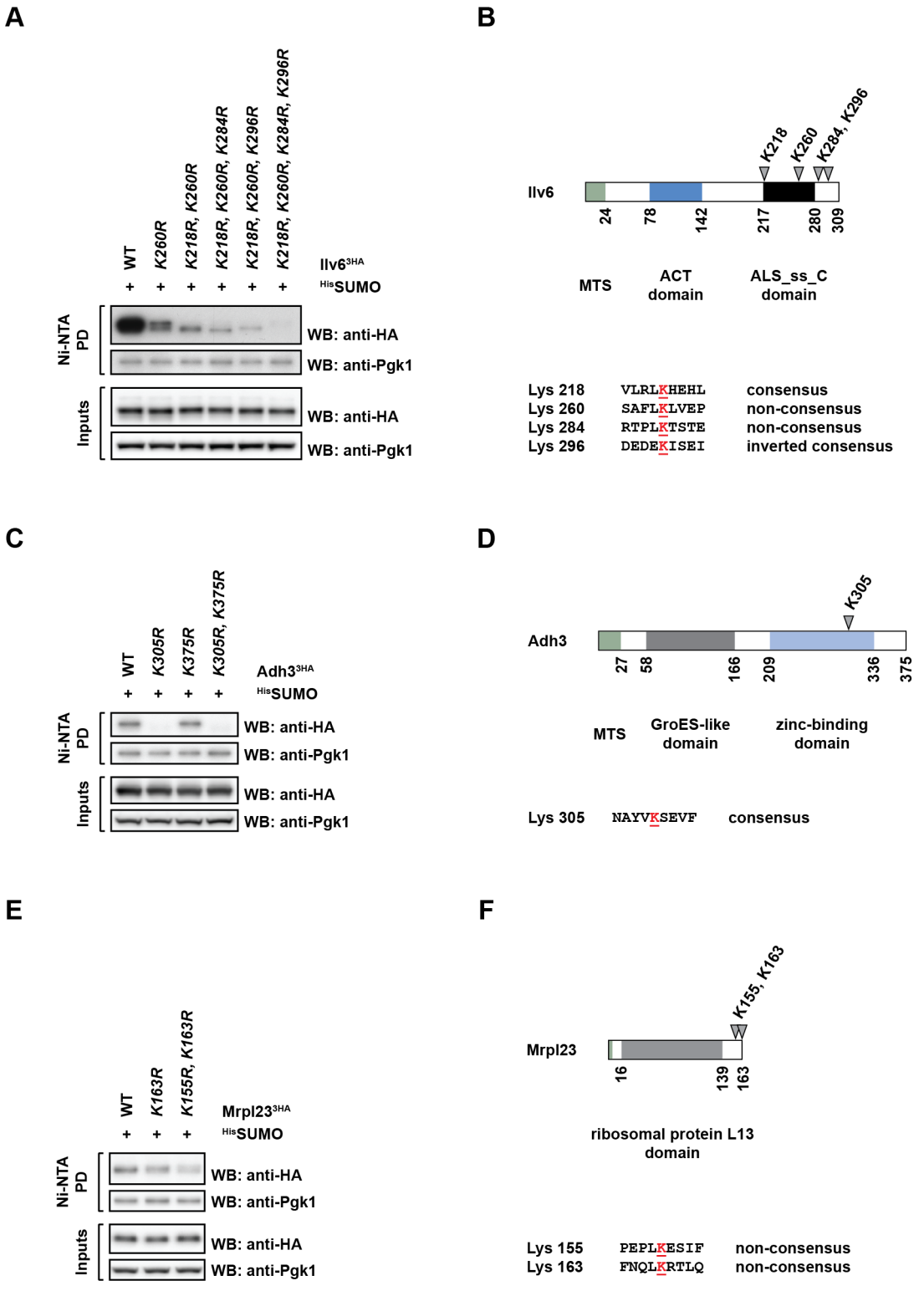


Figure 10. The SUMO acceptor lysines of mitochondria-targeted proteins. (A) Ilv6 SUMOylation occurs at multiple lysine residues. Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from cells harboring plasmids that express C-terminally 3HA-tagged Ilv6 or various *KR* mutant variants as indicated from the endogenous promoter. **(B)** Schematic representation of the Ilv6 protein indicating the positions of four SUMO acceptor lysines (grey triangles). The mitochondrial targeting sequence (MTS) is depicted in green, the ACT (aspartate kinase, chorismate mutase and TyrA) domain in blue and the ALS_ss_C (acetolactate synthase small subunit C-terminus) domain in black. **(C)** Adh3 is SUMOylated at Lys 305 (K305). Similar to (A) but with cells harboring plasmids expressing C-terminally 3HA-tagged Adh3 or the indicated *KR* mutant variants from the *GAL1* promoter. **(continued on next page)**

(legend to Figure 10 continued) (D) Schematic representation of the Adh3 protein and the position of the SUMO acceptor site at Lys 305. The mitochondrial targeting sequence (MTS) is depicted in green, the GroES-like domain in grey and the zinc-binding domain in blue. **(E)** Identification of two SUMO acceptor lysines in Mrpl23. Similar to (A) but with cells harboring plasmids expressing C-terminally 3HA-tagged Mrpl23 or the indicated *KR* mutant variants from the *ADH1* promoter. **(F)** Schematic representation of the Mrpl23 protein and the localization of two SUMO acceptor sites (Lys 155 and Lys 163). The mitochondrial targeting sequence is depicted in green and the ribosomal L13 domain in grey.

3.1.4 SUMOylation of mitochondria-targeted proteins is import-independent

All proteins identified as novel SUMO substrates in this study are nuclear-encoded proteins. These proteins are synthesized on cytosolic ribosomes and subsequently imported into mitochondria. Hence, the important questions arose, at which biogenesis stage the SUMOylation of mitochondria-targeted proteins occurs and whether the modification is linked to their import into the mitochondrial matrix.

To clarify theses question, a series of import-deficient mutant Ilv6 variants lacking the N-terminal MTS were generated (Figure 11A). The design of these variants was based on database annotations (UniProt) and computational MTS prediction, which suggested two potential processing sites of the Ilv6 prepeptide after Cys 24 (UniProt and TargetP) and Val 94 (MitoProt II), respectively. Moreover, as an "intermediate" between the two predictions, an Ilv6 variant lacking amino acid 2-55 was generated.

Microscopic analysis of different Ilv6 constructs fused to GFP confirmed the mitochondrial localization of full-length Ilv6 and demonstrated that removal of an N-terminal 24-amino-acid peptide (Δmts-ilv6^{GFP} variant I) was sufficient to prevent mitochondrial import (Figure 11B). The presumably cytosolic localization of the resulting deletion mutant is in line with a previous study on the catalytic subunit of yeast acetolactate synthase (Ilv2), which reported that MTS deletion causes a similar mislocalization of Ilv2 to the cytosol (Dasari and Kolling, 2011).

To subsequently analyze the SUMOylation of the import-deficient Ilv6 variants, all truncation mutants were fused to C-terminal 3HA epitopes and expressed from the inducible *GAL1* promoter. In the corresponding western blot assays, expression of full-length Ilv6 gave rise to a prominent double band representing the Ilv6 precursor and a faster-migrating mature form of Ilv6 (Figure 12A). As expected, all N-terminally truncated Ilv6 mutants yielded single bands when detected with an HA-specific antibody (Figure 12A). Notably, the Ilv6 variant lacking amino acid 2-24 displayed a similar electrophoretic mobility as the mature form, suggesting that proteolytic processing of Ilv6 by MPP indeed occurs after or in close proximity to Cys 24.

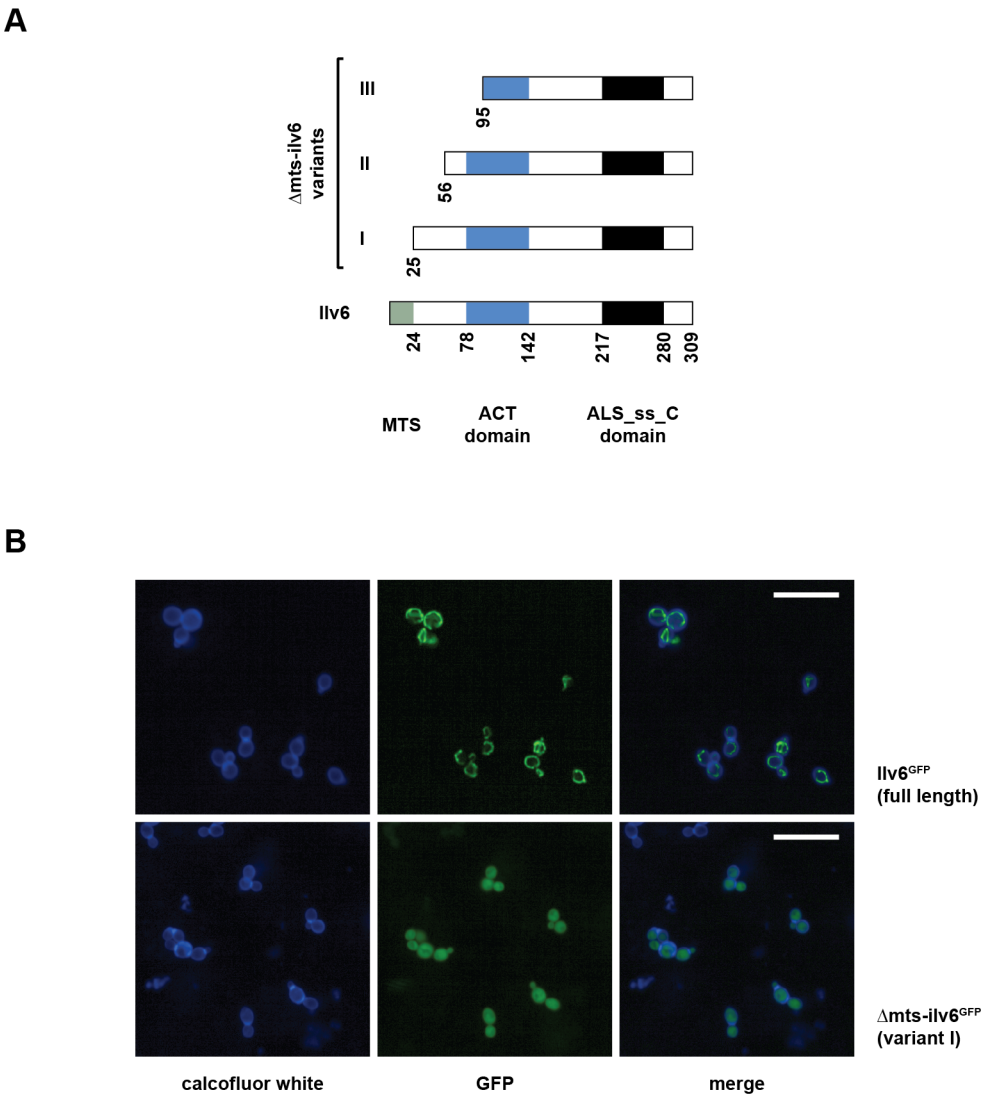


Figure 11. Generation of import-incompetent mutant variants of a mitochondria-targeted protein. **(A)** Schematic representation of the Ilv6 protein and three different N-terminally truncated mutant variants lacking the MTS (Δmts-ilv6 variant I-III). The N-terminal prepeptide (according to UniProt annotation) is depicted in green, the ACT domain in blue and the ALS_ss_C domain in black. **(B)** Deletion of the N-terminal 24-amino-acid prepeptide of Ilv6 is sufficient to prevent mitochondrial import. Microscopic analysis of GFP fusion proteins of full-length Ilv6 and a mutant variant lacking the N-terminal MTS (Δmts-ilv6 variant I). Yeast cell walls were visualized by calcofluor white staining. Scale bars represent 20 µm.

Moreover, strikingly, western blot analysis of subsequently isolated ^{His}SUMO conjugates demonstrated that all import-deficient variants of Ilv6 were efficiently SUMOylated, even at higher levels than the full-length protein (Figure 12A). Thus, the SUMO modification of Ilv6 is in fact independent of mitochondrial import and does not require the presence of an MTS.

Notably, Ilv6^{3HA}-SUMO conjugates isolated from strains expressing full-length Ilv6 exhibited a similar electrophoretic mobility compared to SUMOylated species of the Δmts-ilv6^{3HA} variant I. Assuming that SUMOylated Ilv6 precursors migrate slower

during gel electrophoresis, this suggests that Ilv6-SUMO conjugates possess a proteolytically processed N-terminus also in strains expressing the full-length substrate. Therefore, it is conceivable that C-terminally SUMOylated Ilv6 can initiate import normally, leading to removal of the N-terminal presequence by MPP in the matrix.

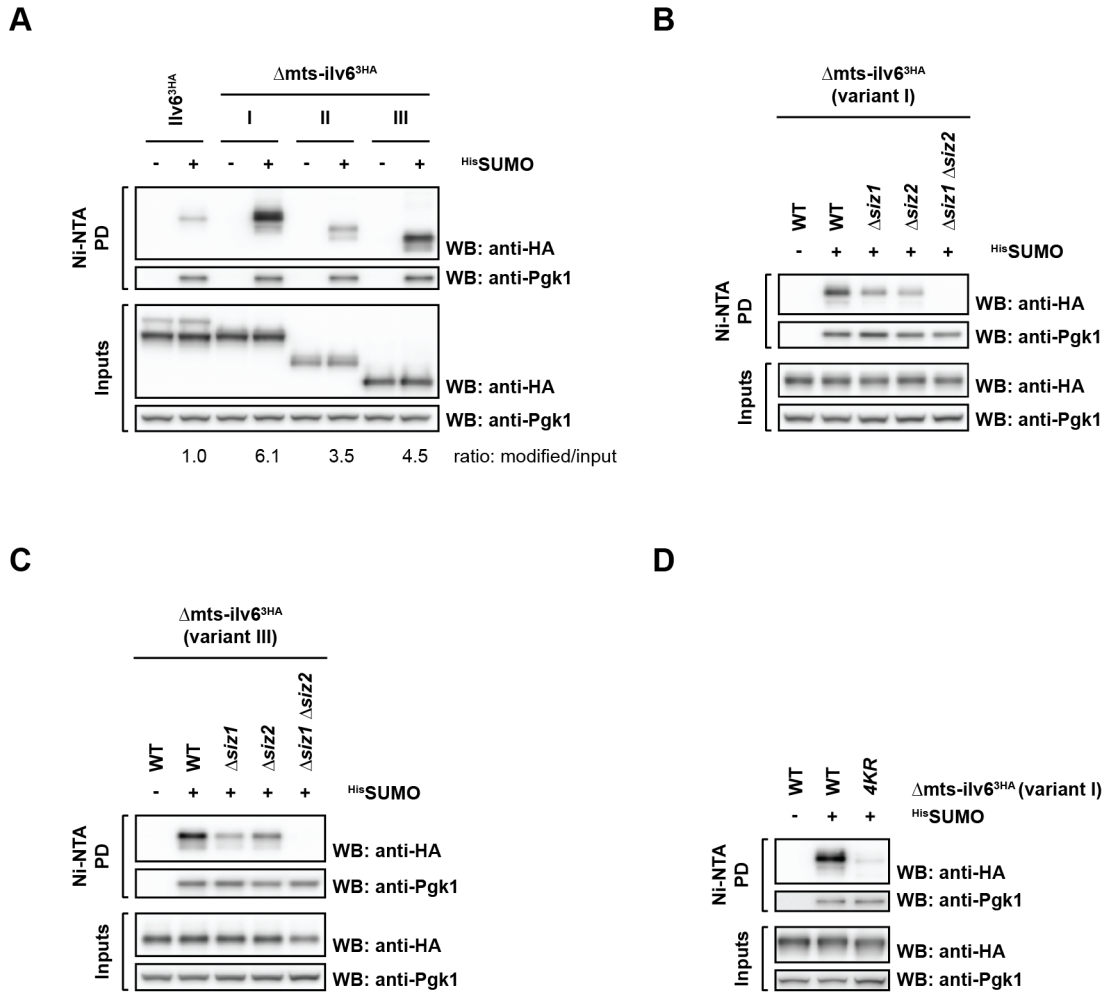


Figure 12. SUMOylation of Ilv6 is independent of mitochondrial import. (A) Import-incompetent Ilv6 mutant variants are efficiently SUMOylated. Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from cells harboring plasmids that express C-terminally 3HA-tagged full-length Ilv6 or one of three N-terminally truncated mutant variants as indicated. All protein variants are expressed from the *GAL1* promoter. To achieve similar protein levels, expression was induced for 30 min (full-length Ilv6) and 60 min (variant I-III), respectively. Cells expressing ^{His}SUMO from the *ADH1* promoter are indicated. Ratios of the levels of SUMOylated vs. unmodified proteins were determined by western blot quantification using ImageJ **(B and C)** SUMOylation of import-deficient Ilv6 is stimulated by the SUMO E3 ligases Siz1 and Siz2. Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from wild type cells, mutants lacking Siz1 (*Δsiz1*), Siz2 (*Δsiz2*) and the double deletion mutant (*Δsiz1 Δsiz2)*. Cells are complemented with plasmids expressing the import-deficient Ilv6 mutant variant I (B) and variant III (C), respectively from the *GAL1* promoter. Protein expression was induced for 60 min. **(D)** Identification of SUMO modification sites of import-incompetent Ilv6. Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from cells harboring plasmids that express import-incompetent Ilv6 (variant I) or a mutant variant, in which four lysine residues are replaced by arginine (corresponding to the positions K218, K260, K284 and K294 of full-length Ilv6).

However, a bulky modification like SUMO might be too large to allow threading through the narrow mitochondrial import pores and therefore could block the completion of mitochondrial import for steric reasons (see section 4.3).

Interestingly, when studied in further detail, the SUMOylation of import-incompetent Ilv6 variants displayed similar characteristics as the modification of the full-length protein. In particular, the modification was also dependent on the E3 ligases Siz1 and Siz2 (Figure 12B and C) and evidently targeted a largely overlapping set of SUMO acceptor sites (Figure 12D).

To further corroborate that the SUMOylation of mitochondria-targeted proteins is independent of mitochondrial import, Adh3 was analyzed as a second SUMO substrate. To this end, a C-terminally HA-tagged Adh3 variant lacking the N-terminal MTS (amino acid 1-27) was generated (Δmts-adh3^{3HA}) (Figure 13A) and expressed from the *GAL1* promoter in yeast. Both, full-length (Adh3^{3HA}) and N-terminally truncated Adh3 (Δmts-adh3^{3HA}) gave rise to a single band in western blot assays. This indicates that also in this experimental system the vast majority of full-length Adh3 species are imported into mitochondria and proteolytically processed by MPP in the matrix (Figure 13B).

Strikingly, Adh3 lacking the MTS was strongly SUMOylated, also at higher levels than the full-length protein (Figure 13B). This further confirmed that SUMOylation efficiently targets import-incompetent mutant variants of mitochondrial proteins. SUMOylation of Δmts-adh3^{3HA} yielded two species with distinctive electrophoretic mobility, suggesting the modification of this mutant variant occurs at two alternative lysine residues. Nevertheless, SUMOylation of both species was dependent on the combined activity of Siz1 and Siz2 (Figure 13C), thus exhibiting the same SUMO E3 ligase requirement as the modification of the full-length protein. Moreover, import-deficient Adh3 was preferentially modified at the same SUMO attachment site as the wild type protein and replacement of this particular lysine residue by arginine caused the loss of the more prominent, faster-migrating Δmts-adh3^{3HA}-SUMO conjugate (Figure 13D).

Taken together, several lines of evidence indicate that the SUMOylation of mitochondria-targeted substrates is generally import-independent. Mutant variants of mitochondrial proteins lacking a functional MTS are strongly SUMOylated and truncation of the MTS in fact enhances their modification. Moreover, in terms of E3 ligase requirement and SUMO attachment sites, the SUMOylation of import-incompetent substrate variants exhibits striking similarities to the modification of the full-length proteins. In conclusion, these findings could have important implications

for the SUMOylation of import-competent mitochondrial proteins and suggest that their modification probably occurs prior to import into the organelle.

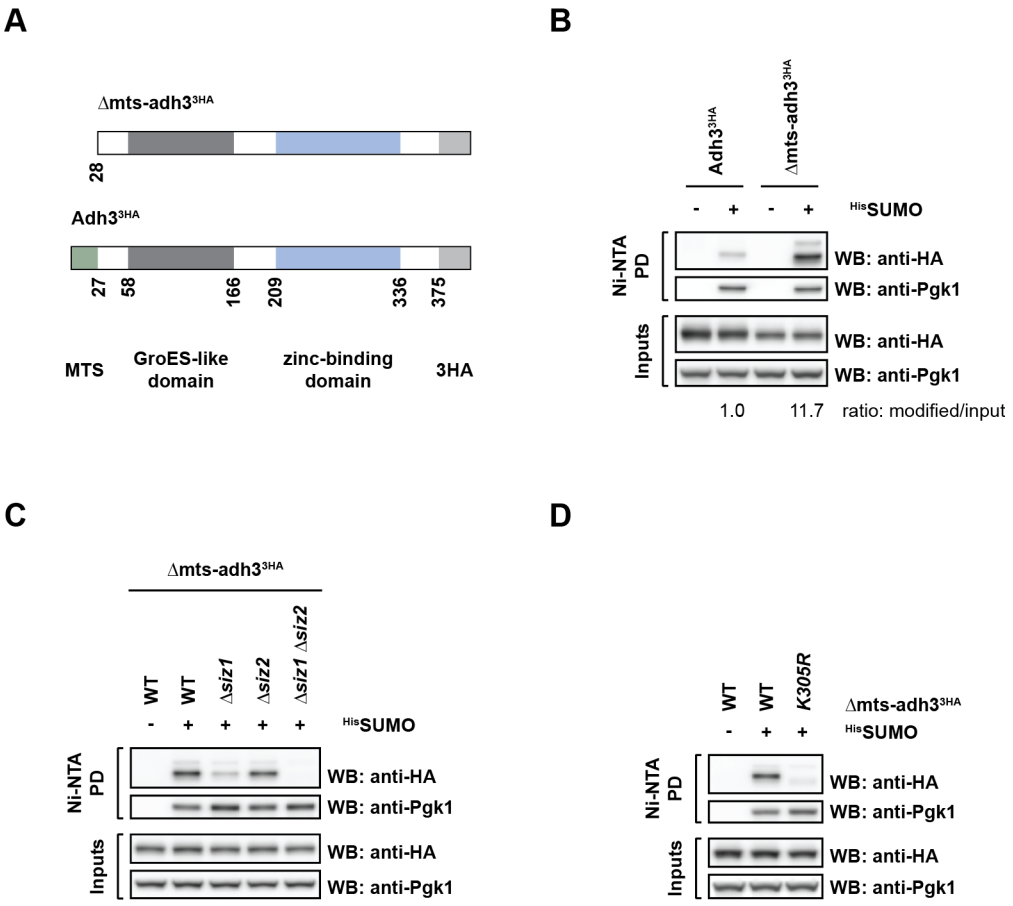


Figure 13. SUMOylation of Adh3 is independent of mitochondrial import. (A) Schematic representation of the Adh3 protein and an N-terminally truncated mutant variant lacking the MTS (Δmts-adh3^{3HA}). The N-terminal prepeptide is depicted in green, the GroES-like domain in dark grey, the zinc-binding domain in blue and the 3HA epitope in light grey. **(B)** Import-incompetent Adh3 is efficiently SUMOylated. Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from cells harboring plasmids that express C-terminally 3HA-tagged full-length Adh3 (Adh3^{3HA}) or a mutant variant lacking the N-terminal MTS (Δmts-adh3^{3HA}). Cells were grown in galactose-containing medium, allowing the constitutive expression of all protein variants from the *GAL1* promoter. Cells expressing ^{His}SUMO from the *ADH1* promoter are indicated. Ratios of the levels of SUMOylated vs. unmodified proteins were determined by western blot quantification using ImageJ **(C)** SUMOylation of import-deficient Adh3 is stimulated by the SUMO E3 ligases Siz1 and Siz2. Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from wild type cells, mutants lacking Siz1 (*Δsiz1*), Siz2 (*Δsiz2*) and the double deletion mutant (*Δsiz1 Δsiz2)*. Cells were complemented with plasmids expressing the import-deficient Adh3 mutant variant under control of the *GAL1* promoter and grown in galactose-containing medium. **(D)** Import-incompetent Adh3 is predominantly SUMOylated on Lys 305 (K305). Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from cells harboring plasmids that express import-incompetent Adh3 (Δmts-adh3^{3HA} WT) or a lysine mutant resulting from the replacement of one lysine residue by arginine (corresponding to position K305 of full-length Adh3) (*Δmts-adh3^{3HA}-K305R*).

3.2 SUMOylation of mitochondria-targeted proteins is regulated by cytosolic factors

Posttranslational import of mitochondrial proteins often involves molecular chaperones or similar factors that maintain the import-competence of precursor proteins after their synthesis in the cytosol. Thus, the idea that the SUMOylation of mitochondria-targeted proteins occurs prior to import at a cytosolic biogenesis stage prompted the question whether the modification is regulated by cytosolic factors as well.

3.2.1 SUMOylation of mitochondria-targeted proteins is linked to the HSP70 system

In yeast, strong evidence indicates an implication of *SSA* family chaperones in protein translocation into mitochondria (Deshaies et al., 1988; Murakami et al., 1988). *SSA* chaperones comprise four homologous proteins, Ssa1-4, and represent a major group of cytosolic HSP70s in *S. cerevisiae*. Although the deletion of all four Ssa proteins is lethal, expression of any single member of this family of chaperones is sufficient to maintain cell viability. Consistently, strains deleted for three out of four *SSA* genes (*Δssa2 Δssa3 Δssa4*) and harboring either wild type *SSA1* or a temperature-sensitive allele (*ssa1-45)* (Becker et al., 1996) have been widely used to study HSP70 functions in yeast.

To test whether mitochondria-targeted protein SUMOylation is affected in *SSA* mutants, ^{His}SUMO together with 3HA-tagged Ilv6 or Adh3 were expressed in wild type (DF5 yeast cells containing the same set of auxotrophic markers but expressing Ssa1-4), *SSA1* and *ssa1-45* cells. Consistent with results described in the previous sections of this study, HA-tagged Ilv6 gave rise to a double band in western blot assays, representing the unprocessed Ilv6 precursor and a faster-migrating mature form (Figure 14A). Notably, particularly *ssa1-45* mutants displayed increased levels of the Ilv6 and Adh3 precursors (Figure 14A and B), indicating that Ssa proteins are required for efficient mitochondrial import of these proteins. This finding is consistent with previous studies, which reported an accumulation of mitochondrial precursor proteins in *SSA* mutant yeast strains (Becker et al., 1996; Deshaies et al., 1988).

Strikingly, the SUMOylation patterns of both Ilv6 and Adh3 were also affected in *ssa1-45* cells, which showed a strong accumulation of the SUMOylated Ilv6 and Adh3 precursor, respectively (Figure 14A and B). In both cases SUMOylation produced multiple slower-migrating species, suggesting that the proteins are perhaps modified at multiple sites.

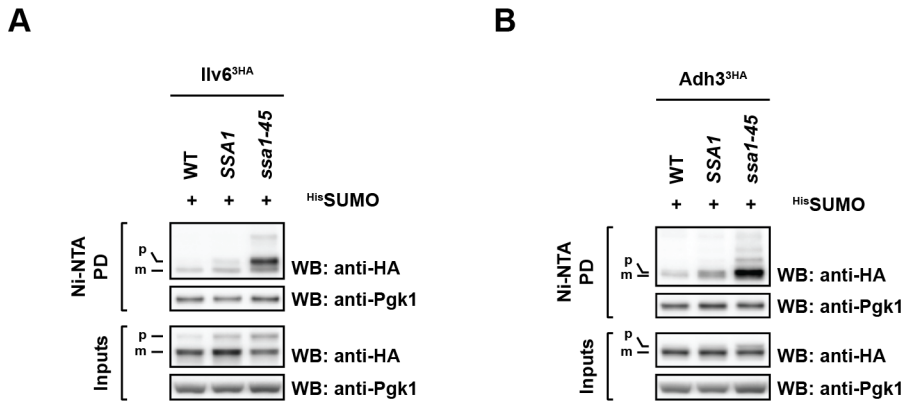


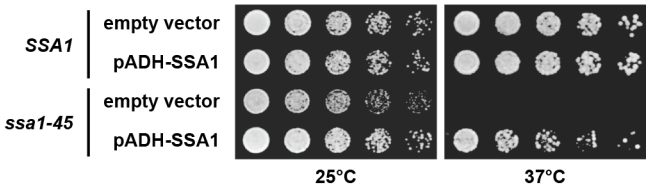
Figure 14. SUMOylation of mitochondria-targeted proteins is increased in mutants of the *SSA* family of HSP70 chaperones. (A) SUMOylated Ilv6 precursor species strongly accumulate in *ssa1-45* chaperone mutant cells. Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from wild type (*SSA1 SSA2 SSA3 SSA4* in DF5 background), *SSA1* (*SSA1 Δssa2 Δssa3 Δssa4*) and *ssa1-45* (*ssa1-45 Δssa2 Δssa3 Δssa4*) cells. C-terminally 3HA-tagged Ilv6 is expressed from the endogenous promoter and ^{His}SUMO from the *ADH1* promoter. Bands corresponding to (monoSUMOylated) precursor protein (p) and the (monoSUMOylated) mature (m) form are labeled. **(B)** Singly and perhaps multiply SUMOylated Adh3 precursor protein species strongly accumulate in *ssa1-45* mutant cells. Similar to (A) but with cells expressing C-terminally 3HA-tagged Adh3 from the endogenous promoter.

In comparison to the entirely cytosolic Δmts-adh3 mutant (Figure 13), the apparent multi-site modification of Adh3 was much more pronounced in *ssa1-45* cells. Thus, the increased levels of SUMOylated mitochondria-targeted substrates were probably not a mere result of the elevated levels of cytosolic precursor proteins. Consistently, in relation to the total Ilv6 precursor levels, the corresponding SUMOylated form also specifically accumulated in the *ssa1-45* background.

To further corroborate that the SUMOylation of mitochondrial substrates is specifically induced in *SSA* mutants, Ilv6 SUMOylation was analyzed in *SSA1* and *ssa1-45* cells reconstituted with a plasmid-borne copy of wild type Ssa1 expressed under control of the *ADH1* promoter. Importantly, expression of Ssa1 rescued the slow-growth phenotype of *ssa1-45* cells at 25°C and largely restored viability at 37°C (Figure 15A). Consistently, Ssa1 expression efficiently reduced Ilv6 precursor SUMOylation in both *SSA1* and *ssa1-45* cells (Figure 15B). By contrast, under the same conditions, total SUMO conjugate levels were largely unaffected, confirming that the SUMOylation of Ilv6 is selectively altered in cells lacking functional *SSA* HSP70s.

In summary, these data indicate that the SUMOylation of mitochondria-targeted proteins is indeed regulated by *SSA* family chaperones, specifically linking this cytosolic HSP70 system to the regulation of protein SUMOylation in yeast.

A



B

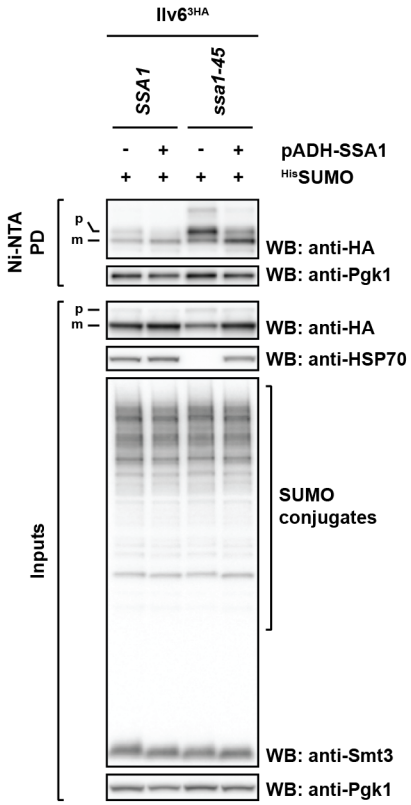


Figure 15. The SUMOylation of Ilv6 is specifically affected by Ssa1. (A) Ectopic expression of Ssa1 rescues the lethality of *ssa1-45* mutant cells at the restrictive temperature (37°C). *SSA1* (*SSA1 Δssa2 Δssa3 Δssa4*) and *ssa1-45* (*ssa1-45 Δssa2 Δssa3 Δssa4*) cells were complemented with plasmids expressing wild type Ssa1 from the *ADH1* promoter. Five-fold serial dilutions of cultures grown over night at 25°C (adjusted to OD_{600} = 1) were spotted on SC-HIS agar plates and incubated at 25°C for 2 days and 37°C for 3 days. **(B)** Expression of wild type Ssa1 reduces Ilv6 precursor SUMOylation in *SSA1* and *ssa1-45* cells. Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from *SSA1* and *ssa1-45* cells complemented with plasmids that express wild type Ssa1 from the *ADH1* promoter. C-terminally 3HA-tagged Ilv6 was expressed from the endogenous promoter and ^{His}SUMO was expressed from the *ADH1* promoter. Total SUMO conjugate levels were analyzed by probing total cell extracts (Inputs) with Smt3-specific polyclonal antibodies. Levels of wild type Ssa1 were analyzed using an HSC70/HSP70-specific monoclonal antibody (BB70) that fails to detect the ssa1-45 mutant protein. Bands corresponding to the (monoSUMOylated) precursor protein (p) and the (monoSUMOylated) mature (m) form are labeled.

3.2.2 Increased range of SUMO acceptor sites in *SSA* mutant cells

The detection of probably multiSUMOylated Ilv6 and Adh3 species in *ssa1-45* cells prompted the question whether these substrates are modified on an extended set of

SUMO acceptor sites. To test this hypothesis, an Ilv6 lysine mutant, which lacks the four major SUMO attachment sites mapped under unperturbed conditions (*ilv6*^{3HA}*-4KR*) (Figure 10A), was expressed in chaperone mutant cells. Strikingly, the precursor form of this Ilv6 variant was indeed considerably SUMOylated in *SSA* mutants, particularly in the *ssa1-45* background (Figure 16A). Moreover, a similar effect could be observed by analysis of Adh3 SUMOylation using a mutant variant lacking Lys 305 (*adh3*^{3HA}*-K305R*) (Figure 16B).

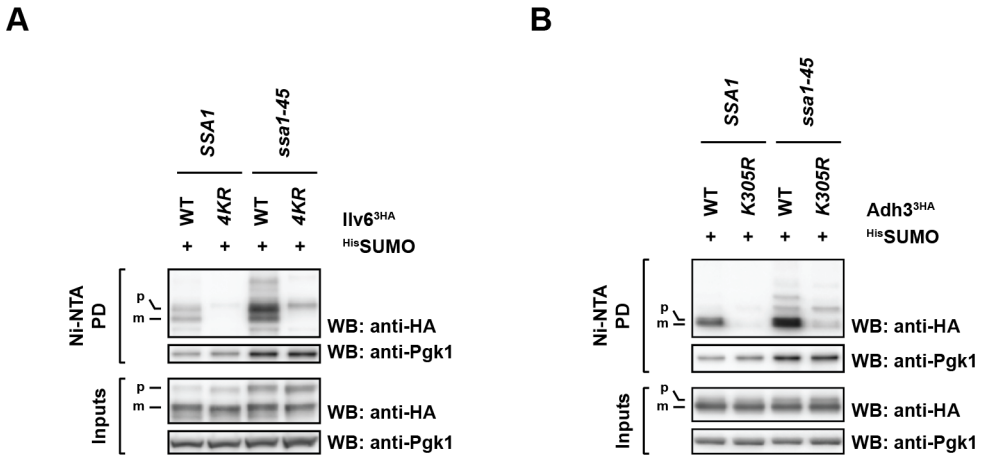


Figure 16. Functional impairment of the *SSA* HSP70 chaperone system increases the range of the SUMO acceptor sites in mitochondria-targeted proteins. (A) The *ilv6*^{3HA}*-4KR* lysine mutant (*K218R, K260R, K284R, K294R*) is substantially SUMOylated at alternative lysine residues in *ssa1-45* cells. Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from *SSA1* and *ssa1-45* mutants. Cells were complemented with plasmids that express C-terminally 3HA-tagged wild type Ilv6 or a corresponding lysine mutant (*4KR*) from the *ADH1* promoter. Expression of ^{His}SUMO under control of the *ADH1* promoter is indicated. Bands corresponding to the (monoSUMOylated) precursor protein (p) and the (monoSUMOylated) mature (m) form are labeled. **(B)** An Adh3 lysine mutant at Lys 305 is SUMOylated at alternative SUMO attachment sites in *ssa1-45* cells. Similar to (A) but with cells harboring plasmids that express C-terminally 3HA-tagged wild type Adh3 or a corresponding lysine mutant at Lys 305 from the *TDH3* promoter.

To gain deeper insights into the positions of Ilv6 SUMO acceptor sites in *ssa1-45* cells, the *4KR* mutant variant was subjected to additional rounds of mutagenesis, thereby further replacing several lysine residues by arginine in a stepwise manner. This approach indeed facilitated the identification of further Ilv6 lysine residues (K116, K158 and K202), which are specifically SUMOylated in the *SSA1* mutant background (Figure 17A and B). Moreover, the electrophoretic mobility of the corresponding SUMO conjugates indicated that exclusively Ilv6 precursors but not the mature form are modified at these sites. Thus, functional impairment of the *SSA* HSP70 system not only causes an increased SUMOylation of mitochondria-targeted substrate precursors but - compared to wild type cells - also leads to modification of an extended set of SUMO attachment sites.

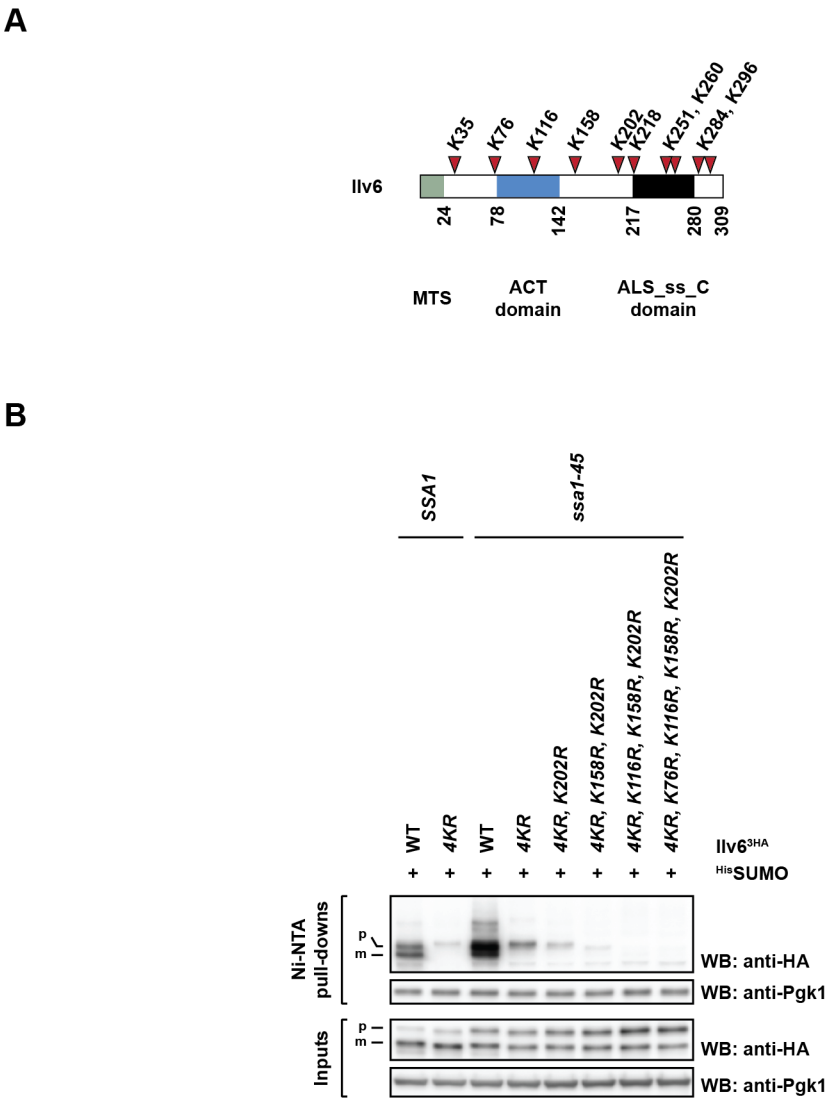


Figure 17. Identification of SUMO attachment sites of Ilv6 modified in chaperone mutant cells. (A) Schematic representation of Ilv6 and the positions of lysine residues within the protein (indicated by red triangles). **(B)** SUMOylation of Ilv6 occurs at multiple and widely distributed lysine residues in *ssa1-45* cells. Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from *SSA1* and *ssa1-45* mutants. Cells were complemented with plasmids that express C-terminally 3HA-tagged wild type Ilv6 or one of several corresponding lysine mutants as indicated from the *ADH1* promoter. Bands corresponding to the (monoSUMOylated) precursor protein (p) and the (monoSUMOylated) mature (m) form are labeled.

3.2.3 Mitochondrial precursor proteins harbor N-terminal HSP70 binding sites

The finding that SUMOylation of mitochondria-targeted proteins is linked to the *SSA* subfamily of HSP70s raised the questions how functional impairment of this chaperone system leads to an increased and apparently less selective modification of multiple lysine residues. Interestingly, it has been reported recently that the phenotypes of the *ssa1-45* allele are caused by an impaired substrate binding activity of the corresponding ssa1-45 mutant protein (Needham et al., 2015). It is therefore conceivable that association of mitochondrial preproteins with chaperones not only

confers import competence but could also shield them from SUMO conjugation. Notably, a further potentially relevant finding in this context was the detection of exclusively C-terminal SUMO attachment sites in wild type cells (Figure 10). These observations gave rise to the hypothesis that chaperones may bind to N-terminal protein segments of mitochondrial preproteins and thereby particularly prevent the SUMOylation of N-terminal lysine residues. Hence, it seemed plausible that mitochondrial precursor proteins harbor N-terminal Ssa1 binding sites.

To further address this idea, peptides derived from the primary structure of Ilv6 were screened for Ssa1 binding. To this end, a cellulose membrane-bound peptide array covering the entire Ilv6 sequence was synthesized using SPOT synthesis. Based on a protocol originally used to determine the substrate specificity of bacterial DnaK (Rudiger et al., 1997), the array was composed of 13mer peptides overlapping by 10 amino acid residues.

To identify peptides harboring Ssa1 binding sites, the peptide scan was then incubated with recombinant GST-Ssa1 under ATP-free conditions followed by the detection of membrane-bound Ssa1 using a GST-specific antibody. This approach indeed revealed Ssa1 binding to multiple Ilv6 peptides, several of them located within N-terminal segments of the protein (Figure 18).

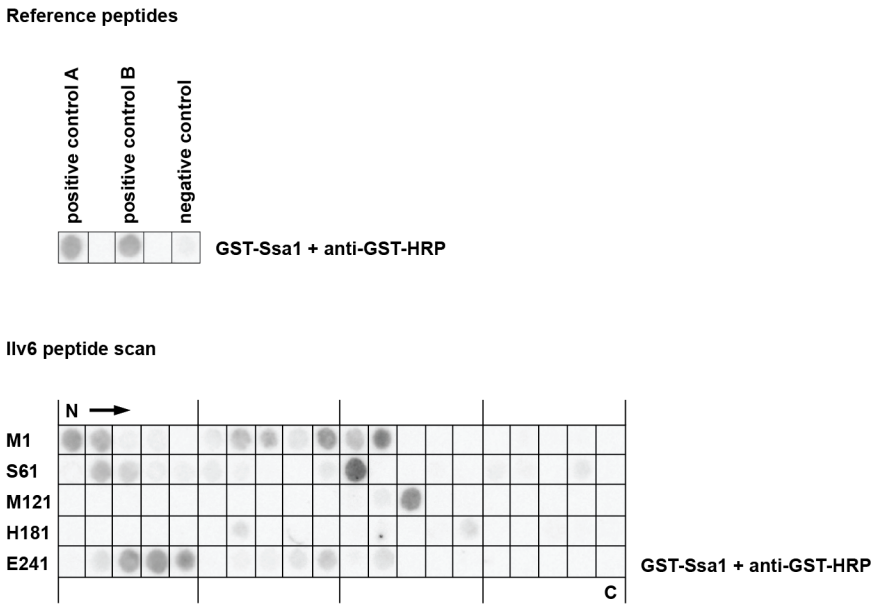


Figure 18. Ilv6 harbors Ssa1 binding sites within its N-terminus. A peptide scan covering the entire Ilv6 amino acid sequence was screened for Ssa1 binding. The peptide array was incubated with recombinant GST-Ssa1 and Ssa1 binding was detected with GST-specific antibodies coupled to HRP. N-terminal residues of the peptides on the first spots of each row (left) are indicated. Two peptides reported to bind the bacterial HSP70 DnaK served as reference peptides (positive control A: AKTLILSHLRFVV and positive control B: VVHIARNYA) (McCarty et al., 1996). A peptide representing the 13 N-terminal amino acids of firefly luciferase served as negative control (Rudiger et al., 1997).

Moreover, remarkably, Ssa1 binding sites were also located at the extreme N-terminus of Ilv6, suggesting that its MTS might directly interact with HSP70s *in vivo*.

Taken together, compelling evidence indicates an involvement of the *SSA* chaperone system in the regulation of mitochondria-targeted protein SUMOylation. Moreover, the data presented in this study support a model in which binding of Ssa proteins to mitochondrial precursors in the cytosol not only restricts their SUMOylation but also affects their modification in terms of site selectivity.

3.3 Degradation of SUMOylated mitochondria-targeted proteins by a proteasome-dependent pathway

3.3.1 SUMO-modified species of mitochondria-targeted proteins accumulate in proteasome mutants

Several results obtained during this study indicate that in cells with impaired *SSA* chaperone activity, SUMOylation particularly targets cytosolic precursors of mitochondrial proteins. Such precursor proteins are generally considered as aggregation-prone (Endo et al., 1995a) and prone to degradation by cellular proteases (Mihara and Omura, 1996a; Neupert, 1997; Pfanner and Neupert, 1990; Schatz and Dobberstein, 1996). Thus, since SUMO has been implicated in aggregate handling and the degradation of aggregation-prone proteins in the past (see section 1.3.3), it seemed conceivable that the SUMOylation of mitochondria-targeted proteins plays a role in protein quality control as well. In particular, the question arose whether SUMOylation targets aggregation-prone pools of mitochondrial precursor proteins and whether the levels of the corresponding SUMO conjugates are regulated by proteasomal degradation.

In a first attempt to evaluate this hypothesis, the SUMOylation of Ilv6 and Adh3 was analyzed in proteasome mutant cells expressing a hypomorphic variant of Rpt6 (*cim3-1*), one of six ATPase subunits within the 19S regulatory particle of the 26S proteasome (Ghislain et al., 1993). Interestingly, *cim3-1* cells showed mildly increased levels of the Ilv6 and Adh3 precursors (Figure 19A and B), suggesting that mitochondrial protein import might be less efficient in proteasome mutants. Alternatively, this could be indicative of a continuous turnover of small pools of mistargeted mitochondrial proteins *in vivo*, which would be blocked upon proteasome inhibition.

Strikingly, proteasome impairment also affected the SUMOylation of mitochondria-targeted proteins and SUMO-modified precursors of Ilv6 and Adh3 strongly accumulated in *cim3-1* cells (Figure 19A and B). SUMOylation of either substrate gave rise to multiple species, highly resembling the SUMO conjugate patterns observed in the *SSA* mutant backgrounds (Figure 14). This suggests that upon proteasome impairment both Ilv6 and Adh3 are perhaps modified at multiple sites. Likewise, the pattern of Mrpl23^{3HA}-SUMO conjugates was noticeably altered in *cim3-1* cells, indicating a strong accumulation of singly and multiply SUMOylated species (Figure 19C). However, since the N-terminal prepeptide of Mrpl23 comprises only four amino acid residues (Figure 10F), a discrimination between the precursor and the processed form could not be made for this particular substrate.

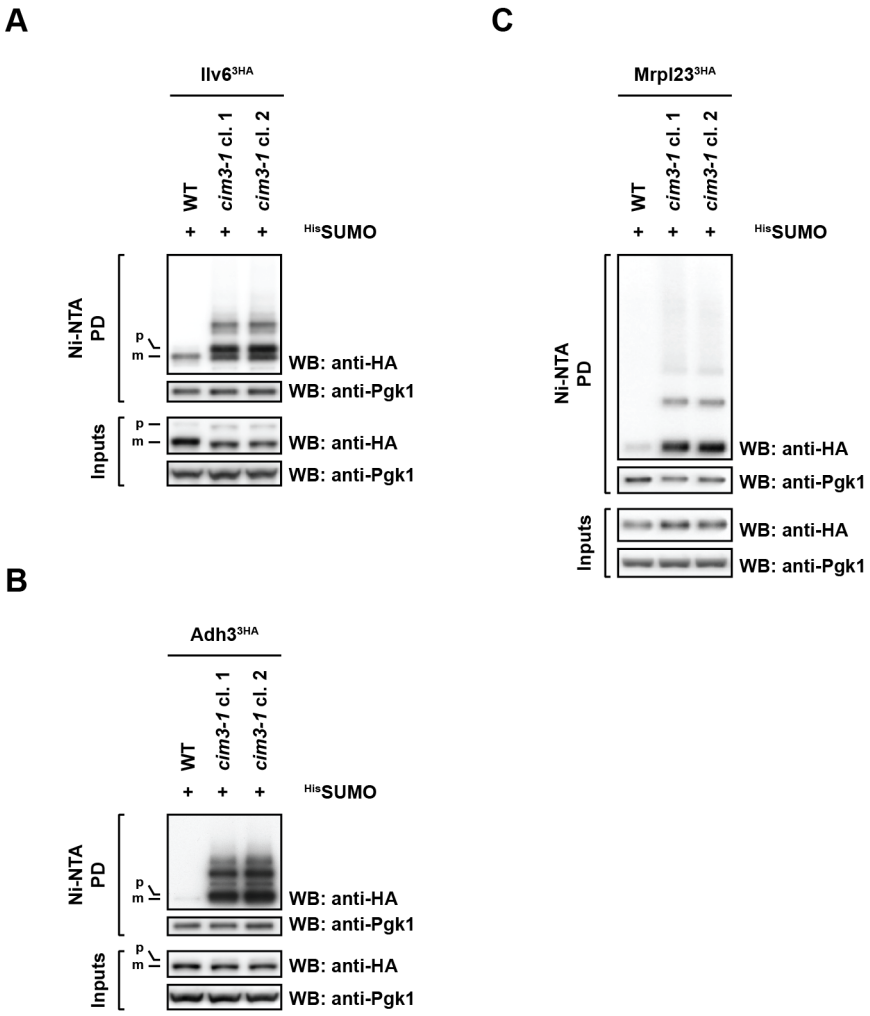


Figure 19. Proteasome impairment causes a strong accumulation of SUMOylated species of mitochondria-targeted proteins. (A) Multiple SUMOylated Ilv6 species, particularly SUMOylated precursor species, accumulate in proteasome mutants (*cim3-1*). Denaturing ^{His}SUMO Ni-NTA pull-downs (Ni-NTA PD) from wild type and *cim3-1* cells expressing C-terminally 3HA-tagged Ilv6 from the endogenous promoter. Expression of ^{His}SUMO from the *ADH1* promoter is indicated. Bands corresponding to the (monoSUMOylated) precursor protein (p) and the (monoSUMOylated) mature (m) form are labeled. **(B-C)** Increased SUMOylation of Adh3 (B) and Mrpl23 (C) in *cim3-1* cells. Similar to (A) but with cells expressing C-terminally 3HA-tagged Adh3 from the endogenous promoter (B) or cells harboring plasmids that express C-terminally 3HA-tagged Mrpl23 from the *ADH1* promoter (C).

In any case, decreased proteasome activity appears to generally result in increased levels of SUMOylated mitochondria-targeted substrates and also leads to the detection of species simultaneously modified at multiple lysine residues.

Several findings described in this study demonstrate that the SUMOylation of mitochondria-targeted substrates is independent of mitochondrial import. Thus, the question arose whether proteasome impairment causes an increased SUMOylation also of import-defective variants of mitochondrial proteins. To this end, an Adh3 mutant variant lacking its MTS (Δmts-adh3^{3HA}) was expressed from the *GAL1* promoter in *cim3-1* cells. Importantly, protein levels of this Adh3 variant were largely

similar in total cell extracts of wild type and proteasome mutant cells (Figure 20A). By contrast, the corresponding SUMO conjugates strongly accumulated upon proteasome impairment (Figure 20A). Similar to the full-length substrate (Figure 19B), SUMOylation produced numerous slower-migrating species, suggesting that the modification occurs simultaneously at multiple sites.

To further investigate site selectivity in this context, the SUMOylation of wild type Δmts-adh3^{3HA} was compared to a lysine mutant, in which Lys 305 (K305) was replaced by arginine. Notably, all substrate variants were again expressed at largely identical levels in wild type and *cim3-1* cells (Figure 20B). Western blot analysis of ^{His}SUMO conjugates isolated from wild type cells confirmed that SUMOylation of Δmts-adh3^{3HA} yields at least two slower-migrating species under unperturbed conditions (Figure 20B, lane 2). One of these species resulted from the modification of Lys 305, which was again accompanied by the SUMOylation of additional lysine residues (Figure 20B, compare lane 2 and lane 3). In proteasome mutant cells (*cim3-1*), SUMOylation at Lys 305 gave rise to two distinct slower-migrating species and contributed to the formation of further high molecular weight species (Figure 20B, compare lane 4 and 5). Moreover, SUMOylation at probably multiple alternative attachment sites was clearly detectable in *cim3-1* cells, even when Lys 305 had been experimentally removed (Figure 20B, lane 5).

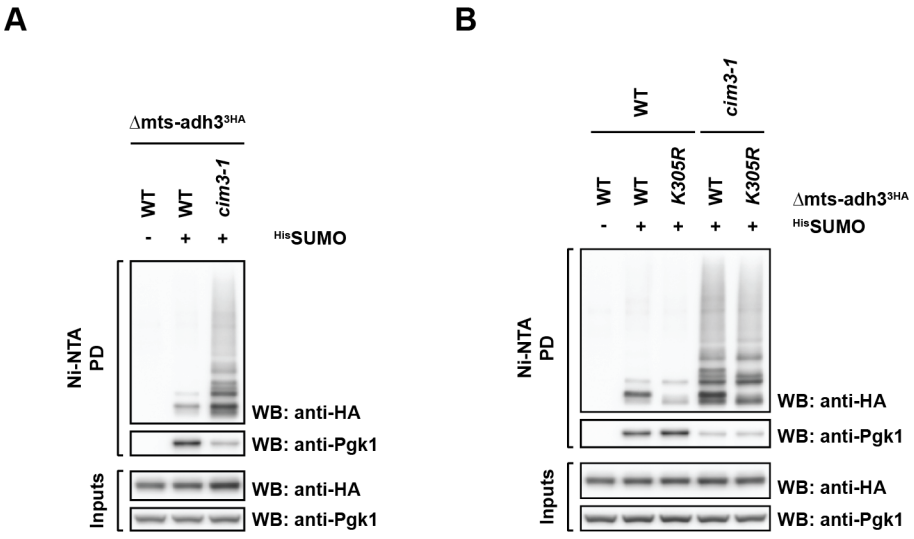


Figure 20. Multiply SUMOylated species of import-incompetent Adh3 accumulate in proteasome mutants. (A-B) The SUMOylation of import-incompetent Adh3 (Δmts-adh3^{3HA}) is strongly affected in proteasome mutant cells, which display a strong accumulation of various SUMOylated species probably modified at multiple sites. Denaturing Ni-NTA pull-downs (Ni-NTA PD) from wild type and *cim3-1* cells harboring plasmids that express Δmts-adh3^{3HA} or a lysine mutant (*K305R*; corresponding to K305 of full-length Adh3) from the *GAL1* promoter. Cells additionally expressing ^{His}SUMO from the *ADH1* promoter are indicated.

Thus, in summary, proteasome impairment affects the SUMOylation of mitochondria-targeted proteins and their import-defective derivatives in two ways: (1) it causes a strong accumulation of SUMO-modified species and (2) results in the robust SUMOylation of sites, which are not noticeably modified in wild type cells.

3.3.2 SUMOylation targets aggregation-prone species of mitochondria-targeted proteins

Mitochondrial preproteins are thought to adopt a rather loosely folded state in the cytosol to maintain import competence (Neupert, 1997). However, protein unfolding often leads to the exposure of hydrophobic peptide stretches, which are usually buried in the native structure of a protein. These structural changes not only provide a basis for the recognition of unfolded substrates by molecular chaperones but also favor non-native protein-protein interactions that cause protein aggregation.

To gain insights into the features of processed and unprocessed mitochondrial proteins in terms of solubility, cellular fractionation assays were performed. To this end, endogenous Ilv6 fused to a C-terminal 3HA-epitope was chosen as a model substrate since low but noticeable levels of the corresponding precursor protein were reproducibly detected in total cell extracts. In brief (see Materials and Methods for details), exponentially growing yeast cells were lysed in buffer containing 1 % of the non-ionic detergent Triton X-100. The lysates were precleared by centrifugation and the resulting total cell extracts (T) were fractionated to separate soluble (S) and insoluble pellet (P) fractions. Interestingly, when compared to the mature mitochondrial form, the Ilv6 precursor showed a substantially increased aggregation propensity (Figure 21) and the levels of insoluble precursor species were further increased when HSP70 (*SSA1* and *ssa1-45* cells) (Figure 21) or proteasome activity (*cim3-1* cells) (Figure 22) was impaired.

Figure 21. Increased aggregation propensity of a mitochondrial precursor protein. Ilv6 precursors are pronouncedly more aggregation-prone than the mature mitochondrial form. Total cell extracts (T) from wild type (DF5 background), *SSA1* and *ssa1-45* cells were fractionated by centrifugation to obtain soluble (S) and insoluble pellet (P) fractions. All strains used in the fractionation assay express C-terminally 3HA-tagged Ilv6 from the endogenous and ^{His}SUMO from the *ADH1* promoter. Proteins were separated on 12 % Bis-Tris gels and analyzed by western blotting using HA epitope-specific, Smt3-specific and Dpm1-specific antibodies. Bands corresponding to the precursor protein (p) and the mature (m) form are labeled.

Remarkably, after a longer exposure, western blot analysis also indicated the presence of a single distinct, slower-migrating form of Ilv6 in the pellet fractions of *cim3-1* cells (Figure 22A, black triangle). As judged from its electrophoretic mobility, the molecular weight of this species exactly matched the modification of HA-tagged Ilv6 with a single ^{His}SUMO moiety. Indeed, expression of a ^{GFP}SUMO fusion protein as the only source of SUMO increased its apparent molecular weight by about 30 kDa (Figure 22B), suggesting that substantial levels of SUMOylated Ilv6 accumulate in the insoluble protein pool in *cim3-1* cells. Thus, SUMO might act on aggregation-prone species of mitochondria-targeted proteins, which accumulate in insoluble cellular protein fractions when proteasomal clearance mechanisms are defective.

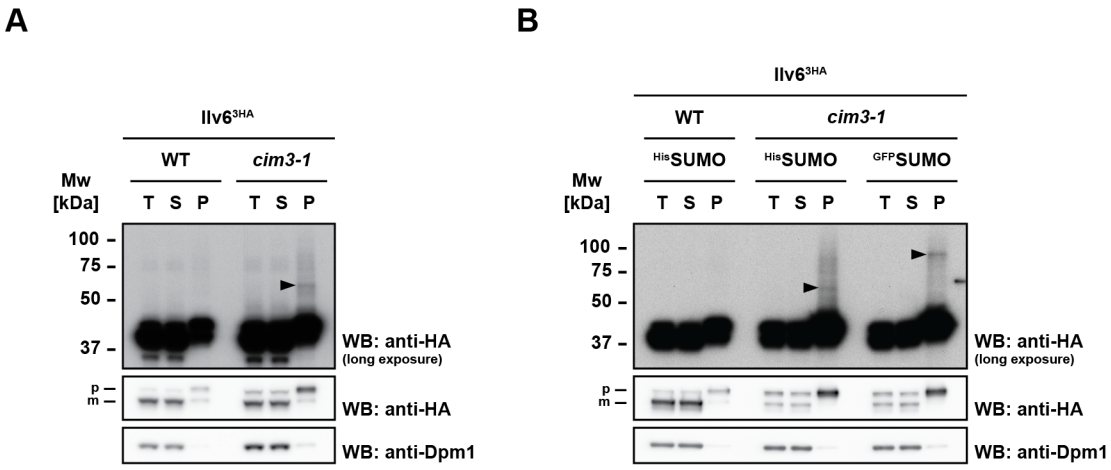


Figure 22. Accumulation of SUMOylated Ilv6 in insoluble protein fractions of proteasome mutant cells. (A-B) Ilv6-SUMO conjugates (indicated by black triangles) are detectable in the insoluble pellet fractions of proteasome mutant cells (*cim3-1*). Total cell extracts (T) from wild type and *cim3-1* cells were fractioned into soluble (S) and insoluble pellet (P) fractions. The strains used in (A) express ^{His}SUMO from the *ADH1* promoter and are derived from the original CMY826 (WT) and CMY763 (*cim3-1)* backgrounds (Ghislain et al., 1993). Strains used in (B) were obtained by backcrossing the *cim3-1* allele into the DF5 background and either express ^{His}SUMO or ^{GFP}SUMO from the *ADH1* promoter. All strains carry a genomic allele encoding C-terminally 3HA-tagged Ilv6 under control of the endogenous promoter. Proteins were separated on 12 % Bis-Tris gels and analyzed by western blotting using HA epitope-specific and Dpm1-specific antibodies. Bands corresponding to the precursor protein (p) and the mature (m) form are labeled.

3.3.3 Proteasome impairment affects the turnover of Ilv6-SUMO conjugates

Recently, SUMO has been proposed to function as a modulator of protein aggregation, which facilitates the proteasomal degradation of insoluble proteins (Guo et al., 2014). To assess a potential turnover of Ilv6-SUMO conjugates *in vivo*, expression shut-off assays were combined with denaturing Ni-NTA pull-downs to directly monitor the levels of SUMOylated Ilv6 over time. In brief, exponentially

growing yeast cells expressing ^{His}SUMO and epitope-tagged Ilv6 were treated with the translational inhibitor cycloheximide and ^{His}SUMO conjugates were isolated from cells sampled after different times. In wild type cells, SUMOylated Ilv6 was indeed instable, showing a time-dependent turnover after the block of protein synthesis by cycloheximide (Figure 23A). By contrast, strikingly, Ilv6^{3HA}-SUMO conjugates were almost completely stabilized in cells defective in proteasomal degradation (*cim3-1*) (Figure 23B). Thus, proteasome activity is a crucial determinant for the SUMOylation dynamics of mitochondria-targeted proteins, suggesting the possibility that the corresponding SUMO-protein conjugates are directly targeted for proteasomal degradation.

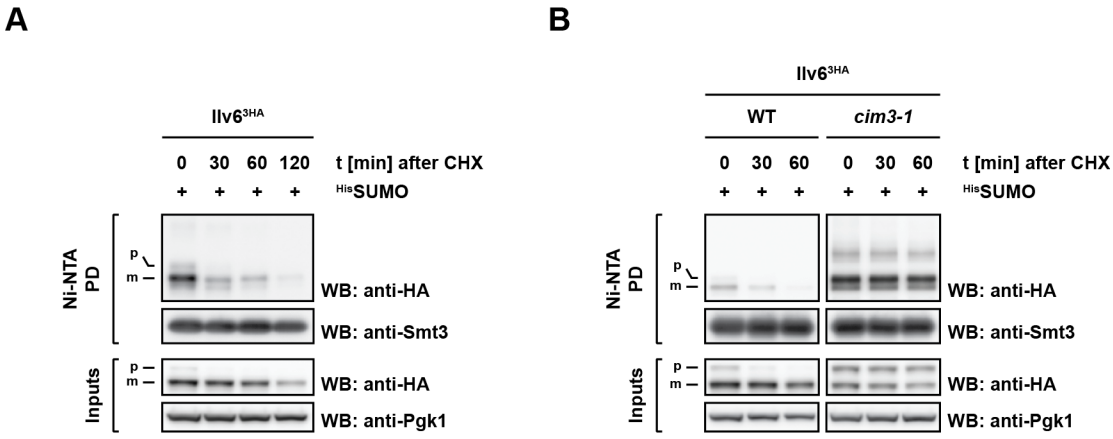


Figure 23. Dynamic, proteasome-dependent turnover of Ilv6-SUMO conjugates upon translation shut-off. (A) Time-dependent decrease of Ilv6-SUMO conjugate levels in wild type (WT) cells analyzed by expression shut-off assays. Cells were grown at 25°C and shifted to 37°C for 60 min prior to the addition of cycloheximide (CHX). Subsequently, samples for the isolation of ^{His}SUMO conjugates by denaturing Ni-NTA pull-downs (Ni-NTA PD) and the preparation of total cells extracts (Inputs) were taken at the indicated time points. Pull-down efficiency was controlled by monitoring the levels of unconjugated ^{His}SUMO using Smt3-specific antibodies. Bands corresponding to the (monoSUMOylated) precursor protein (p) and the (monoSUMOylated) mature (m) form are labeled. **(B)** Stabilization of Ilv6-SUMO conjugate levels in proteasome mutants. Similar as in (A) but including proteasome mutant cells (*cim3-1*). All strains used in (A) and (B) express C-terminally 3HA-tagged Ilv6 from the endogenous and ^{His}SUMO from the *ADH1* promoter.

3.3.4 Isopeptidase-resistant Ilv6-SUMO conjugates are degraded by a proteasome-dependent pathway

Protein modification by SUMO is dynamic and in many cases regulated by SUMO-specific isopeptidases. Additional layers of control are provided by proteasomal degradation pathways, which sometimes involve the action of SUMO-targeted ubiquitin ligases (STUbLs) (Tatham et al., 2008; Uzunova et al., 2007). Hence, the levels of SUMO-protein conjugates can be dynamically regulated by the balance of SUMO conjugation, deconjugation and proteasomal degradation.

In the further course of this study, the question arose how SUMO deconjugation and proteasomal degradation each contribute to the SUMOylation dynamics of mitochondria-targeted proteins. Analysis of SUMO conjugates in yeast cells defective in SUMO deconjugation is, however, complicated by the fact that deletion of SUMO-specific isopeptidases is lethal (*Δulp1*) (Li and Hochstrasser, 1999) or confers strong pleiotropic phenotypes (*Δulp2*) (Li and Hochstrasser, 2000). Thus, it seemed reasonable to interfere with SUMO deconjugation by an alternative experimental strategy.

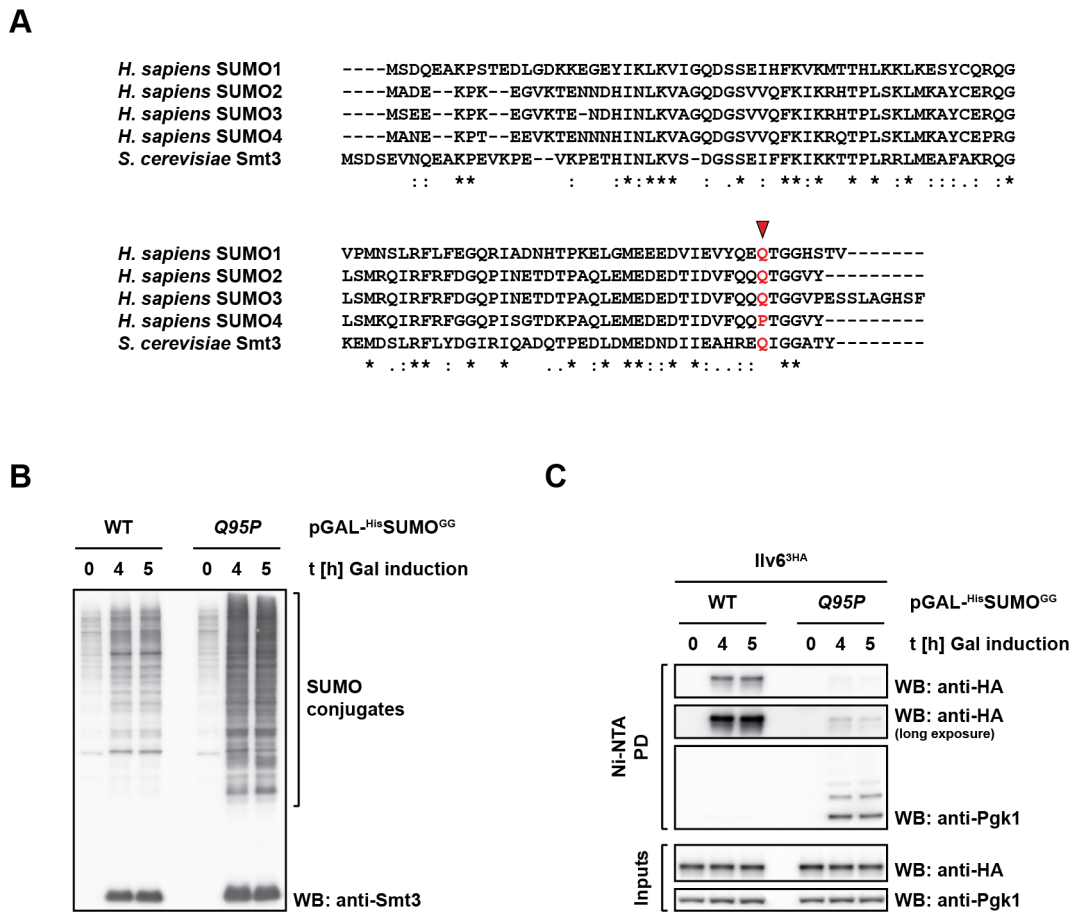
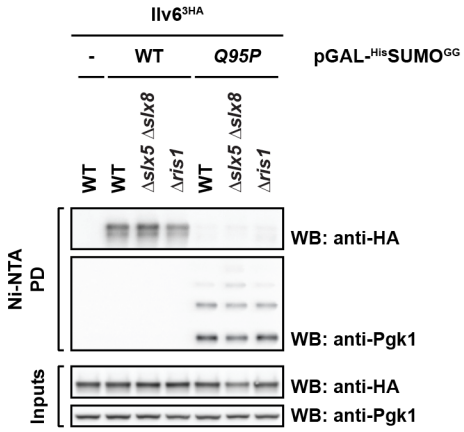


Figure 24. Generation and expression of an isopeptidase-resistant yeast SUMO variant. (A) Multiple sequence alignment of human SUMO1-4 and yeast Smt3. The position in which human SUMO4 displays a Q-to-P replacement compared to other SUMO isoforms is indicated (red letters marked by a red triangle). Multiple sequence alignment assembly was carried out using the EBI Clustal Omega web tool. **(B)** Expression of a mature *SUMO-Q95P* variant leads to an accumulation of multiple SUMO conjugates in yeast. Plasmid constructs expressing His-tagged yeast Smt3 with C-terminal double glycine motif ($^{His}SUMO^{GG}$) from the *GAL1* promoter are genomically integrated at the *URA3* locus. The amino acids corresponding to Gln 95 of wild type Smt3 are either Gln (WT) or Pro (*Q95P*). Cells were grown in raffinose-containing medium and expression of SUMO variants was induced by addition of galactose for the indicated periods of time. Total cell extracts were prepared and analyzed by western blotting using Smt3-specific antibodies. **(C)** Isopeptidase-resistant Ilv6-SUMO conjugates are highly unstable. Similar to (B) but including the western blot analysis of $^{His}SUMO$ conjugates isolated by denaturing Ni-NTA pull-downs (Ni-NTA PD). C-terminally 3HA-tagged Ilv6 expressed from the endogenous promoter was detected with an HA epitope-specific antibody.

Interestingly, it has been reported that human SUMO4 is refractory to the processing by SUMO isopeptidases due to the presence of a critical proline residue at position 90 (Owerbach et al., 2005) (Figure 24A). Likewise, introduction of homologous amino acid changes allows the transfer of isopeptidase resistance to other SUMO isoforms (Mukherjee et al., 2009; Owerbach et al., 2005). Expression of such SUMO variants with a mature C-terminus bypasses the requirement for SUMO proteases for the initial SUMO maturation and leads to the formation of "isopeptidase-resistant" SUMO-protein conjugates in (Mukherjee et al., 2009).

A



B

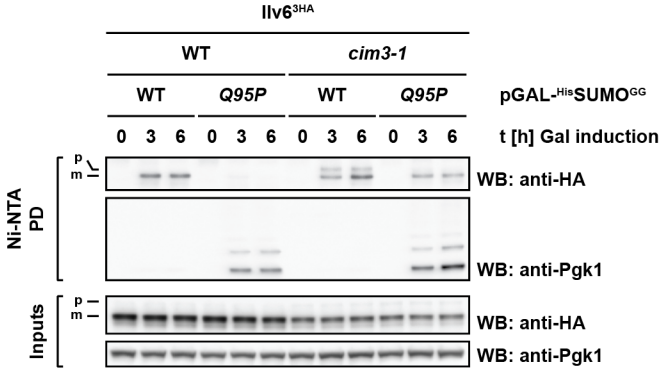


Figure 25. SUMOylation mediates the degradation of mitochondria-targeted substrates by a proteasome-dependent pathway. (A) Ilv6-*SUMO-Q95P* conjugates are highly unstable in cells lacking known STUbLs. Wild type (WT) or isopeptidase-resistant (*Q95P*) $^{His}SUMO^{GG}$ variants (as described in Fig. 24) were expressed from the *GAL1* promoter for the indicated periods of time in wild type (WT) cells and cells lacking Slx5/Slx8 (*Δslx5 Δslx8*) or Ris1 (*Δris1*). Cells were grown in raffinose-containing medium and protein expression was induced by the addition of galactose. Total cell extracts were prepared by TCA precipitation (Inputs) and $^{His}SUMO$ conjugates were isolated by denaturing Ni-NTA pull-downs (Ni-NTA PD). **(B)** Pronounced stabilization of isopeptidase-resistant Ilv6-SUMO conjugates in proteasome mutant cells. Similar to (A) but including wild type (WT) and proteasome mutant cells (*cim3-1*). Expression of $^{His}SUMO^{GG}$ variants from the *GAL1* promoter was induced for the indicated periods of time. Bands corresponding to the (monoSUMOylated) precursor protein (p) and the (monoSUMOylated) mature (m) form are labeled.

Indeed, expression of an analogous mutant variant of mature yeast SUMO (*^{His}SUMO^{GG}-Q95P*) from the galactose-inducible *GAL1* promoter led to a remarkable increase in SUMO conjugate levels, including Pgk1-SUMO (Figure 24B and C). By contrast, strikingly, SUMOylated Ilv6 species did not accumulate under these conditions but were hardly detectable (Figure 24C), indicating that the modification of mitochondria-targeted proteins by isopeptidase-resistant SUMO could trigger their degradation. Since the proteasomal degradation of SUMO conjugates often involves SUMO-targeted ubiquitin ligases (STUbLs), it seemed plausible that this could be the case for mitochondria-targeted proteins as well. However, levels of SUMO-modified Ilv6 were not altered in yeast cells lacking known STUbLs (*Δslx5 Δslx8* and *Δris1*) (Figure 25A), suggesting that the degradation of mitochondria-targeted SUMO substrates is based on an alternative mechanism. Indeed, Ilv6-*SUMO-Q95P* conjugates were substantially stabilized in proteasome mutant cells (*cim3-1*) (Figure 25B), indicating that SUMOylated Ilv6 is degraded by a STUbL-independent proteasomal pathway.

In summary, the SUMOylation of Ilv6 and perhaps mitochondria-targeted proteins in general appears to be a dynamic PTM that ultimately leads to the degradation of the modified substrate pool by the proteasome. The underlying degradation mechanism does not require known STUbLs, suggesting the existence of a novel proteasomal pathway that mediates the clearance of specific SUMO conjugates in a STUbL-independent manner.

4 DISCUSSION

This study identifies mitochondrial-targeted proteins as a novel group of SUMO substrates and provides a first in-depth analysis of the modification and its functional consequences. The SUMOylation of mitochondria-targeted proteins is enhanced in response to certain proteotoxic stresses and appears to ultimately serve as a trigger for proteasomal degradation. Hence, the present study not only provides unique insights into the SUMO-modified proteome but also reveals novel links of the SUMO system to cellular protein quality control.

4.1 An unexpected group of novel SUMO substrates

Posttranslational modification by SUMO has been extensively studied in the past. Generally, the detection of SUMOylated proteins is complicated by the fact that the modification frequently affects only a small percentage of a given target. However, mass spectrometry-based technologies have strongly boosted the field of SUMO proteomics and facilitated the identification a plethora of SUMO substrates in yeast and human cells (Hendriks and Vertegaal, 2016; Makhnevych et al., 2009). Consistent with early studies on the predominantly nuclear activities of the SUMO system (Rodriguez et al., 2001; Sternsdorf et al., 1999), the majority of SUMO targets appears to be nuclear proteins (Hendriks and Vertegaal, 2016; Wohlschlegel et al., 2004). Additionally, multiple lines of evidence indicate a function of SUMO enzymes in the cytosol and several cytosolic SUMO substrates have been reported (Geiss-Friedlander and Melchior, 2007; Martin et al., 2007b). By contrast, only a minute number of potential mitochondrial SUMO substrates have been suggested by previous studies (Denison et al., 2005; Hannich et al., 2005; Panse et al., 2004; Wohlschlegel et al., 2004; Wykoff and O'Shea, 2005; Zhou et al., 2004). In fact, at the onset of this study, the modification of mitochondrial proteins, particularly proteins of inner subcompartments, seemed unexpected for two major reasons: First, current knowledge about the SUMO system strongly indicates that intramitochondrial proteins are inaccessible to SUMO enzymes upon sorting (Flotho and Melchior, 2013). Second, import of mitochondrial proteins occurs promptly and efficiently after their synthesis (Ungermann et al., 1996). Hence, hardly any pools of non-imported mitochondrial preproteins are detected in the cytosol *in vivo* (Ades and Butow, 1980b; Fujiki and Verner, 1993; Hallermayer et al., 1977; Reid and Schatz, 1982).

Despite these apparent restrictions, this study provides striking evidence that several mitochondria-targeted proteins are modified by SUMO *in vivo* (Figure 7). Interestingly, these proteins differ substantially in terms of structure and function,

suggesting that SUMOylation does not specifically target a single, functionally distinct group of mitochondrial proteins. Notably, mitochondria-targeted substrates are modified at both ΨKX(D/E)-type consensus and non-consensus SUMOylation sites. The SUMOylation of non-consensus attachment sites has been reported previously for several yeast proteins (Hoege et al., 2002; Psakhye and Jentsch, 2012; Sacher et al., 2006), indicating that it might be a widespread phenomenon. At any rate, the discovery of mitochondria-targeted protein SUMOylation reveals unique new aspects of the SUMO system and its substrates.

4.2 SUMOylation of mitochondria-targeted proteins occurs prior to import

Consistent with SUMO's known activities in the cytosol, it can be envisioned that a small pool of mitochondrial preproteins is accessible to SUMO enzymes prior to import. As a consequence, SUMOylation would be restricted to proteins that are posttranslationally sorted into mitochondria. Indeed, although evidence for cotranslational import has been provided for some proteins (Ades and Butow, 1980a; Fox, 2012; Fujiki and Verner, 1993), protein transport into mitochondria is thought to occur in a predominantly posttranslational manner (Chen and Douglas, 1987; Eilers and Schatz, 1986; Rassow et al., 1989; Wienhues et al., 1991). This concept is largely confirmed by a recent study, which globally assessed mitochondrial protein import by proximity-specific ribosome profiling (Williams et al., 2014). Although cotranslational translocation might be of particular relevance for inner membrane proteins, it has been reported that the majority of mitochondrial proteins, including Ilv6, Adh3 and Mrpl23, follows a predominantly posttranslational import route. Thus, it is generally conceivable that small pools of mitochondrial precursor proteins are targets of SUMO modifications in the cytosol.

Strong support for a model in which the SUMOylation of mitochondria-targeted proteins occurs prior to import is also provided by data presented in this study, which demonstrate that their modification is independent of N-terminal MTSs. MTSs are known to form amphipathic alpha helices capable of interacting with the Tom20 import receptor at the outer mitochondrial membrane (Abe et al., 2000). Hence, MTS removal allows for the generation of import-incompetent mutant variants of mitochondrial proteins (Dasari and Kolling, 2011). Remarkably, compared to the full-length proteins, such variants (derived from Ilv6 and Adh3) are SUMOylated with strikingly similar characteristics in terms of E3 ligase requirement and SUMO attachment sites (Figure 12 and Figure 13). Thus, it is conceivable that, regarding their SUMOylation, MTS-lacking mutant proteins mimic an early biogenesis stage of

mitochondrial proteins and undergo a similar recognition by SUMO enzymes as their full-length counterparts. Moreover, for all substrates and substrate variants, SUMOylation is strongly dependent on the SUMO E3 ligase Siz1 (Figure 9, Figure 12 and Figure 13). Since nuclear export of Siz1 into the cytosol is well-characterized in the context of septin SUMOylation (Makhnevych et al., 2007; Takahashi et al., 2008), it appears plausible that a cytosolic pool of Siz1 is responsible for the SUMOylation of mitochondria-targeted substrates as well. However, the modification of mitochondria-targeted substrates additionally involves the E3 ligase Siz2, suggesting a cytosolic function also for this particular enzyme. Consistently, small cytosolic pools of Siz2 have been reported by a study, which globally analyzed the localization of GFP fusion proteins in budding yeast (Huh et al., 2003).

Taken together, several lines of evidence indicate that SUMOylation of mitochondria-targeted proteins occurs prior to import, where the E3 ligases Siz1 and Siz2 modify small pool of substrates “en route” to mitochondria. Accordingly, the present study not only identifies an unanticipated group of SUMO substrates but also provides novel insights into the functions of SUMO E3 ligases in the cytosol.

4.3 SUMOylation as a potential mechanism to regulate mitochondrial protein import

Protein import into mitochondria involves the translocation of polypeptides through narrow proteinaceous channels. Virtually all types of preproteins enter mitochondria via a general entry gate, the TOM complex. The hydrophilic pores of this complex are formed by Tom40 subunits and have been determined to span a diameter of 2.0-2.5 nm (Kunkele et al., 1998; Model et al., 2002; Schwartz and Matouschek, 1999). This is in line with the finding that mitochondrial proteins are imported in an unfolded and extended conformation (Rassow et al., 1990). Notably, proteins destined for the mitochondrial matrix have to additionally pass through the Tim23 channel in the inner mitochondrial membrane. With a diameter of 1.3-2.4 nm (Schwartz and Matouschek, 1999; Truscott et al., 2001), this channel represents a further barrier restricting the mitochondrial import of folded proteins. Indeed, even small proteins such as model substrates containing dihydrofolate reductase fused to a mitochondrial targeting sequence are unable to complete import when their three-dimensional structure is stabilized (Eilers and Schatz, 1986; Rassow et al., 1989; Wienhues et al., 1991).

Given the fact that the SUMOylation of mitochondria-targeted proteins occurs most likely at a cytosolic biogenesis stage, the modification might have striking consequences for protein import. Ubiquitin family proteins are spherical molecules with diameters of approximately 2.5 nm (Renatus et al., 2006). Thus, it can be

envisioned that a modifier such as SUMO, which is tightly folded and covalently conjugated via a branched peptide, could stall the translocation of a modified substrate protein for steric reasons. Remarkably, a similar scenario has been reported for a model substrate obtained by crosslinking a folded 6 kDa protein moiety to the C-terminus of a mitochondrial precursor protein (Vestweber and Schatz, 1988). Moreover, importantly, this hypothesis is not contradictory to the observation that SUMO-modified species of substrates such as Ilv6 and Adh3 display mature N-termini, which result from proteolytic processing by MPP in the mitochondrial matrix. Because these species are exclusively modified at C-terminal lysine residues (Figure 10), it is possible that their N-termini reach the matrix-resident MPP while the SUMOylated C-termini of the proteins remain exposed to the cytosol (Figure 26). Notably, such partly imported translocation intermediates spanning both mitochondrial membranes have been described previously by several independent studies (Chen and Douglas, 1987; Cyr et al., 1995; Eilers and Schatz, 1986; Endo et al., 1995b; Kubrich et al., 1995; Rassow et al., 1989; Schleyer and Neupert, 1985; Schwaiger et al., 1987; Vestweber and Schatz, 1988). Moreover, it has been reported that a polypeptide segment comprising roughly 50 amino acid residues is sufficient to span both mitochondrial membranes (Rassow et al., 1990).

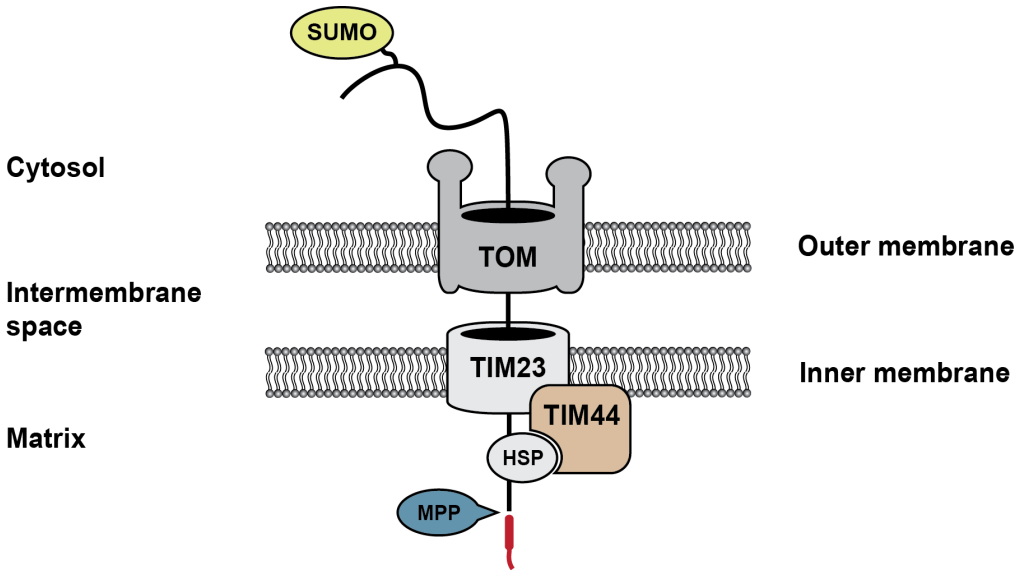


Figure 26. Potential scenario explaining the N-terminal processing of C-terminally SUMOylated mitochondrial proteins. Due to the size of a folded SUMO moiety, it is conceivable that SUMOylation interferes with the complete import of mitochondria-targeted proteins. Nevertheless, SUMOylated species of mitochondria-targeted proteins display processed N-termini. This finding might be explained by the formation of translocation intermediates, for which the N-terminus reaches the mitochondrial processing peptidase (MPP) in the matrix while the SUMOylated C-terminus remains in the cytosol. TOM complex, translocase of the outer membrane; TIM23 complex, presequence translocase of the inner membrane; TIM44, membrane anchor for mitochondrial HSP70 (HSP); TIM44 and mtHSP70 are components of the PAM complex, the presequence translocase-associated motor.

Thus, a distance of more than 100 amino acid residues between the presequence cleavage site and the SUMO-modified lysine residues would allow the initiation of protein translocation into the matrix. Such a positioning of SUMO acceptor sites is indeed the case for all substrates described in this study (Figure 10) and SUMOylated lysine residues in closer proximity to the N-terminus were exclusively detected on precursor proteins (Figure 17).

Assuming that C-terminal SUMOylation arrests the translocation of a modified protein, two fates are conceivable for the stalled translocation intermediate: (1) the completion of import after the removal of the SUMO moiety by an isopeptidase or (2) the retrograde translocation into the cytosol after cleavage of the N-terminal presequence by MPP. Remarkably, such a retrotranslocation mechanism indeed exists and is involved in the biogenesis of certain enzymes such as fumarase in yeast. Fumarase is synthesized as a single translation product harboring an N-terminal 24-amino-acid presequence. However, it is dually distributed between the mitochondrial matrix and the cytosol *in vivo* (Stein et al., 1994). Importantly, the cytosolic and mitochondrial enzyme populations display identical N-termini, which result from processing by MPP (Sass et al., 2001). Thus, initially all polypeptides are targeted to mitochondria, leading to the proteolytic removal of the N-terminal MTS. However, not all fumarase molecules are completely imported into the matrix. Following translation termination, rapid folding of fumarase's C-terminus impedes import and induces the retrograde translocation of a fraction of polypeptides into the cytosol (Karniely and Pines, 2005; Knox et al., 1998; Stein et al., 1994).

Taken together, it appears conceivable that SUMOylation of mitochondria-targeted proteins arrests their translocation at the import pore and that such intermediates have to be cleared by SUMO removal followed by the completion of import or by retrograde translocation into the cytosol. However, further experimental evidence is required to clarify whether one of these scenarios indeed arises *in vivo*.

4.4 A partially hypothetical model for the regulation of mitochondria-targeted protein SUMOylation by HSP70 chaperones

Cytosolic factors, particularly chaperones of the HSP70 system, have been widely implicated in mitochondrial protein import (Hoogenraad et al., 2002; Mihara and Omura, 1996b; Mori and Terada, 1998). In yeast, HSP70s of the *SSA* family together with HSP40 co-chaperones are of particular importance for this process (see section 1.2.4). In addition to their role in protein translocation, this study describes a novel function of *SSA* family chaperones in regulating the SUMOylation of mitochondria-targeted proteins. In *SSA* mutant cells, SUMOylation of mitochondria-targeted

proteins is affected in two ways: (1) The levels of SUMOylated species, particularly of precursor proteins, are strongly increased and (2) the modification occurs at multiple attachment sites, including lysine residues, which are not detectably SUMOylated in wild type cells. Importantly, these alterations could be observed for different substrates, suggesting a general effect.

In combination, the analysis of SUMO attachment sites and Ssa1 binding sites within the amino acid sequence of one substrate (Ilv6) allows to propose a partially hypothetical model of how SUMOylation of mitochondria-targeted proteins is regulated by molecular chaperones. In particular, the detection of potential chaperone binding sites within the N-terminal presequence of Ilv6 suggests that MTS peptides may directly interact with HSP70s *in vivo*. The resulting formation of precursor-chaperone complexes might not only maintain the import competence of mitochondria-targeted proteins but perhaps also reduces their accessibility to SUMO enzymes. Moreover, a direct association of HSP70 proteins with the N-terminus of preproteins could additionally account for the preferential SUMOylation of C-terminal lysine residues under unperturbed conditions.

Notably, it has been shown recently that the ssa1-45 mutant protein, which has been used in the course of this study, is deficient in substrate binding (Needham et al., 2015). It is therefore conceivable that in *ssa1-45* cells, SUMO enzymes target a pool of “free” mitochondrial precursor proteins, which are not incorporated into precursor-chaperone complexes. For these substrates, SUMOylation is not restricted by chaperone binding to N-terminal protein segments, thus potentially allowing the modification of a more extended set of lysine residues.

HSP70 chaperones are generally thought to preferentially interact with short hydrophobic peptide segments, which are usually buried in the native structure of a protein (Flynn et al., 1991; Rudiger et al., 1997; Zhu et al., 1996). Hence, at a first glance, HSP70 binding to N-terminal, positively charged signal peptides might appear unexpected. However, remarkably, it has been reported that the yeast HSP70 Ssa1 indeed binds mitochondrial presequences and that the interaction depends on the amphiphilicity of the presequence (Endo et al., 1996). Moreover, enrichment of basic amino acid residues has been shown for peptides interacting with multiple HSP70 proteins (Fourie et al., 1994; Rudiger et al., 1997). Accordingly, binding to HSP70 chaperones might be a general feature of mitochondrial presequences, which thereby not only mediate protein targeting but perhaps also contribute to the formation of precursor-chaperone complexes.

In summary, this study reveals novels aspects of HSP70 chaperones as central regulators of the early biogenesis of mitochondrial proteins. Besides their role

in the maintenance of import competence, data presented here also indicate a function of HSP70s in the regulation of mitochondria-targeted protein SUMOylation.

4.5 Proteasomal degradation of SUMO conjugates by a STUbL-independent mechanism

It has become evident that SUMO and ubiquitin not always act independently of each other but that a complex interplay between the two conjugation systems exists (Liebelt and Vertegaal, 2016; Schimmel et al., 2008; Tatham et al., 2011). Crosstalk between the two pathways has been particularly revealed by the identification of SUMO-targeted ubiquitin ligases (STUbLs), which specifically mediate the ubiquitylation and proteasomal degradation of SUMO conjugates (Tatham et al., 2008; Uzunova et al., 2007). Moreover, further levels of interdependent control may exist, for instance the regulation of ubiquitin conjugating enzymes (E2) (Pichler et al., 2005) and ubiquitin E3 ligases (Novoselova et al., 2013) by SUMOylation.

In line with the discovery of proteasome-dependent pathways mediating the proteolytic turnover of SUMO conjugates, this study identifies mitochondrial proteins as a novel group of SUMO substrates regulated by proteasomal degradation. Notably, proteasomal protein degradation might be generally involved in the clearance of mislocalized or retrotranslocated mitochondrial proteins in the cytosol (Bragoszewski et al., 2013; Habelhah et al., 2004; Pearce and Sherman, 1997; Wrobel et al., 2015). However, SUMO appears to confer additional specificity for the recognition of a small pool of mitochondria-targeted proteins by the proteasome system. Experimental evidence supporting this notion is given by the finding that upon proteasome impairment, particularly SUMOylated mitochondrial precursor proteins strongly accumulate (Figure 19). Even more strikingly affected is the SUMOylation of import-incompetent Adh3. Overall protein levels of this substrate are largely similar in total cell lysates of wild type and proteasome mutant cells (Figure 20). However, the corresponding SUMO conjugates strongly and specifically accumulate upon proteasome impairment (Figure 20).

Further evidence for the proteasomal degradation of SUMOylated pools of mitochondria-targeted proteins is demonstrated by the finding that SUMO-modified species become highly unstable when SUMO deconjugation is blocked (Figure 24). This is in stark contrast to multiple other SUMO conjugates including Pgk1, which strongly accumulate in cells expressing the isopeptidase-resistant *SUMO-Q95P* variant (Figure 24). Interestingly, these data also suggest that the SUMO conjugate levels of mitochondrial substrates observed in wild type cells are a result of dynamic SUMOylation and deSUMOylation. Additionally, it seems plausible that irreversible

SUMO attachment to mitochondria-targeted proteins leads to the rapid degradation of the corresponding SUMO conjugates. The underlying mechanism requires proteasome function, however, is evidently independent of known yeast STUbLs (Figure 25). At first glance, the degradation of SUMO conjugates independent of SUMO-specific STUbLs might appear unexpected. However, even in proteasome mutant cells, predominantly mono- and only to a minor degree diSUMOylated species of mitochondria-targeted proteins are detectable (Figure 19), suggesting that the degradation of these SUMO-protein conjugates does not involve SUMO chains. By contrast, most STUbLs harbor multiple SIMs that mediate cooperative binding of multiple SUMO units, thereby facilitating the preferential recognition of polySUMO chains (Rojas-Fernandez et al., 2014; Tatham et al., 2008; Uzunova et al., 2007).

In a model in which SUMO conjugates are degraded by a STUbL-independent pathway, it remains unclear by which mechanism SUMO-modified substrates are recognized. One potential explanation is the existence of a novel, yet to be identified, ubiquitin E3 ligase, which possesses specificity for SUMOylated substrates. Alternatively, an intriguing possibility is the proteasomal clearance of SUMO conjugates independent of ubiquitylation. Notably, ubiquitin-independent proteasomal degradation has indeed been described for some substrates (Erales and Coffino, 2014). A further, potentially relevant, fact might be that mitochondrial precursor proteins are known to adopt a loosely folded state in the cytosol (Neupert, 1997). It is therefore conceivable that mitochondria-targeted substrates become SUMOylated in an unfolded state and that the resulting branched peptide impairs further folding for steric reasons. The presence of SUMO attachment sites within functional domains (Figure 10) might indeed support this hypothesis. Thus, SUMOylation of mitochondria-targeted proteins could promote the exposure of degrons, which are recognized by ubiquitin E3 ligases generally involved in protein quality control, for instance Ubr1/2 (Eisele and Wolf, 2008; Nillegoda et al., 2010), San1 (Heck et al., 2010; Prasad et al., 2010), Rsp5 (Fang et al., 2014), Hul5 (Fang et al., 2011) or Doa10 (Metzger et al., 2008).

4.6 SUMOylation of mitochondria-targeted proteins as example for SUMO-mediated protein quality control

Data presented in this study support a model of a novel stress-induced SUMO pathway involved in the quality control of mitochondria-targeted proteins (Figure 27). In a first step, substrates of this pathway are recognized by the SUMO E3 ligases Siz1 and Siz2. Substrate selection occurs most probably prior to import and targets a small pool of proteins “en route” to mitochondria. As for many SUMO substrates, the

SUMOylation of mitochondria-targeted proteins appears to be dynamic, allowing the reversal of the modification and potentially the return to the usual import route into the organelle. In fact, under unperturbed conditions, the major pool of detectable SUMO-modified species appears to remain targeting-competent, leading to the initiation of import and cleavage of the N-terminal mitochondrial targeting signal. By contrast, upon stress, particularly impairment of the cytosolic HSP70 chaperone or the proteasome system, the SUMOylation of mitochondrial precursor proteins is strongly increased. Remarkably, under these conditions, the modification appears less stringent in terms of site-selectivity (Figure 16 and Figure 20). Interestingly, similar findings have been reported previously for the SUMOylation of c-Myc upon proteasome inhibition (Gonzalez-Prieto et al., 2015). Accordingly, it has been suggested that SUMOylation might generally act more promiscuously during stress (Hendriks and Vertegaal, 2016).

Altogether, these findings indicate a role for SUMO in general cytosolic protein quality control, which targets import-incompetent mitochondrial preproteins accumulating in response to specific stress conditions. Notably, mitochondrial precursor proteins are generally thought to be prone to aggregation and degradation (Neupert, 1997) and experimental evidence indeed indicates a pronounced presence of SUMO-modified precursor species in insoluble cell fractions of proteasome mutant cells (Figure 22). It is therefore conceivable that SUMOylation might chiefly target unfolded and potentially aggregation-prone mitochondrial preproteins. Finally, biochemical data presented in this study support a model in which SUMO attachment to mitochondria-targeted proteins ultimately serves as a degradative mark, which mediates the proteasomal clearance of the modified substrate pool.

Stepwise proteasome-dependent degradation mechanisms involving initial substrate SUMOylation have been reported by several previous studies (Guo et al., 2014; Her et al., 2015; Kohler et al., 2015; Wang and Prelich, 2009). However, a largely unanswered question concerning these pathways is why SUMO is required in addition to ubiquitin. Intriguingly, a potential answer to this issue, at least in the context of protein quality control, might be provided by the finding that SUMO specifically promotes the proteasomal degradation of insoluble fractions of aggregation-prone proteins (Guo et al., 2014). It has been proposed that the SUMO system acts by a sequential mechanism based on the specific recognition of misfolded proteins by a SUMO E3 ligase and SUMO's ability to increase the solubility of strongly aggregating proteins (Abeywardana and Pratt, 2015; Janer et al., 2010; Krumova et al., 2011; Mukherjee et al., 2009; Steffan et al., 2004; Zhang and Sarge, 2008).

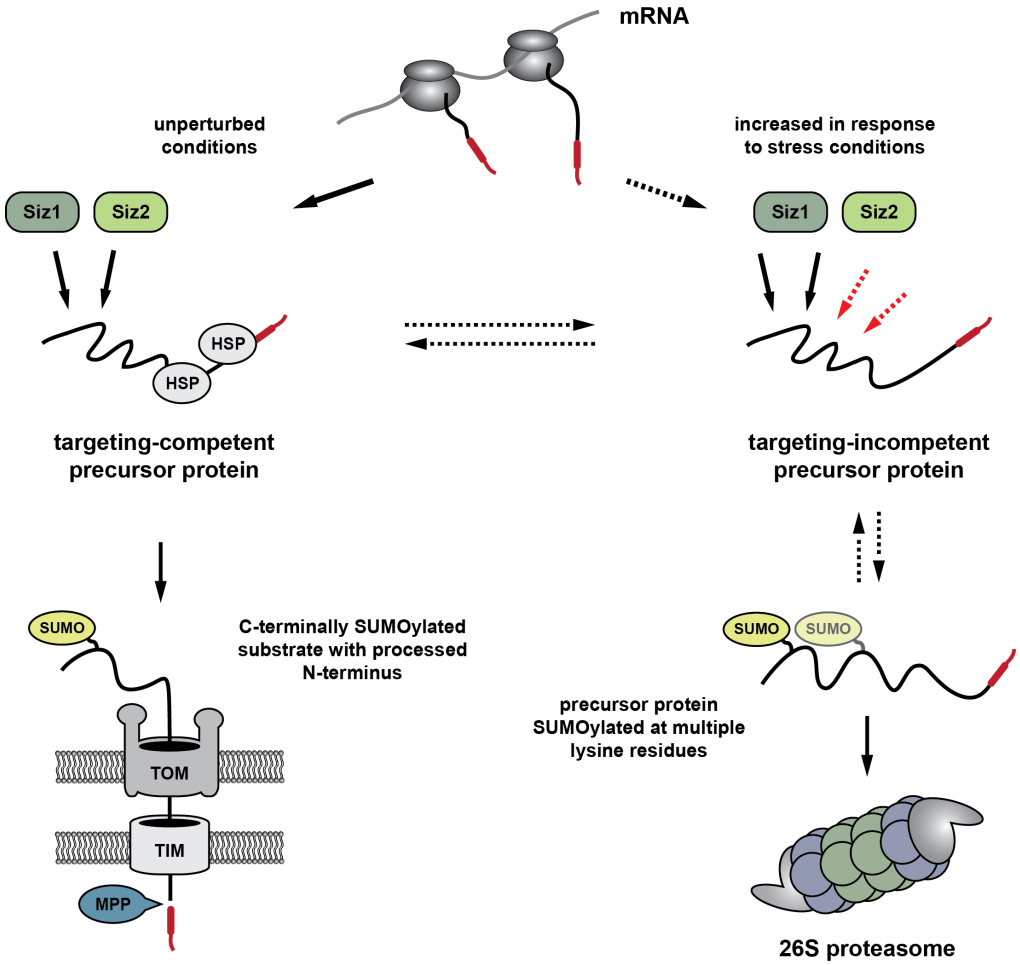


Figure 27. Model of a SUMO-mediated protein quality control pathway acting on mitochondria-targeted substrates. Mitochondria-targeted SUMO substrates are nuclear-encoded proteins, which are synthesized on cytosolic ribosomes and subsequently imported into mitochondria. Under unperturbed conditions, mitochondrial protein import is efficient and the major pools of SUMO substrates with N-terminal MTSs (depicted in red) are targeted to the organelle. In the cytosol, such proteins are probably bound by molecular chaperones (HSPs), which may directly interact with N-terminal MTSs. Consequently, only minor protein fractions are recognized by the SUMO E3 ligases Siz1 and Siz2 and SUMOylation occurs predominantly in the form of single SUMO moieties attached to C-terminal lysine residues. In response to certain proteotoxic stresses, the SUMOylation of mitochondria-targeted substrates is strongly increased. Under these conditions, SUMOylated precursor species of mitochondria-targeted proteins accumulate and the modification occurs at multiple sites, including lysine residues located in closer proximity to the N-termini of the substrates. Ultimately, the SUMOylation of mitochondria-targeted proteins mediates their degradation by a proteasome-dependent pathway.

Thereby, SUMO could facilitate the clearance of insoluble protein aggregates, which are otherwise not efficiently degraded by the proteasome (Verhoef et al., 2002). Notably, the function as a solubility-promoting modification might be unique for SUMO and different from ubiquitin, which typically targets insoluble protein aggregates for degradation via autophagy (Kirkin et al., 2009a; Kirkin et al., 2009b; Lu et al., 2014; Pankiv et al., 2007). Moreover, the reversibility of SUMOylation could be the basis of the triage decision whether a misfolded substrate is selected for deSUMOylation and subsequent refolding or for degradation. In this regard, SUMO

appears to display striking parallels to the function of molecular chaperones. In fact, remarkably, it has been speculated that SUMO acts as a chaperone-like factor under certain circumstances (Seifert et al., 2015).

In conclusion, a role for SUMO as a solubility-promoting or chaperone-like factor is an intriguing new concept with strong implications for cellular protein quality control. Moreover, particular relevance is given for neurodegenerative diseases, which are widely associated with protein aggregation. Thus, future research will not only shed further light on SUMO's role in proteostasis but might also provide possibilities for therapeutic intervention.

5 MATERIALS AND METHODS

Common chemicals and reagents were purchased from BD Biosciences (San Jose, USA), Bio-Rad Laboratories (Hercules, USA), Cayman Chemical Company (Ann Arbor, USA), Merck Millipore (Darmstadt, Germany), MP Biomedicals (Santa Ana, USA), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany), Seikagaku Corporation (Tokyo, Japan), Sigma-Aldrich (St. Louis, USA), Thermo Fisher Scientific (Waltham, USA) and VWR (Radnor, USA). Restriction endonucleases and deoxynucleotide triphosphates (dNTPs) were from New England Biolabs (Ipswitch, USA). DNA polymerases were obtained from Agilent Technologies (Santa Clara, USA), New England Biolabs and Thermo Fisher Scientific and alkaline phosphatases were from Roche Life Science (Penzberg, Germany) and Thermo Fisher Scientific. Custom-made DNA oligonucleotides for PCR applications were from Eurofins Genomics (Ebersberg, Germany).

5.1 Microbiological methods

5.1.1 *Escherichia coli* (*E. coli*) techniques

E. coli strains

Name	Genotype	Source
XL1-blue	*recA1 andA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]	Agilent
Rosetta	F^{-} *ompT hsd*S$_{B}$(r_{B}^{-} m_{B}^{-}) *gal dcm* pRARE (Cam^{R})	Merck Millipore

E. coli plasmids

Plasmid constructs encoding GST fusion proteins were based on the vector pGEX-4T-1 (GE Healthcare, Chicago, USA). For the expression of GST-Ssa1, the full-length *SSA1* open reading frame was amplified by PCR using yeast genomic DNA (DF5 background) as template and cloned into pGEX-4T-1.

Media, buffers and solutions

LB medium (plates)	1 % tryptone 0.5 % yeast extract 1 % NaCl (1.5 % agar) sterilized by autoclaving

TFB-I	30 mM KOAc 50 mM $MnCl_2$ 100 mM KCl 15 % glycerol pH 5.8 (adjusted with HOAc)
TFB-II	10 mM MOPS 7.5 mM $CaCl_2$ 10 mM KCl pH 7 (adjusted with NaOH)

Cultivation and storage of *E. coli* cells

E. coli cells harboring plasmids were grown overnight at 37°C on LB agar plates containing appropriate antibiotics (100 µg/ml ampicillin or 30 µg/ml kanamycin). Plates were sealed with parafilm and stored at 4°C for up to one week. For long-term storage, stationary cultures were frozen as glycerol stocks containing 15 % (v/v) glycerol and stored at -80°C. Liquid cultures containing appropriate antibiotics (100 µg/ml ampicillin or 30 µg/ml kanamycin) were usually inoculated from single colonies and grown at 37°C with constant shaking. For protein expression, liquid cultures were shifted to 25°C.

Preparation of competent *E. coli* cells

For the preparation of competent *E. coli* cells, 200 ml LB medium were inoculated with 1 ml of a fresh overnight culture grown at 37°C. The main culture was grown at 37°C until an OD_{600} of 0.45-0.55 was reached. The flasks were then chilled on ice for 10 min and the cells were harvested by centrifugation (5000 *g*, 10 min, 4°C). Subsequently, the supernatant was removed and the cell pellet was resuspended in 30 ml TFB-I solution. After further incubation on ice for 10 min, the bacteria were pelleted by centrifugation and resuspended in 6 ml TFB-II solution. Competent *E. coli* cells were frozen as 100 µl aliquots on dry ice and stored at -80°C.

Transformation of *E. coli* cells

Chemically competent *E. coli* cells were thawed on ice and 50 µl cells were mixed with an appropriate amount of plasmid DNA or 2 µl of a ligation sample. After incubation on ice for 20-30 min, the cells were heat-shocked at 42°C for 45 s. The reaction tubes were then cooled on ice for 2 min and 300 µl LB medium were added followed by recovery at 37°C for 20 min. Subsequently, the cells were plated on LB agar plates containing an appropriate antibiotic and transformants were grown overnight at 37°C.

Recombinant protein expression

Expression of recombinant GST fusion proteins was performed using Rosetta *E. coli* cells. Chemically competent cells were transformed with pGEX-4T-1-based expression vectors carrying the gene of interest and transformants were selected for on LB agar plates containing ampicillin following overnight growth. To start growth in liquid cultures, 25 ml LB medium were inoculated with a single colony and grown overnight at 37°C. The next day, precultures were diluted 1:100 with fresh LB medium and the cells were grown at 37°C until an OD_{600} of 0.5 was reached. The cultures were then cooled down to 25°C and protein expression was induced by addition of 1 mM IPTG. The cultures were further incubated over night with shaking and cells were harvested by centrifugation (10 min, 5000 *g*, 4°C). If required, cell pellets were frozen in liquid N_2 and stored at -80°C.

5.1.2 *Saccharomyces cerevisiae* (*S. cerevisiae*) techniques

***S. cerevisiae* strains**

Name	Genotype	Source
DF5	*his3-Δ200, leu2-3, 2-112, lys2-801, trp1-1, ura3-52*	D. Finley
W303	*leu2-3,112, ade2-1, can1-100, his3-11,15, ura3-1, trp1-1, RAD5*	X.Zhao
Y0002	DF5, *Matα*	D. Finley
Y2725	W303*, Matα*	X. Zhao
JN516	*Matα, SSA1 Δssa2::LEU2 Δssa3::TRP1 Δssa4::LYS2*	E. Craig
ssa1-45	*Matα, ssa1-45 Δssa2::LEU2 Δssa3::TRP1 Δssa4::LYS2*	E. Craig
CMY826	*Mata, ura3-52, leu2Δ1, his3Δ-200, trp1Δ63, lys2-801, ade2-101, Δbar1::HIS3*	C. Mann
CMY763	*Matα, cim3-1, ura3-52, leu2Δ1*	C. Mann
YFP162	CMY826, *Mata, YIplac211-pADH-*^{His}*SMT3::URA3*	this study
YFP140	CMY763, *Matα, YIplac211-pADH-*^{His}*SMT3::URA3* (cl.1)	this study
YFP141	CMY763, *Matα, YIplac211-pADH-*^{His}*SMT3::URA3* (cl. 2)	this study
YFP167	CMY826, *Mata, YIplac211-pADH-*^{His}*SMT3::URA3 ADH3*^{3HA}*::kanMX4*	this study
YFP154	CMY763, *Matα, YIplac211-pADH-*^{His}*SMT3::URA3 ADH3*^{3HA}*::kanMX4* (cl. 1)	this study
YFP155	CMY763, *Matα, YIplac211-pADH-*^{His}*SMT3::URA3 ADH3*^{3HA}*::kanMX4* (cl. 2)	this study
YFP171	CMY826, *Mata, YIplac211-pADH-*^{His}*SMT3::URA3 ILV6*^{3HA}*::kanMX4*	this study
YFP156	CMY763, *Matα, YIplac211-pADH-*^{His}*SMT3::URA3 ILV6*^{3HA}*::kanMX4* (cl. 1)	this study
YFP157	CMY763, *Matα, YIplac211-pADH-*^{His}*SMT3::URA3 ILV6*^{3HA}*::kanMX4* (cl. 2)	this study

(continued on next page)

Name	Genotype	Source
YFP516	JN516, *Matα, YIplac211-pADH-^{His}SMT3::URA3*	this study
YFP519	*ssa1-45*, *Matα, YIplac211-pADH-^{His}SMT3::URA3*	this study
YFP602	JN516, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ADH3^{3HA}::kanMX4*	this study
YFP606	JN516, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ILV6^{3HA}::kanMX4*	this study
YFP612	*ssa1-45*, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ADH3^{3HA}::kanMX4*	this study
YFP616	*ssa1-45*, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ILV6^{3HA}::kanMX4*	this study
YFP594	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ILV6^{3HA}::kanMX4 Δzip3::hphNT1*	this study
YFPX4-3A	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3*	this study
YFPX244-5C	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 Δsiz1::hphNT1*	this study
YFPX245-7C	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 Δsiz2::natNT2*	this study
YFPX248-2C	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 Δsiz1::hphNT1 Δsiz2::natNT2*	this study
YFPX10-5C	DF5, *Matα, ADH3^{3HA}::kanMX4*	this study
YFPX103-3D	DF5, *Matα, ADH3^{3HA}::TRP1*	this study
YFPX12-9B	DF5, *Matα, ILV6^{3HA}::kanMX4*	this study
YFPX112-16D	DF5, *Matα, ILV6^{3HA}::TRP1*	this study
YFPX14-1C	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ADH3^{3HA}::kanMX4*	this study
YFPX115-11A	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ADH3^{3HA}::TRP1*	this study
YFPX149-12D	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ADH3^{3HA}::kanMX4 Δsiz1::hphNT1*	this study
YFPX147-4B	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ADH3^{3HA}::kanMX4 Δsiz2::HIS3MX6*	this study
YFPX153-2D	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ADH3^{3HA}::kanMX4 Δsiz1::hphNT1 Δsiz2::HIS3MX6*	this study
YFPX16-6C	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ILV6^{3HA}::kanMX4*	this study
YFPX16-2D	DF5, *Mata, YIplac211-pADH-^{His}SMT3::URA3 ILV6^{3HA}::kanMX4*	this study
YFPX116-3B	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ILV6^{3HA}::TRP1*	this study
YFPX150-7C	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ILV6^{3HA}::kanMX4 Δsiz1::hphNT1*	this study
YFPX164-3C	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ILV6^{3HA}::kanMX4 Δsiz2::natNT2*	this study
YFPX165-14B	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ILV6^{3HA}::kanMX4 Δsiz1::hphNT1 Δsiz2::natNT2*	this study
YFPX71-2A	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ILV6^{3HA}::kanMX4 mms21-11::natNT2*	this study
YFPX246-12A	DF5, *Matα, YIplac211-pADH-^{His}SMT3::URA3 ILV6^{3HA}::kanMX4 cim3-1*	this study

(continued on next page)

Name	Genotype	Source
YFPX266-20C	DF5, *Matα, pADH-^{GFP}SMT3::natNT2 ILV6^{3HA}::kanMX4 cim3-1*	this study
YFP339	W303, *Matα, Ylplac211-pADH-^{His}SMT3::URA3*	this study
YFPX212-7D	W303, *Matα, Ylplac211-pADH-^{His}SMT3::URA3 Δsiz1::hphNT1*	this study
YFPX213-7D	W303, *Matα, Ylplac211-pADH-^{His}SMT3::URA3 Δsiz2::natNT2*	this study
YFP627	W303, *Matα, pRS306-pGAL-ADH3^{3HA}-tCYC1::URA3*	this study
YFP630	W303, *Matα, pRS306-pGAL-adh3$_{28-375}$^{3HA}-tCYC1::URA3*	this study
YFPX251-15C	W303, *Matα, Ylplac128-pADH-^{His}SMT3::LEU2 pRS306-pGAL-ADH3^{3HA}-tCYC1::URA3*	this study
YFPX256-9C	W303, *Matα, Ylplac128-pADH-^{His}SMT3::LEU2 pRS306-pGAL-adh3$_{28-375}$^{3HA}-tCYC1::URA3*	this study
YFPX255-3B	W303, *Matα, Ylplac128-pADH-^{His}SMT3::LEU2 pRS306-pGAL-adh3$_{28-375}$^{3HA}-K305R-tCYC1::URA3*	this study
YFPX254-6D	W303, *Matα, Ylplac128-pADH-^{His}SMT3::LEU2 pRS306-pGAL-adh3$_{28-375}$^{3HA}-tCYC1::URA3 cim3-1*	this study
YFPX255-7D	W303, *Matα, Ylplac128-pADH-^{His}SMT3::LEU2 pRS306-pGAL-adh3$_{28-375}$^{3HA}-K305R-tCYC1::URA3 cim3-1*	this study
YFPX259-2D	W303, *Matα, Ylplac211-pGAL-^{His}SMT3^{GG}::URA3 ILV6^{3HA}::TRP1*	this study
YFPX260-7C	W303, *Matα, Ylplac211-pGAL-^{His}SMT3^{GG}-Q95P::URA3 ILV6^{3HA}::TRP1*	this study
YFPX205-3B	W303, *Matα, Ylplac211-pGAL-^{His}SMT3^{GG}::URA3 ILV6^{3HA}::TRP1 cim3-1*	this study
YFPX206-13B	W303, *Matα, Ylplac211-pGAL-^{His}SMT3^{GG}-Q95P::URA3 ILV6^{3HA}::TRP1 cim3-1*	this study
YFPX283-4A	W303, *Matα, Ylplac211-pGAL-^{His}SMT3^{GG}::URA3 ILV6^{3HA}::TRP1 Δslx5::natNT2 Δslx8::HIS3MX6*	this study
YFPX284-3D	W303, *Matα, Ylplac211-pGAL-^{His}SMT3^{GG}-Q95P::URA3 ILV6^{3HA}::TRP1 Δslx5::natNT2 Δslx8::HIS3MX6*	this study
YFPX288-3D	W303, *Matα, Ylplac211-pGAL-^{His}SMT3^{GG}::URA3 ILV6^{3HA}::TRP1 Δris1::natNT2*	this study
YFPX292-7C	W303, *Matα, Ylplac211-pGAL-^{His}SMT3^{GG}-Q95P::URA3 ILV6^{3HA}::TRP1 Δris1::natNT2*	this study

S. cerevisiae vectors

Name	Plasmid type	Source
Ylplac128 Ylplac211	integrative	Gietz and Sugino, 1988
pRS306	integrative	Sikorski and Hieter, 1989
YCplac22	centromeric	Gietz and Sugino, 1988
p413ADH p415ADH p415GAL	centromeric	Mumberg et al., 1994, 1995
pRS306-pGAL	integrative	this study
p413TDH3	centromeric	this study

S. cerevisiae plasmids

Plasmids for the expression of ^{His}SUMO under control of the *ADH1* promoter were based on the integrative vectors YIplac211 and YIplac128 (Gietz and Sugino, 1988), respectively and have been described previously (Hoege et al., 2002; Psakhye and Jentsch, 2012; Sacher et al., 2006). ORFs encoding mature ^{His}SUMO variants (wild type and *Q95P*) were cloned into YIplac211 under control of the *GAL1* promoter.

For subsequent cloning into different types of expression vectors, ORFs encoding C-terminally 3HA-tagged proteins (and N-terminally truncated mutant variants) were amplified by PCR using specific primer pairs. The respective template DNA was isolated from yeast cells, in which individual genes were chromosomally fused to cassettes encoding C-terminal 3HA epitopes. These ORFs were then cloned into vectors of the p41XADH or p41XGAL series (Mumberg et al., 1994, 1995) for expression under control of the *ADH1* and *GAL1* promoter, respectively. For the expression of wild type Ilv6^{3HA} and various *KR* mutant variants under control of the endogenous promoter, the *ILV6* promoter, the *ILV6^{3HA}* ORF and the *ILV6* terminator were cloned into YCplac22 (Gietz and Sugino, 1988).

For expression of wild type Adh3^{3HA} and ∆mts-adh3^{3HA} from the *GAL1* or the TDH3 promoter, the respective ORFs were cloned into p415GAL, pRS306-pGAL or p413TDH3. pRS306-pGAL was generated by subcloning a DNA fragment comprising the *GAL1* promoter, the multiple cloning site and the *CYC1* terminator from p415GAL into pRS306 (Sikorski and Hieter, 1989). For the generation of p413TDH3, the *GAL1* promoter of p413GAL was replaced by the *TDH3* promoter.

In all constructs, *KR* mutations were introduced by site-directed mutagenesis using specific primer pairs.

Media, buffers and solutions

YPD/YPGal (plates)	1 % yeast extract 2 % bacto peptone 2 % D-glucose/D-galactose (2 % agar) sterilized by autoclaving
YPD selection plates	YPD agar was autoclaved, cooled down to 50°C and the respective antibiotic was added: geneticin (G418 sulfate, Thermo Fisher Scientific) to 200 mg/l nourseothricin (NAT, HKI, Jena) to 100 mg/l hygromycin (Hygro, Cayman Chemical) to 500 mg/l

SC-media (plates)	0.67 % yeast nitrogen base 0.2 % amino acid drop-out mix (lacking one or multiple compounds if required) 2 % carbon source (D-glucose, D-raffinose or D-galactose)
Amino acid drop-out mix	4.0 g Leu 2.0 g Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val 2.0 g myo-inositol 2.0 g uracil 0.5 g adenine 0.2 g p-aminobenzoic acid
SORB solution	100 mM LiOAc 10 mM Tris-HCl pH 8.0 1 mM EDTA pH 8.0 1 M sorbitol sterilized by filtration
PEG solution	100 mM LiOAc 10 mM Tris-HCl pH 8.0 1 mM EDTA pH 8.0 40 % (w/v) PEG-3350 sterilized by filtration stored at 4°C
Sporulation medium	2 % (w/v) KOAc sterilized by autoclaving
Sporulation plates	0.25 % yeast extract 0.1 % D-glucose 2 % KOAc 0.168 % CSM powder 2 % agar adjusted to pH 7 with KOH/HOAc
Zymolyase solution	0.9 M sorbitol 0.1 M Tris-HCl pH 8.0 0.2 M EDTA pH 8.0 50 mM DTT 0.5 mg/ml Zymolyase-100T (Seikagaku Corporation)

Cultivation and storage of *S. cerevisiae* cells

S. cerevisiae cells were grown on agar plates and in liquid cultures, respectively. For growth on plates, yeast cells were streaked from glycerol stocks using a sterile 2 ml glass pipette and incubated at 30°C for 2-3 days. Plates with temperature-sensitive strains were kept at the permissive temperature (typically 25°C) for 3-4 days.

Liquid cultures were inoculated with cells from freshly streaked agar plates and grown overnight at 25°C or 30°C on a shaking platform. Main cultures were obtained by diluting fresh overnight cultures to an OD_{600} of 0.1-0.2. These cultures were incubated in baffle-flasks (flask volume ≥ 5x liquid culture volume) with constant shaking at 110-150 rpm until the mid log growth phase was reached (OD_{600} = 0.6-1.0). Notably, all hypomorphic mutants (*cim3-1* and *ssa1-45*) used in this study were grown at 25°C. A temperature shift to 37°C is not required for these strains. Culture densities (OD_{600}) were determined photometrically and yeast cells were harvested by centrifugation. Yeast cultures on agar plates were sealed with parafilm and stored at 4°C for 1-4 weeks. For long-term storage, stationary cultures were frozen as glycerol stocks containing 15 % (v/v) glycerol and stored at -80°C.

Preparation of competent *S. cerevisiae* cells

Yeast cells from a fresh overnight culture were inoculated in 50 ml medium (usually YPD) to an OD_{600} of 0.1-0.2 and grown until an OD_{600} of 0.5-0.7 was reached. The cells were harvested by centrifugation (500 *g*, 5 min, room temperature), washed first with 25 ml sterile dH_2O and subsequently with 5 ml sterile SORB solution. The cell pellet was then resuspended in 360 µl SORB solution, mixed with 40 µl carrier DNA (10 mg/ml salmon or herring sperm DNA, Thermo Fisher Scientific) and stored at -80°C.

Transformation of *S. cerevisiae* cells

For transformation, 200 ng of circular plasmid DNA and 2 µg of linear DNA (linearized plasmid DNA or PCR products) were added to 10 µl and 50 µl of competent yeast cells, respectively. The cells were mixed with 6 volumes of PEG solution and incubated at room temperature for 30 min. After addition of 10 % sterile DMSO the cells were heat-shocked at 42°C for 8-15 min and pelleted by centrifugation (500 *g*, 3 min, room temperature). For the selection of transformants containing auxotrophic markers, cells were resuspended in 100 µl sterile dH_2O and directly plated on the respective SC agar plates. For the selection of transformants on plates containing antibiotics, cells were resuspended 700 µl YPD and incubated at 30°C (25°C for temperature-sensitive strains) for 3-4 h prior to plating. Stable transformants were grown at 30°C (25°C for temperature-sensitive strains) for 2-4

days. If required, replica plating using sterile velvet was performed to remove background.

Genetic manipulation of *S. cerevisiae*

S. cerevisiae is a highly recombination-proficient organism and therefore ideally suited for genetic manipulation. Gene deletion mutants and chromosomally tagged strains were constructed using a PCR-based strategy (Janke et al., 2004; Knop et al., 1999). In brief, targeting cassettes containing selection markers (and optionally sequences encoding epitope tags) were generated by PCR and transformed into competent yeast cells. Upon integration of the cassettes into the yeast genome, stable transformants were selected on appropriate agar plates. Subsequently, integration of deletion constructs at the correct genomic locations was verified by yeast colony PCR using specific primer pairs. In case of epitope taggings, expression of the respective fusion proteins was additionally confirmed by western blot analysis using epitope-specific antibodies.

Integrative yeast plasmids (based on the vectors YIplac128, YIplac211 and pRS306) were linearized by treatment with restriction endonucleases. Selected cut sites were located within inserts (*Bgl*II for the YIplac211-pADH-^{His}SMT3 construct) and marker genes (*Eco*RV for *LEU2* in the YIplac128-pADH-^{His}SMT3 construct and *Nco*I for *URA3* in pRS306-based constructs), respectively. Linearized plasmids were then transformed into competent yeast cells and stable transformants were selected on appropriate agar plates. Plasmid integration at the correct chromosomal loci was verified by yeast colony PCR and expression of encoded proteins was confirmed by western blot analysis.

Mating of haploid *S. cerevisiae* strains

For mating of haploid yeast strains, freshly streaked cells of opposite mating type (Mat a and Mat α) were mixed on a YPD plate using sterile toothpicks. The plate was then incubated over night at 30°C (25°C for temperature-sensitive strains) and diploid cells were identified by growth on double selection plates. In cases, for which the use of double selection plates was not applicable, diploid cells were identified by consecutive streaking on different selection plates.

Sporulation and tetrad analysis of diploid *S. cerevisiae* strains

For sporulation in liquid medium, diploid yeast cells from 500 µl of a saturated overnight culture (typically grown in YPD at 30°C) were harvested by centrifugation (500 *g*, 3 min, room temperature). The cells were washed three times with 1 ml sterile dH_2O and once with sporulation medium. Subsequently, the cells were

resuspended in 4 ml sporulation medium and incubated at room temperature on a shaker for 3-6 days. Prior to tetrad dissection, 10 µl of a sporulated culture were mixed with 10 µl zymolyase solution and incubated at room temperature for 5 min. The cells were then transferred to a YPD agar plate, dried and tetrad dissection was performed using a MSM400 micromanipulator (Singer Instruments, Roadwater, UK).

For sporulation on plate, diploid cells were streaked on a sporulation plate and incubated at 30°C (25°C for temperature-sensitive strains) for 3-4 days. A patch of cells from a sporulation plate was resuspended in 1 ml sterile dH_2O and 10 µl of the suspension was mixed with 10 µl zymolyase solution. After incubation for 5 min at room temperature the cells were transferred to a YPD agar plate and tetrad dissection was performed as described above.

Tetrads were grown at 30°C (25°C for temperature-sensitive strains) for 2-3 days and genotypic analysis was performed by replica plating on selection plates. Temperature-sensitive spores were identified by replica plating on YPD plates and incubation at the non-permissive temperature (typically 37°C).

Mating type analysis

Mating types of haploid yeast cells were analyzed using the tester strains RC634a and RC75-7α (Dietzel and Kurjan, 1987). These strains display a strong sensitivity towards mating pheromones secreted by cells of the opposite mating type. To generate tester plates for mating type analysis, patches of freshly streaked tester cells were resuspended in 1 % agar (cooled to max. 40°C) and poured onto YPD plates. Tetrads were replica plated onto tester plates and incubated at 30°C (25°C for temperature-sensitive strains) for 1-2 days. Growth inhibition of the tester strain in the top layer agar in close proximity to cells of the opposite mating type leads to formation of a so called “halo” and thereby allows the determination of the corresponding mating type. Diploid cells do not secrete mating type pheromones and can be identified by the absence of a ‘halo’ on both types of tester plates.

Spotting assays

Spotting assays were used to analyze the growth phenotypes of yeast strains at different temperatures. Cells grown in liquid cultures were diluted to an OD_{600} of 1 and six 5-fold serial dilutions in sterile dH_2O were prepared. These dilutions were spotted on agar plates using a custom-made stamping device followed by incubation at different temperatures for 2-5 days.

5.2 Methods in molecular biology

General buffers and solutions

TBE buffer	90 mM Tris 90 mM boric acid 2.5 mM EDTA
10x DNA sample buffer	0.25 % bromophenol blue 0.25 % xylene cyanol FF 50 % glycerol

5.2.1 Nucleic acid purification and analysis

Isolation of plasmid DNA from *E. coli* cells

Isolation of plasmid DNA from *E. coli* cells was performed with the AccuPrep Plasmid Mini Extraction Kit (Bioneer Corporation, Daejeon, South Korea) and the QIAGEN Plasmid Mini Kit (Qiagen, Hilden, Germany), respectively. 5 ml LB medium containing 100 µg/ml ampicillin or 30 µg/ml kanamycin were inoculated with a single *E. coli* colony and grown over night at 37°C. Cells were pelleted by centrifugation and plasmid isolation was performed according to the manufacturers' instructions.

Purification of genomic DNA from *S. cerevisiae* cells

Purification of genomic DNA from yeast cells was performed using the MasterPure Yeast DNA Purification Kit (Epicentre, Madison, USA) according to the manufacturer's instructions. Typically, cells from 500 µl of a fresh overnight culture were used.

Purification of PCR products

Linear DNA fragments generated by PCR were purified using the QIAquick PCR Purification Kit (Qiagen) and the AccuPrep PCR Purification Kit (Bioneer Corporation), respectively. Both kits were used according to the manufacturers' instructions.

Agarose gel electrophoresis of DNA molecules

DNA fragments generated by PCR or by digestion of plasmid DNA using restriction endonucleases were resolved using agarose gel electrophoresis. Gels were prepared by dissolving 1-2 % agarose in TBE buffer in a microwave. Ethidiumbromide was added to a final concentration of 0.5 µg/ml and the solution

was poured into a gel casting form. Before loading, DNA samples were mixed with an appropriate volume of 10x DNA sample buffer. Electrophoretic separation was carried out in TBE buffer at a constant voltage of 80-120 V. DNA bands were visualized by UV illumination.

Extraction of DNA fragments from agarose gels

For the isolation of DNA from agarose gels, the required fragment was visualized by ethidium bromide staining and excised from the gel on a UV transilluminator using a clean razor blade. The gel block was transferred to a sterile 1.5 ml reaction tube and the DNA was purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

Determination of DNA concentrations

Concentrations of DNA in aqueous solutions were determined photometrically using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Measurements were based on the following calculation:

$$1\ A_{260}\ \text{unit of dsDNA} \approx 50\ \mu g/ml\ \text{in}\ dH_2O$$

The purity of DNA solutions was estimated by the following value:

$$A_{260} / A_{280} \geq 1.8$$

DNA sequence analysis

DNA sequencing was performed by at the MPIB microchemistry core facility using an ABI 3730 DNA analyzer (Applied Biosystems/Thermo Fisher Scientific) and the ABI Big Dye 3.1 sequencing system. Alternatively, DNA samples were sent to Eurofins Genomics for custom DNA sequencing.

5.2.2 DNA amplification by polymerase chain reaction (PCR)

Amplification of DNA fragments for molecular cloning

For molecular cloning, DNA fragments were amplified using *Phusion* high fidelity DNA polymerase (Thermo Fisher Scientific). Primers with restriction sites within the 5'-overhangs were designed to consist of 20-23 nucleotides complementary to the DNA sequence of interest. PCR reactions were set up on ice in a total volume of 50 µl.

PCR reaction mix:
5.0 µl 5x *Phusion* HF buffer
1.0 µl genomic DNA
2.5 µl primer 1 (10 µM)
2.5 µl primer 2 (10 µM)
1.0 µl dNTP mix (10 mM each)
0.5 µl *Phusion* High-Fidelity DNA polymerase
31.5 µl dH_2O

Thermocycler program (34 amplification cycles):

PCR step	T [°C]	Time
Initial denaturation	98	30 s
Denaturation	98	10 s
Annealing	50-55	30 s
Elongation	72	15-30 s/kb
Final elongation	72	10 min
Cooling	4	∞

Amplification of yeast targeting cassettes

Targeting cassettes for gene deletions and chromosomal epitope taggings in yeast were amplified using a mixture of *Taq* (purified by U. Cramer, Department of Molecular Cell Biology, MPIB) and *Vent* DNA polymerases (Thermo Fisher Scientific). The PCR cycling parameters have been described previously (Janke et al., 2004). Primers were designed in a way that the resulting PCR products containing the selection marker (and optionally a sequence encoding the epitope tag) were flanked by 55 bp-long targeting arms on both sides. The sequences of these targeting arms were homologous to the genomic loci of interest. PCR reactions were set up on ice in a total volume of 50 µl.

PCR reaction mix:
5.0 µl 10x ThermoPol reaction buffer
2.0 µl plasmid DNA (app. 50 ng/µl)
3.2 µl primer 1 (10 µM)
3.2 µl primer 2 (10 µM)
1.75 µl dNTP mix (10 mM each)
0.4 µl *Taq* DNA polymerase
0.2 µl *Vent* DNA polymerase
34.25 µl dH_2O

Verification of genomic recombination events (yeast colony PCR)

The integration of plasmids or targeting cassettes at the correct genomic loci was confirmed by yeast colony PCR using the Whole Cell Yeast PCR Kit (MP Biomedicals) according to the manufacturer's instructions. In brief, a small portion of

a single yeast colony was resuspended in 2.5 µl lysis solution using a sterile pipette and lysed by incubation for 1h at 37°C. Subsequently, the lysate was mixed with 22.5 µl PCR master mix and the reaction tubes were transferred into a PCR thermo cycler.

PCR reaction mix:
5.0 µl 10x ThermoPol reaction buffer
2.5 µl genomic DNA (cell extract)
5.0 µl primer 1 (10 µM)
5.0 µl primer 2 (10 µM)
1.75 µl dNTP mix (10 mM each)
1.25 µl *Taq* DNA polymerase
29.5 µl dH_2O

Thermocycler program (40 amplification cycles):

PCR step	T [°C]	Time
Initial denaturation	94	3 min
Denaturation	94	30 s
Annealing	50	30 s
Elongation	72	2 min
Final elongation	72	10 min
Cooling	4	∞

Site-directed mutagenesis

PCR-based site-directed mutagenesis related to the QuickChange method (Stratagene/Agilent Technologies) was used to introduce specific mutations, insertions or deletions into plasmid DNA. The approach requires two complementary primers, which consist of one or multiple central nucleotides harboring the desired mutation(s), flanked by 15 nucleotides of the correct target sequence on both sides. Dam-methylated circular plasmid DNA served as template for the PCR reaction and PCR amplification was performed using *PfuTurbo* DNA polymerase (Agilent Technologies) in a total volume of 25 µl.

PCR reaction mix:
2.5 µl 10x Cloned *Pfu* DNA polymerase reaction buffer
0.5 µl template DNA (app. 500 ng/µl)
0.5 µl primer 1 (10 µM)
0.5 µl primer 2 (10 µM)
0.6 µl dNTP mix (10 mM each)
0.5 µl *PfuTurbo* DNA polymerase
19.9 µl dH_2O

Thermocycler program (19 amplification cycles):

PCR step	T [°C]	Time
Initial denaturation	94	3 min
Denaturation	94	30 s
Annealing	49	45 s
Elongation	68	16 min
Final elongation	68	16 min
Cooling	4	∞

Subsequent to PCR, the methylated template DNA was selectively digested by *Dpn*I treatment for 3-4 h at 37°C. The resulting PCR product was transformed into competent *E. coli* cells and plasmid DNA was isolated from several individual clones. Plasmids harboring the desired mutation(s) were identified by DNA sequencing.

5.2.3 Molecular cloning

Cleavage of DNA with restriction endonucleases

Restriction endonucleases (New England Biolabs) were used for the sequence-specific cleavage of DNA molecules. For analytical purposes, app. 1 µg of circular plasmid DNA isolated from *E. coli* was incubated with the respective restriction endonucleases for 1-3h at 37°C. For molecular cloning, vector DNA and DNA fragments obtained by PCR were usually digested over night at 37°C.

Dephosphorylation of vector DNA

To prevent the religation of vector DNA during ligation reactions, 5' end dephosphorylation was performed using FastAP (Thermo Fisher Scientific) and rAPid Alkaline Phosphatase (Roche), respectively. 2 µl phosphatase were mixed directly with the restriction digest and incubated at 37°C for 2-4h. Subsequently, the linearized vector DNA was purified using agarose gel electrophoresis followed by gel extraction.

Ligation of DNA fragments

Ligation of DNA fragments with linearized (dephosphorylated) vector DNA was performed using T4 DNA ligase and the Quick DNA Ligation Kit (New England Biolabs), respectively. Typically, vector DNA and inserts were mixed in a 1:3 molar ratio. Ligation reactions using Quick T4 DNA ligase were incubated at 25°C for 5 min. Ligation reactions using T4 DNA ligase were incubated at 25°C for 10 min or overnight at 16°C. Subsequently, the reaction tubes were chilled on ice and transformed into competent *E. coli* cells.

5.3 Biochemical and cell biological methods

General buffers and solutions

HU sample buffer	8 M urea 5 % SDS 1 mM EDTA 1.5 % DTT 0.025 % bromophenolblue 200 mM Tris-HCl pH 6.8
MOPS running buffer	50 mM MOPS 50 mM Tris base 3.5 mM SDS 1 mM EDTA
Transfer buffer	250 mM Tris base 1.92 M glycine 0.1 % SDS 20 % (v/v) methanol
TBST	25 mM Tris-HCl pH 7.5 137 mM NaCl 2.6 mM KCl 0.1 % Tween 20
PBS	137 mM NaCl 2.7 mM KCl 4.3 mM Na_2HPO_4 1.47 mM KH_2PO_4 pH 7.5
Blocking solution	5 % (w/v) skim milk powder in TBST

5.3.1 Protein methods

Preparation of total cell extracts by trichloroacetic acid (TCA) precipitation

Trichloroacetic acid (TCA) precipitation was used for the preparation of small-scale denatured total protein extracts from yeast cells. Typically, 1 OD of cells were resuspended in 1 ml ice-cold dH_2O and lysed by addition of 150 µl 1.85 M NaOH/7.5 % β-mercaptoethanol. After 15 min incubation on ice, proteins were

precipitated by addition of 150 µl 55 % TCA and further incubation on ice for 10 min. The denatured material was recovered by two sequential centrifugation steps (14 krpm, 10 min, 4°C) and aspiration of the supernatant. Subsequently, the pellet was resuspended in 100 µl HU sample buffer by vigorous agitation for 10 min at 65°C.

Purification of ^{His}SUMO conjugates from denatured yeast extracts

Purification of ^{His}SUMO conjugates from yeast was performed by Ni-NTA pull-downs under denaturing conditions (Hoege et al., 2002; Sacher et al., 2006). Typically, 200 OD of yeast cells from logarithmically growing cultures were harvested by centrifugation (2500 *g*, 5 min, 4°C), washed once with ice-cold dH_2O and flash-frozen in liquid N_2. Optionally, cell pellets were stored at -80°C. Yeast cells were then lysed by resuspending the pellets in 6 ml 1.85 M NaOH containing 7.5 % β-mercaptoethanol and incubation on ice for 15 min. Subsequently, the cellular material was precipitated by addition of 6 ml 55 % TCA and further incubation on ice for 15 min. The precipitated material was pelleted by centrifugation (3000 *g*, 30 min, 4°C), washed twice with 50 ml ice-cold dH_2O and solubilized in 12 ml buffer A (6 M guanidinium hydrochloride, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8) containing 0.05 % Tween-20. The samples were incubated on a shaking platform for 1-2h (220 rpm, RT) and insoluble material was removed by centrifugation (23,000 *g*, 20 min, 4°C). The supernatants were transferred to 15 ml Falcon tubes, mixed with imidazole to a final concentration of 20 mM and 50-100 µl of magnetic Ni-NTA agarose beads (Qiagen) were added. After overnight incubation at 4°C on a tube roller, the beads were recovered by centrifugation (1000 rpm, 5 min, 4°C), transferred to 1.5 ml reaction tubes and washed three times with buffer A containing 20 mM imidazole/0.05 % Tween-20 and five times with buffer C (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 6.3) containing 0.05 % Tween-20. To remove detergents, the beads were then transferred to a fresh 1.5 ml reaction tube using 100 µl buffer C without Tween-20. Eventually, proteins bound to the beads were eluted by shaking in 30 µl 1 % SDS for 10 min at 65°C. The samples were dried in a SpeedVac centrifuge (Eppendorf, Hamburg, Germany) at 45°C for 30 min, dissolved in 10 µl dH_2O and 15 µl HU buffer for 10 min at 65°C and analyzed by SDS-PAGE and western blotting.

Preparation of native yeast cell lysates and cellular fractionation

For the preparation of native cell lysates from yeast, usually 100-200 OD of yeast cells were harvested by centrifugation (2000 *g*, 5 min, 4°C), washed once with ice-cold PBS and resuspended in 700 µl ice-cold lysis buffer in a 2 ml reaction tube.

Zirconia/silica beads (BioSpec Inc., Bartlesville, USA) were added until a 2 mm liquid phase was visible on top of the beads. Cells were then lysed at 4°C in a MM301 multi-tube bead-beater (Retsch Technology, Haan, Germany) using 6 disruption intervals of 1 min (frequency = 30 Hz), each followed by 5 min incubation on ice. The piggyback method was used to separate cellular lysates from beads and to transfer the lysates into fresh 15 ml Falcon tubes.

Cellular fractionations assessing the solubility of proteins were performed as described previously (Fang et al., 2011). In brief, yeast cells were lysed by bead-beating in lysis buffer (100 mM HEPES pH 7.5, 1 % Triton X-100, 300mM NaCl, 1x complete EDTA-free protease inhibitor cocktail (Roche), 1 mg/ml Pefabloc SC (Roche)). Subsequently, the lysates were pre-cleared by centrifugation (2000 *g*, 10 min, 4°C) and the resulting total cell extracts (T fraction) were fractionated by a second centrifugation step (16000 *g*, 10 min, 4°C) to yield soluble (S) and insoluble pellet (P) fractions. 10 µl of each, the total and soluble fractions, were mixed with 50 µl HU sample buffer and denatured at 65°C for 10 min in a thermo shaker. The pellet was washed three times with 1 ml ice cold lysis buffer and resolubilized in 50 µl HU sample buffer at 65°C for 10 min.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Size-dependent separation of proteins by denaturing SDS polyacrylamide gel electrophoresis was performed using pre-cast 12 % or 4-12 % NuPAGE Novex Bis-Tris gels (Thermo Fisher Scientific). Protein samples were prepared in HU buffer and denatured by shaking for 10 min at 65°C. Electrophoretic separation was carried out in MOPS running buffer at a constant voltage of 110-140V. The All Blue Precision Plus pre-stained protein standard (Bio-Rad Laboratories) served as molecular weight marker.

Western blot analysis

Western blotting was performed using a wet tank blotting system (Hoefer Inc. Holliston, USA). Proteins from polyacrylamide gels were transferred to Immobilon-P PVDF membranes (Merck Millipore) in fresh transfer buffer at a constant voltage of 75 V for 90 min. Subsequently, the membranes were briefly washed in TBST and blocked by shaking in blocking solution for 60 min. Incubation with primary antibodies diluted in blocking solution containing 0.05 % sodium azide was performed over night at 4°C. The next day, the membranes were washed four times for 5 min with TBST and incubated with specific horseradish peroxidase (HRP)-coupled secondary antibodies (Dianova, Hamburg, Germany) in blocking solution at room temperature

for 1-3 h. The membranes were washed four times for 10 min with TBST and protein detection was performed using the ECL or ECL Plus chemiluminescence systems (Thermo Fisher Scientific) according to the manufacturer's instructions. Chemiluminescence signals were detected using a CCD-based LAS-3000 imaging system (Fujifilm, Tokyo, Japan). Alternatively, western blot membranes were exposed to Hyperfilm ECL chemiluminescence films (GE Healthcare) followed by automated film development.

Primary antibodies

Name	Dilution	Type	Source
anti-HA (F-7)	1:2000	mouse monoclonal	Santa Cruz Biotechnology
anti-HA (Y-11)	1:1000	rabbit polyclonal	Santa Cruz Biotechnology
anti-Pgk1 (22C5D8)	1:15000	mouse monoclonal	Thermo Fisher Scientific
anti-Dpm1 (5C5)	1:2000	mouse monoclonal	Thermo Fisher Scientific
anti-HSP70 (BB70)	1:10000	mouse monoclonal	Enzo Life Sciences
anti-Smt3	1:10000	rabbit polyclonal	Hoege et al., 2002
anti-GST (B-14)	1:1000	mouse monoclonal (HRP-coupled)	Santa Cruz Biotechnology

Secondary antibodies

Name	Dilution	Type	Source
goat anti-mouse	1:5000	HRP-coupled	Dianova
goat anti-rabbit	1:5000	HRP-coupled	Dianova

Stripping of PVDF membranes

For incubation with alternative primary antibodies, PVDF membranes were stripped using the Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) according to the manufacturer's protocol. The membranes were washed three times for 10 min with TBST, incubated in blocking solution for 60 min and probed with an alternative primary antibody of choice.

Analysis of HSP70 binding sites on peptide arrays

Peptide arrays on cellulose membranes were generated using automated SPOT synthesis on a MultiPep peptide synthesizer (INTAVIS Bioanalytical Instruments, Cologne, Germany). Peptides covering the Ilv6 primary sequence were designed to comprise a length of 13 amino acids overlapping by 10 amino acids.

Before incubation with recombinant GST-Ssa1, the membrane was washed for 10 min with MeOH and three times for 15 min with TBST. Subsequently, the membrane was blocked for 60 min with blocking solution and washed four times for 5-10 min with TBS containing 0.05 % Tween-20. The peptide scan was then incubated for 90 min with 100 nM GST-Ssa1 in TBS (containing 0.05 % Tween-20) at room temperature followed by four 5 min washes with TBS (containing 0.05 % Tween-20). For the detection of GST-Ssa1 on the peptide array, the membrane was incubated at room temperature for 120 min with an anti-GST-HRP conjugate (B14) (Santa Cruz Biotechnology, Dallas, USA) in TBS (containing 0.05 % Tween-20). Subsequently, the membrane was washed four times for 5 min with TBST and subjected to chemiluminescence detection using ECL (Thermo Fisher Scientific) and a CCD-based LAS-3000 imaging system (Fujifilm).

Purification of recombinant GST fusion proteins

GST-Ssa1 was purified from *E. coli* cells (Rosetta) using standard protocols. In brief, cells were resuspended in lysis buffer (40 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM DTT, 1x complete EDTA-free protease inhibitor cocktail (Roche), 1 mg/ml Pefabloc SC (Roche)) and lysed in an EmulsiFlex-C5 homogenizer (Avestin, Ottawa, Canada). Lysates were then cleared by centrifugation for 30 min at 20 krpm and 4°C. Glutathione Sepharose 4B beads (GE Healthcare) were washed twice with dH_2O, three times with PBS and equilibrated in lysis buffer. Subsequently, the beads were added to the lysates and protein binding was performed for 4 h at 4°C on a rotating wheel. The beads were then washed twice with lysis buffer and four times with lysis buffer containing 450 mM NaCl. Finally, GST-tagged proteins were eluted in four steps with 0.5 ml elution buffer (40 mM Tris-HCl pH 7.5, 50 mM reduced glutathione, 5 mM DTT) and dialyzed overnight at 4°C against 5 l PBS using Slyde-A-Lyser dialysis cassettes (Thermo Fisher Scientific). Protein samples were frozen in liquid N_2 and stored at -80°C.

5.3.2 Microscopy techniques

Confocal fluorescence microscopy

To analyze the subcellular localization of GFP fusion proteins by fluorescence microscopy, yeast cells were grown at 30°C to exponential phase in synthetic complete (SC) medium. In case of import-incompetent Ilv6 variants expressed from the *GAL1* promoter, cells were grown in raffinose-containing medium and protein expression was induced by addition of 2 % galactose for 60 min. The cells were then

transferred to a CellCarrier-96 black polystyrene microplate (Perkin Elmer, Waltham, USA) and stained using calcofluor white. Subsequently, images were captured using an OperaPhenix HCS confocal microscope (Perkin Elmer) equipped with an Olympus 63x water NA 1.15 objective.

Calcofluor white staining

To visualize yeast cells during microscopy analysis, yeast cell walls were stained by calcofluor white staining using a dye solution that contains 1 g/l calcofluor white and 0.5 g/l Evans blue (Sigma-Aldrich). The solution was directly added to yeast cultures to a final dilution of 1:10, incubated at room temperature for at least 1 min and images were captured by confocal microscopy.

5.4 Database searches, computational analysis and software

Literature search was performed using the PubMed search engine of the United States National Library of Medicine (https://www.ncbi.nlm.nih.gov/pubmed). Nucleic acid and protein sequence searches as well as protein domain analysis was performed using electronic databases of the Saccharomyces Genome Database (http://www.yeastgenome.org) and the UniProt consortium (http://www.uniprot.org). Multiple sequence alignments were assembled using the Clustal Omega webserver of the European Bioinformatics Institute (https://www.ebi.ac.uk/Tools/msa/clustalo).

For the *in silico* prediction of SUMO attachment sites, the GPS-SUMO 2.0 software of the Cuckoo workgroup was used (http://sumosp.biocuckoo.org). Mitochondrial targeting sequences were predicted using MitoProt II (https://ihg.gsf.de/ihg/mitoprot.html) (Claros and Vincens, 1996) and TargetP (http://www.cbs.dtu.dk/services/TargetP) (Emanuelsson et al., 2000; Nielsen et al., 1997).

DNA sequence analysis and *in silico* cloning was carried out using the DNASTAR Lasergene software package (DNASTAR Inc., Madison, USA). For the presentation of statistical data, GraphPad Prism (GraphPad Software, La Jolla, USA) was used. Linear adjustment of western blot contrasts and preparation of figures, illustrations and cartoons was performed using Adobe Photoshop and Illustrator (Adobe Systems Inc., San Jose, USA). ImageJ software (https://imagej.nih.gov/ij) was used for western blot quantification. Text processing and generation of tables were carried out using Microsoft Office (Microsoft Corporation, Redmond, USA). EndNote X7 (Thomson Reuters, New York City, USA) was used for reference management.

6 REFERENCES

Abe, Y., Shodai, T., Muto, T., Mihara, K., Torii, H., Nishikawa, S., Endo, T., and Kohda, D. (2000). Structural basis of presequence recognition by the mitochondrial protein import receptor Tom20. Cell *100*, 551-560.

Abeywardana, T., and Pratt, M.R. (2015). Extent of inhibition of alpha-synuclein aggregation in vitro by SUMOylation is conjugation site- and SUMO isoform-selective. Biochemistry *54*, 959-961.

Ades, I.Z., and Butow, R.A. (1980a). The products of mitochondria-bound cytoplasmic polysomes in yeast. J Biol Chem *255*, 9918-9924.

Ades, I.Z., and Butow, R.A. (1980b). The transport of proteins into yeast mitochondria. Kinetics and pools. J Biol Chem *255*, 9925-9935.

Agarwal, S., and Roeder, G.S. (2000). Zip3 provides a link between recombination enzymes and synaptonemal complex proteins. Cell *102*, 245-255.

Ahting, U., Thieffry, M., Engelhardt, H., Hegerl, R., Neupert, W., and Nussberger, S. (2001). Tom40, the pore-forming component of the protein-conducting TOM channel in the outer membrane of mitochondria. J Cell Biol *153*, 1151-1160.

Alam, R., Hachiya, N., Sakaguchi, M., Kawabata, S., Iwanaga, S., Kitajima, M., Mihara, K., and Omura, T. (1994). cDNA cloning and characterization of mitochondrial import stimulation factor (MSF) purified from rat liver cytosol. J Biochem *116*, 416-425.

Ammon, T., Mishra, S.K., Kowalska, K., Popowicz, G.M., Holak, T.A., and Jentsch, S. (2014). The conserved ubiquitin-like protein Hub1 plays a critical role in splicing in human cells. J Mol Cell Biol *6*, 312-323.

Argan, C., Lusty, C.J., and Shore, G.C. (1983). Membrane and cytosolic components affecting transport of the precursor for ornithine carbamyltransferase into mitochondria. J Biol Chem *258*, 6667-6670.

Atencio, D.P., and Yaffe, M.P. (1992). MAS5, a yeast homolog of DnaJ involved in mitochondrial protein import. Mol Cell Biol *12*, 283-291.

Ballinger, C.A., Connell, P., Wu, Y., Hu, Z., Thompson, L.J., Yin, L.Y., and Patterson, C. (1999). Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. Mol Cell Biol *19*, 4535-4545.

Baumeister, W., Walz, J., Zuhl, F., and Seemuller, E. (1998). The proteasome: paradigm of a self-compartmentalizing protease. Cell *92*, 367-380.

Becker, J., Walter, W., Yan, W., and Craig, E.A. (1996). Functional interaction of cytosolic hsp70 and a DnaJ-related protein, Ydj1p, in protein translocation in vivo. Mol Cell Biol *16*, 4378-4386.

Bencsath, K.P., Podgorski, M.S., Pagala, V.R., Slaughter, C.A., and Schulman, B.A. (2002). Identification of a multifunctional binding site on Ubc9p required for Smt3p conjugation. J Biol Chem *277*, 47938-47945.

Benson, M.D., Li, Q.J., Kieckhafer, K., Dudek, D., Whorton, M.R., Sunahara, R.K., Iniguez-Lluhi, J.A., and Martens, J.R. (2007). SUMO modification regulates inactivation of the voltage-gated potassium channel Kv1.5. Proc Natl Acad Sci U S A *104*, 1805-1810.

Bernier-Villamor, V., Sampson, D.A., Matunis, M.J., and Lima, C.D. (2002). Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. Cell *108*, 345-356.

Bossis, G., and Melchior, F. (2006). Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. Mol Cell *21*, 349-357.

Bracher, A., and Verghese, J. (2015a). GrpE, Hsp110/Grp170, HspBP1/Sil1 and BAG domain proteins: nucleotide exchange factors for Hsp70 molecular chaperones. Subcell Biochem *78*, 1-33.

Bracher, A., and Verghese, J. (2015b). The nucleotide exchange factors of Hsp70 molecular chaperones. Front Mol Biosci *2*, 10.

Bragoszewski, P., Gornicka, A., Sztolsztener, M.E., and Chacinska, A. (2013). The ubiquitin-proteasome system regulates mitochondrial intermembrane space proteins. Mol Cell Biol *33*, 2136-2148.

Braschi, E., Zunino, R., and McBride, H.M. (2009). MAPL is a new mitochondrial SUMO E3 ligase that regulates mitochondrial fission. EMBO Rep *10*, 748-754.

Breitschopf, K., Bengal, E., Ziv, T., Admon, A., and Ciechanover, A. (1998). A novel site for ubiquitination: the N-terminal residue, and not internal lysines of MyoD, is essential for conjugation and degradation of the protein. EMBO J *17*, 5964-5973.

Caplan, A.J., Cyr, D.M., and Douglas, M.G. (1992). YDJ1p facilitates polypeptide translocation across different intracellular membranes by a conserved mechanism. Cell *71*, 1143-1155.

Castoralova, M., Brezinova, D., Sveda, M., Lipov, J., Ruml, T., and Knejzlik, Z. (2012). SUMO-2/3 conjugates accumulating under heat shock or MG132 treatment result largely from new protein synthesis. Biochim Biophys Acta *1823*, 911-919.

Chacinska, A., Koehler, C.M., Milenkovic, D., Lithgow, T., and Pfanner, N. (2009). Importing mitochondrial proteins: machineries and mechanisms. Cell *138*, 628-644.

Chacinska, A., Pfannschmidt, S., Wiedemann, N., Kozjak, V., Sanjuan Szklarz, L.K., Schulze-Specking, A., Truscott, K.N., Guiard, B., Meisinger, C., and Pfanner, N. (2004). Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins. EMBO J *23*, 3735-3746.

Chan, N.C., Likic, V.A., Waller, R.F., Mulhern, T.D., and Lithgow, T. (2006). The C-terminal TPR domain of Tom70 defines a family of mitochondrial protein import receptors found only in animals and fungi. J Mol Biol *358*, 1010-1022.

Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science *243*, 1576-1583.

Chen, W.J., and Douglas, M.G. (1987). The role of protein structure in the mitochondrial import pathway. Unfolding of mitochondrially bound precursors is required for membrane translocation. J Biol Chem *262*, 15605-15609.

Cheng, C.H., Lo, Y.H., Liang, S.S., Ti, S.C., Lin, F.M., Yeh, C.H., Huang, H.Y., and Wang, T.F. (2006). SUMO modifications control assembly of synaptonemal complex and polycomplex in meiosis of Saccharomyces cerevisiae. Genes Dev *20*, 2067-2081.

Claros, M.G., and Vincens, P. (1996). Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem *241*, 779-786.

Connell, P., Ballinger, C.A., Jiang, J., Wu, Y., Thompson, L.J., Hohfeld, J., and Patterson, C. (2001). The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. Nat Cell Biol *3*, 93-96.

Cooper, H.J., Tatham, M.H., Jaffray, E., Heath, J.K., Lam, T.T., Marshall, A.G., and Hay, R.T. (2005). Fourier transform ion cyclotron resonance mass spectrometry for the analysis of small ubiquitin-like modifier (SUMO) modification: identification of lysines in RanBP2 and SUMO targeted for modification during the E3 autoSUMOylation reaction. Anal Chem *77*, 6310-6319.

Cullin, C., Baudin-Baillieu, A., Guillemet, E., and Ozier-Kalogeropoulos, O. (1996). Functional analysis of YCL09C: evidence for a role as the regulatory subunit of acetolactate synthase. Yeast *12*, 1511-1518.

Curran, S.P., Leuenberger, D., Oppliger, W., and Koehler, C.M. (2002). The Tim9p-Tim10p complex binds to the transmembrane domains of the ADP/ATP carrier. EMBO J *21*, 942-953.

Cyr, D.M., Ungermann, C., and Neupert, W. (1995). Analysis of mitochondrial protein import pathway in Saccharomyces cerevisiae with translocation intermediates. Methods Enzymol *260*, 241-252.

Dadke, S., Cotteret, S., Yip, S.C., Jaffer, Z.M., Haj, F., Ivanov, A., Rauscher, F., 3rd, Shuai, K., Ng, T., Neel, B.G., *et al.* (2007). Regulation of protein tyrosine phosphatase 1B by sumoylation. Nat Cell Biol *9*, 80-85.

Dasari, S., and Kolling, R. (2011). Cytosolic localization of acetohydroxyacid synthase Ilv2 and its impact on diacetyl formation during beer fermentation. Appl Environ Microbiol *77*, 727-731.

Demand, J., Alberti, S., Patterson, C., and Hohfeld, J. (2001). Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. Curr Biol *11*, 1569-1577.

Denison, C., Rudner, A.D., Gerber, S.A., Bakalarski, C.E., Moazed, D., and Gygi, S.P. (2005). A proteomic strategy for gaining insights into protein sumoylation in yeast. Mol Cell Proteomics *4*, 246-254.

Deshaies, R.J., and Joazeiro, C.A. (2009). RING domain E3 ubiquitin ligases. Annu Rev Biochem *78*, 399-434.

Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A., and Schekman, R. (1988). A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature *332*, 800-805.

Desterro, J.M., Rodriguez, M.S., and Hay, R.T. (1998). SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. Mol Cell *2*, 233-239.

Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994). A 26 S protease subunit that binds ubiquitin conjugates. J Biol Chem *269*, 7059-7061.

Diamant, S., Ben-Zvi, A.P., Bukau, B., and Goloubinoff, P. (2000). Size-dependent disaggregation of stable protein aggregates by the DnaK chaperone machinery. J Biol Chem *275*, 21107-21113.

Dietzel, C., and Kurjan, J. (1987). Pheromonal regulation and sequence of the Saccharomyces cerevisiae SST2 gene: a model for desensitization to pheromone. Mol Cell Biol *7*, 4169-4177.

Donaghue, C., Bates, H., and Cotterill, S. (2001). Identification and characterisation of the Drosophila homologue of the yeast Uba2 gene. Biochim Biophys Acta *1518*, 210-214.

Eichinger, C.S., and Jentsch, S. (2010). Synaptonemal complex formation and meiotic checkpoint signaling are linked to the lateral element protein Red1. Proc Natl Acad Sci U S A *107*, 11370-11375.

Eilers, M., and Schatz, G. (1986). Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. Nature *322*, 228-232.

Eisele, F., and Wolf, D.H. (2008). Degradation of misfolded protein in the cytoplasm is mediated by the ubiquitin ligase Ubr1. FEBS Lett *582*, 4143-4146.

Eliyahu, E., Pnueli, L., Melamed, D., Scherrer, T., Gerber, A.P., Pines, O., Rapaport, D., and Arava, Y. (2010). Tom20 mediates localization of mRNAs to mitochondria in a translation-dependent manner. Mol Cell Biol *30*, 284-294.

Elsasser, S., Chandler-Militello, D., Muller, B., Hanna, J., and Finley, D. (2004). Rad23 and Rpn10 serve as alternative ubiquitin receptors for the proteasome. J Biol Chem *279*, 26817-26822.

Elsasser, S., and Finley, D. (2005). Delivery of ubiquitinated substrates to protein-unfolding machines. Nat Cell Biol *7*, 742-749.

Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol *300*, 1005-1016.

Endo, T., Mitsui, S., Nakai, M., and Roise, D. (1996). Binding of mitochondrial presequences to yeast cytosolic heat shock protein 70 depends on the amphiphilicity of the presequence. J Biol Chem *271*, 4161-4167.

Endo, T., Mitsui, S., and Roise, D. (1995a). Mitochondrial presequences can induce aggregation of unfolded proteins. FEBS Lett *359*, 93-96.

Endo, T., Nakayama, Y., and Nakai, M. (1995b). Avidin fusion protein as a tool to generate a stable translocation intermediate spanning the mitochondrial membranes. J Biochem *118*, 753-759.

Erales, J., and Coffino, P. (2014). Ubiquitin-independent proteasomal degradation. Biochim Biophys Acta *1843*, 216-221.

Fang, N.N., Chan, G.T., Zhu, M., Comyn, S.A., Persaud, A., Deshaies, R.J., Rotin, D., Gsponer, J., and Mayor, T. (2014). Rsp5/Nedd4 is the main ubiquitin ligase that targets cytosolic misfolded proteins following heat stress. Nat Cell Biol *16*, 1227-1237.

Fang, N.N., Ng, A.H., Measday, V., and Mayor, T. (2011). Hul5 HECT ubiquitin ligase plays a major role in the ubiquitylation and turnover of cytosolic misfolded proteins. Nat Cell Biol *13*, 1344-1352.

Feliciangeli, S., Bendahhou, S., Sandoz, G., Gounon, P., Reichold, M., Warth, R., Lazdunski, M., Barhanin, J., and Lesage, F. (2007). Does sumoylation control K2P1/TWIK1 background K+ channels? Cell *130*, 563-569.

Finley, D., Bartel, B., and Varshavsky, A. (1989). The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. Nature *338*, 394-401.

Finley, D., Ulrich, H.D., Sommer, T., and Kaiser, P. (2012). The ubiquitin-proteasome system of Saccharomyces cerevisiae. Genetics *192*, 319-360.

Flotho, A., and Melchior, F. (2013). Sumoylation: a regulatory protein modification in health and disease. Annu Rev Biochem *82*, 357-385.

Flynn, G.C., Pohl, J., Flocco, M.T., and Rothman, J.E. (1991). Peptide-binding specificity of the molecular chaperone BiP. Nature *353*, 726-730.

Folsch, H., Gaume, B., Brunner, M., Neupert, W., and Stuart, R.A. (1998). C- to N-terminal translocation of preproteins into mitochondria. EMBO J *17*, 6508-6515.

Fourie, A.M., Sambrook, J.F., and Gething, M.J. (1994). Common and divergent peptide binding specificities of hsp70 molecular chaperones. J Biol Chem *269*, 30470-30478.

Fox, T.D. (2012). Mitochondrial protein synthesis, import, and assembly. Genetics *192*, 1203-1234.

Fujiki, M., and Verner, K. (1993). Coupling of cytosolic protein synthesis and mitochondrial protein import in yeast. Evidence for cotranslational import in vivo. J Biol Chem *268*, 1914-1920.

Funakoshi, M., Sasaki, T., Nishimoto, T., and Kobayashi, H. (2002). Budding yeast Dsk2p is a polyubiquitin-binding protein that can interact with the proteasome. Proc Natl Acad Sci U S A *99*, 745-750.

Gakh, O., Cavadini, P., and Isaya, G. (2002). Mitochondrial processing peptidases. Biochim Biophys Acta *1592*, 63-77.

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

Garrido, C., Paul, C., Seigneuric, R., and Kampinga, H.H. (2012). The small heat shock proteins family: the long forgotten chaperones. Int J Biochem Cell Biol *44*, 1588-1592.

Gartner, F., Voos, W., Querol, A., Miller, B.R., Craig, E.A., Cumsky, M.G., and Pfanner, N. (1995). Mitochondrial import of subunit Va of cytochrome c oxidase characterized with yeast mutants. J Biol Chem *270*, 3788-3795.

Geiss-Friedlander, R., and Melchior, F. (2007). Concepts in sumoylation: a decade on. Nat Rev Mol Cell Biol *8*, 947-956.

Ghislain, M., Udvardy, A., and Mann, C. (1993). S. cerevisiae 26S protease mutants arrest cell division in G2/metaphase. Nature *366*, 358-362.

Gietz, R.D., and Sugino, A. (1988). New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene *74*, 527-534.

Giorgino, F., de Robertis, O., Laviola, L., Montrone, C., Perrini, S., McCowen, K.C., and Smith, R.J. (2000). The sentrin-conjugating enzyme mUbc9 interacts with GLUT4 and GLUT1 glucose transporters and regulates transporter levels in skeletal muscle cells. Proc Natl Acad Sci U S A *97*, 1125-1130.

Glaser, S.M., Miller, B.R., and Cumsky, M.G. (1990). Removal of a hydrophobic domain within the mature portion of a mitochondrial inner membrane protein causes its mislocalization to the matrix. Mol Cell Biol *10*, 1873-1881.

Glick, B.S., Brandt, A., Cunningham, K., Muller, S., Hallberg, R.L., and Schatz, G. (1992). Cytochromes c1 and b2 are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism. Cell *69*, 809-822.

Glover, J.R., and Lindquist, S. (1998). Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell *94*, 73-82.

Goldstein, G., Scheid, M., Hammerling, U., Schlesinger, D.H., Niall, H.D., and Boyse, E.A. (1975). Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. Proc Natl Acad Sci U S A *72*, 11-15.

Golebiowski, F., Matic, I., Tatham, M.H., Cole, C., Yin, Y., Nakamura, A., Cox, J., Barton, G.J., Mann, M., and Hay, R.T. (2009). System-wide changes to SUMO modifications in response to heat shock. Sci Signal *2*, ra24.

Goloubinoff, P., Mogk, A., Zvi, A.P., Tomoyasu, T., and Bukau, B. (1999). Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. Proc Natl Acad Sci U S A *96*, 13732-13737.

Gonzalez-Prieto, R., Cuijpers, S.A., Kumar, R., Hendriks, I.A., and Vertegaal, A.C. (2015). c-Myc is targeted to the proteasome for degradation in a SUMOylation-dependent manner, regulated by PIAS1, SENP7 and RNF4. Cell Cycle *14*, 1859-1872.

Grumbt, B., Stroobant, V., Terziyska, N., Israel, L., and Hell, K. (2007). Functional characterization of Mia40p, the central component of the disulfide relay system of the mitochondrial intermembrane space. J Biol Chem *282*, 37461-37470.

Grunwald, M., and Bono, F. (2011). Structure of Importin13-Ubc9 complex: nuclear import and release of a key regulator of sumoylation. EMBO J *30*, 427-438.

Guo, L., Giasson, B.I., Glavis-Bloom, A., Brewer, M.D., Shorter, J., Gitler, A.D., and Yang, X. (2014). A cellular system that degrades misfolded proteins and protects against neurodegeneration. Mol Cell *55*, 15-30.

Habelhah, H., Laine, A., Erdjument-Bromage, H., Tempst, P., Gershwin, M.E., Bowtell, D.D., and Ronai, Z. (2004). Regulation of 2-oxoglutarate (alpha-ketoglutarate) dehydrogenase stability by the RING finger ubiquitin ligase Siah. J Biol Chem *279*, 53782-53788.

Hachiya, N., Alam, R., Sakasegawa, Y., Sakaguchi, M., Mihara, K., and Omura, T. (1993). A mitochondrial import factor purified from rat liver cytosol is an ATP-dependent conformational modulator for precursor proteins. EMBO J *12*, 1579-1586.

Hachiya, N., Komiya, T., Alam, R., Iwahashi, J., Sakaguchi, M., Omura, T., and Mihara, K. (1994). MSF, a novel cytoplasmic chaperone which functions in precursor targeting to mitochondria. EMBO J *13*, 5146-5154.

Hachiya, N., Mihara, K., Suda, K., Horst, M., Schatz, G., and Lithgow, T. (1995). Reconstitution of the initial steps of mitochondrial protein import. Nature *376*, 705-709.

Hallermayer, G., Zimmermann, R., and Neupert, W. (1977). Kinetic studies on the transport of cytoplasmically synthesized proteins into the mitochondria in intact cells of Neurospora crassa. Eur J Biochem *81*, 523-532.

Hannich, J.T., Lewis, A., Kroetz, M.B., Li, S.J., Heide, H., Emili, A., and Hochstrasser, M. (2005). Defining the SUMO-modified proteome by multiple approaches in Saccharomyces cerevisiae. J Biol Chem *280*, 4102-4110.

Harder, Z., Zunino, R., and McBride, H. (2004). Sumo1 conjugates mitochondrial substrates and participates in mitochondrial fission. Curr Biol *14*, 340-345.

Hartl, F.U., Bracher, A., and Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. Nature *475*, 324-332.

Hartl, F.U., Schmidt, B., Wachter, E., Weiss, H., and Neupert, W. (1986). Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome c reductase. Cell *47*, 939-951.

Heck, J.W., Cheung, S.K., and Hampton, R.Y. (2010). Cytoplasmic protein quality control degradation mediated by parallel actions of the E3 ubiquitin ligases Ubr1 and San1. Proc Natl Acad Sci U S A *107*, 1106-1111.

Hecker, C.M., Rabiller, M., Haglund, K., Bayer, P., and Dikic, I. (2006). Specification of SUMO1- and SUMO2-interacting motifs. J Biol Chem *281*, 16117-16127.

Hell, K., Herrmann, J., Pratje, E., Neupert, W., and Stuart, R.A. (1997). Oxa1p mediates the export of the N- and C-termini of pCoxII from the mitochondrial matrix to the intermembrane space. FEBS Lett *418*, 367-370.

Hell, K., Herrmann, J.M., Pratje, E., Neupert, W., and Stuart, R.A. (1998). Oxa1p, an essential component of the N-tail protein export machinery in mitochondria. Proc Natl Acad Sci U S A *95*, 2250-2255.

Hendriks, I.A., D'Souza, R.C., Yang, B., Verlaan-de Vries, M., Mann, M., and Vertegaal, A.C. (2014). Uncovering global SUMOylation signaling networks in a site-specific manner. Nat Struct Mol Biol *21*, 927-936.

Hendriks, I.A., and Vertegaal, A.C. (2016). A comprehensive compilation of SUMO proteomics. Nat Rev Mol Cell Biol *17*, 581-595.

Her, J., Jeong, Y.Y., and Chung, I.K. (2015). PIAS1-mediated sumoylation promotes STUbL-dependent proteasomal degradation of the human telomeric protein TRF2. FEBS Lett *589*, 3277-3286.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu Rev Biochem *67*, 425-479.

Hickey, C.M., Wilson, N.R., and Hochstrasser, M. (2012). Function and regulation of SUMO proteases. Nat Rev Mol Cell Biol *13*, 755-766.

Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G., and Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature *419*, 135-141.

Hoogenraad, N.J., Ward, L.A., and Ryan, M.T. (2002). Import and assembly of proteins into mitochondria of mammalian cells. Biochim Biophys Acta *1592*, 97-105.

Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., and O'Shea, E.K. (2003). Global analysis of protein localization in budding yeast. Nature *425*, 686-691.

Husnjak, K., Elsasser, S., Zhang, N., Chen, X., Randles, L., Shi, Y., Hofmann, K., Walters, K.J., Finley, D., and Dikic, I. (2008). Proteasome subunit Rpn13 is a novel ubiquitin receptor. Nature *453*, 481-488.

Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M.*, et al.* (2000). A ubiquitin-like system mediates protein lipidation. Nature *408*, 488-492.

Janer, A., Werner, A., Takahashi-Fujigasaki, J., Daret, A., Fujigasaki, H., Takada, K., Duyckaerts, C., Brice, A., Dejean, A., and Sittler, A. (2010). SUMOylation attenuates the aggregation propensity and cellular toxicity of the polyglutamine expanded ataxin-7. Hum Mol Genet *19*, 181-195.

Janke, C., Magiera, M.M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E.*, et al.* (2004). A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast *21*, 947-962.

Jentsch, S., and Psakhye, I. (2013). Control of nuclear activities by substrate-selective and protein-group SUMOylation. Annu Rev Genet *47*, 167-186.

Jiang, J., Ballinger, C.A., Wu, Y., Dai, Q., Cyr, D.M., Hohfeld, J., and Patterson, C. (2001). CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. J Biol Chem *276*, 42938-42944.

Jin, L., Williamson, A., Banerjee, S., Philipp, I., and Rape, M. (2008). Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. Cell *133*, 653-665.

Johnson, E.S. (2004). Protein modification by SUMO. Annu Rev Biochem *73*, 355-382.

Johnson, E.S., Ma, P.C., Ota, I.M., and Varshavsky, A. (1995). A proteolytic pathway that recognizes ubiquitin as a degradation signal. J Biol Chem *270*, 17442-17456.

Kagey, M.H., Melhuish, T.A., and Wotton, D. (2003). The polycomb protein Pc2 is a SUMO E3. Cell *113*, 127-137.

Kaiser, S.E., Riley, B.E., Shaler, T.A., Trevino, R.S., Becker, C.H., Schulman, H., and Kopito, R.R. (2011). Protein standard absolute quantification (PSAQ) method for the measurement of cellular ubiquitin pools. Nat Methods *8*, 691-696.

Kaminsky, R., Denison, C., Bening-Abu-Shach, U., Chisholm, A.D., Gygi, S.P., and Broday, L. (2009). SUMO regulates the assembly and function of a cytoplasmic intermediate filament protein in C. elegans. Dev Cell *17*, 724-735.

Kamitani, T., Nguyen, H.P., and Yeh, E.T. (1997). Preferential modification of nuclear proteins by a novel ubiquitin-like molecule. J Biol Chem *272*, 14001-14004.

Kampinga, H.H., and Craig, E.A. (2010). The HSP70 chaperone machinery: J proteins as drivers of functional specificity. Nat Rev Mol Cell Biol *11*, 579-592.

Kanazawa, M., Terada, K., Kato, S., and Mori, M. (1997). HSDJ, a human homolog of DnaJ, is farnesylated and is involved in protein import into mitochondria. J Biochem *121*, 890-895.

Kaplun, L., Tzirkin, R., Bakhrat, A., Shabek, N., Ivantsiv, Y., and Raveh, D. (2005). The DNA damage-inducible UbL-UbA protein Ddi1 participates in Mec1-mediated degradation of Ho endonuclease. Mol Cell Biol *25*, 5355-5362.

Karniely, S., and Pines, O. (2005). Single translation--dual destination: mechanisms of dual protein targeting in eukaryotes. EMBO Rep *6*, 420-425.

Kellems, R.E., Allison, V.F., and Butow, R.A. (1974). Cytoplasmic type 80 S ribosomes associated with yeast mitochondria. II. Evidence for the association of cytoplasmic ribosomes with the outer mitochondrial membrane in situ. J Biol Chem *249*, 3297-3303.

Kellems, R.E., Allison, V.F., and Butow, R.A. (1975). Cytoplasmic type 80S ribosomes associated with yeast mitochondria. IV. Attachment of ribosomes to the outer membrane of isolated mitochondria. J Cell Biol *65*, 1-14.

Kellems, R.E., and Butow, R.A. (1972). Cytoplasmic-type 80 S ribosomes associated with yeast mitochondria. I. Evidence for ribosome binding sites on yeast mitochondria. J Biol Chem *247*, 8043-8050.

Kellems, R.E., and Butow, R.A. (1974). Cytoplasmic type 80 S ribosomes associated with yeast mitochondria. 3. Changes in the amount of bound ribosomes in response to changes in metabolic state. J Biol Chem *249*, 3304-3310.

Kerscher, O. (2007). SUMO junction-what's your function? New insights through SUMO-interacting motifs. EMBO Rep *8*, 550-555.

Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu Rev Cell Dev Biol *22*, 159-180.

Khaminets, A., Behl, C., and Dikic, I. (2016). Ubiquitin-Dependent And Independent Signals In Selective Autophagy. Trends Cell Biol *26*, 6-16.

Kim, W., Bennett, E.J., Huttlin, E.L., Guo, A., Li, J., Possemato, A., Sowa, M.E., Rad, R., Rush, J., Comb, M.J.*, et al.* (2011). Systematic and quantitative assessment of the ubiquitin-modified proteome. Mol Cell *44*, 325-340.

Kim, Y.E., Hipp, M.S., Bracher, A., Hayer-Hartl, M., and Hartl, F.U. (2013). Molecular chaperone functions in protein folding and proteostasis. Annu Rev Biochem *82*, 323-355.

Kirkin, V., Lamark, T., Sou, Y.S., Bjorkoy, G., Nunn, J.L., Bruun, J.A., Shvets, E., McEwan, D.G., Clausen, T.H., Wild, P.*, et al.* (2009a). A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Mol Cell *33*, 505-516.

Kirkin, V., McEwan, D.G., Novak, I., and Dikic, I. (2009b). A role for ubiquitin in selective autophagy. Mol Cell *34*, 259-269.

Kitakawa, M., Graack, H.R., Grohmann, L., Goldschmidt-Reisin, S., Herfurth, E., Wittmann-Liebold, B., Nishimura, T., and Isono, K. (1997). Identification and characterization of the genes for mitochondrial ribosomal proteins of Saccharomyces cerevisiae. Eur J Biochem *245*, 449-456.

Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K., and Schiebel, E. (1999). Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast *15*, 963-972.

Knox, C., Sass, E., Neupert, W., and Pines, O. (1998). Import into mitochondria, folding and retrograde movement of fumarase in yeast. J Biol Chem *273*, 25587-25593.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell *96*, 635-644.

Kohler, J.B., Tammsalu, T., Jorgensen, M.M., Steen, N., Hay, R.T., and Thon, G. (2015). Targeting of SUMO substrates to a Cdc48-Ufd1-Npl4 segregase and STUbL pathway in fission yeast. Nat Commun *6*, 8827.

Komander, D., Clague, M.J., and Urbe, S. (2009). Breaking the chains: structure and function of the deubiquitinases. Nat Rev Mol Cell Biol *10*, 550-563.

Komander, D., and Rape, M. (2012). The ubiquitin code. Annu Rev Biochem *81*, 203-229.

Komiya, T., Hachiya, N., Sakaguchi, M., Omura, T., and Mihara, K. (1994). Recognition of mitochondria-targeting signals by a cytosolic import stimulation factor, MSF. J Biol Chem *269*, 30893-30897.

Komiya, T., Rospert, S., Schatz, G., and Mihara, K. (1997). Binding of mitochondrial precursor proteins to the cytoplasmic domains of the import receptors Tom70 and Tom20 is determined by cytoplasmic chaperones. EMBO J *16*, 4267-4275.

Komiya, T., Sakaguchi, M., and Mihara, K. (1996). Cytoplasmic chaperones determine the targeting pathway of precursor proteins to mitochondria. EMBO J *15*, 399-407.

Kotaja, N., Karvonen, U., Janne, O.A., and Palvimo, J.J. (2002). PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. Mol Cell Biol *22*, 5222-5234.

Kraft, C., Peter, M., and Hofmann, K. (2010). Selective autophagy: ubiquitin-mediated recognition and beyond. Nat Cell Biol *12*, 836-841.

Krumova, P., Meulmeester, E., Garrido, M., Tirard, M., Hsiao, H.H., Bossis, G., Urlaub, H., Zweckstetter, M., Kugler, S., Melchior, F*., et al.* (2011). Sumoylation inhibits alpha-synuclein aggregation and toxicity. J Cell Biol *194*, 49-60.

Krumova, P., and Weishaupt, J.H. (2013). Sumoylation in neurodegenerative diseases. Cell Mol Life Sci *70*, 2123-2138.

Kubrich, M., Dietmeier, K., and Pfanner, N. (1995). Genetic and biochemical dissection of the mitochondrial protein-import machinery. Curr Genet *27*, 393-403.

Kunkele, K.P., Heins, S., Dembowski, M., Nargang, F.E., Benz, R., Thieffry, M., Walz, J., Lill, R., Nussberger, S., and Neupert, W. (1998). The preprotein translocation channel of the outer membrane of mitochondria. Cell *93*, 1009-1019.

Lain, B., Iriarte, A., Mattingly, J.R., Jr., Moreno, J.I., and Martinez-Carrion, M. (1995). Structural features of the precursor to mitochondrial aspartate aminotransferase responsible for binding to hsp70. J Biol Chem *270*, 24732-24739.

Lee, C.M., Sedman, J., Neupert, W., and Stuart, R.A. (1999). The DNA helicase, Hmi1p, is transported into mitochondria by a C-terminal cleavable targeting signal. J Biol Chem *274*, 20937-20942.

Lee, G.W., Melchior, F., Matunis, M.J., Mahajan, R., Tian, Q., and Anderson, P. (1998). Modification of Ran GTPase-activating protein by the small ubiquitin-related modifier SUMO-1 requires Ubc9, an E2-type ubiquitin-conjugating enzyme homologue. J Biol Chem *273*, 6503-6507.

Leggett, D.S., Hanna, J., Borodovsky, A., Crosas, B., Schmidt, M., Baker, R.T., Walz, T., Ploegh, H., and Finley, D. (2002). Multiple associated proteins regulate proteasome structure and function. Mol Cell *10*, 495-507.

Li, S.J., and Hochstrasser, M. (1999). A new protease required for cell-cycle progression in yeast. Nature *398*, 246-251.

Li, S.J., and Hochstrasser, M. (2000). The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein. Mol Cell Biol *20*, 2367-2377.

Li, Y., Wang, H., Wang, S., Quon, D., Liu, Y.W., and Cordell, B. (2003). Positive and negative regulation of APP amyloidogenesis by sumoylation. Proc Natl Acad Sci U S A *100*, 259-264.

Liebelt, F., and Vertegaal, A.C. (2016). Ubiquitin-dependent and independent roles of SUMO in proteostasis. Am J Physiol Cell Physiol *311*, C284-296.

Lill, R., and Muhlenhoff, U. (2008). Maturation of iron-sulfur proteins in eukaryotes: mechanisms, connected processes, and diseases. Annu Rev Biochem *77*, 669-700.

Lu, K., Psakhye, I., and Jentsch, S. (2014). Autophagic clearance of polyQ proteins mediated by ubiquitin-Atg8 adaptors of the conserved CUET protein family. Cell *158*, 549-563.

Luders, J., Pyrowolakis, G., and Jentsch, S. (2003). The ubiquitin-like protein HUB1 forms SDS-resistant complexes with cellular proteins in the absence of ATP. EMBO Rep *4*, 1169-1174.

Lutstorf, U., and Megnet, R. (1968). Multiple forms of alcohol dehydrogenase in Saccharomyces cerevisiae. I. Physiological control of ADH-2 and properties of ADH-2 and ADH-4. Arch Biochem Biophys *126*, 933-944.

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

Makhnevych, T., Ptak, C., Lusk, C.P., Aitchison, J.D., and Wozniak, R.W. (2007). The role of karyopherins in the regulated sumoylation of septins. J Cell Biol *177*, 39-49.

Makhnevych, T., Sydorskyy, Y., Xin, X., Srikumar, T., Vizeacoumar, F.J., Jeram, S.M., Li, Z., Bahr, S., Andrews, B.J., Boone, C.*, et al.* (2009). Global map of SUMO function revealed by protein-protein interaction and genetic networks. Mol Cell *33*, 124-135.

Margeot, A., Blugeon, C., Sylvestre, J., Vialette, S., Jacq, C., and Corral-Debrinski, M. (2002). In Saccharomyces cerevisiae, ATP2 mRNA sorting to the vicinity of mitochondria is essential for respiratory function. EMBO J *21*, 6893-6904.

Martin, S., Nishimune, A., Mellor, J.R., and Henley, J.M. (2007a). SUMOylation regulates kainate-receptor-mediated synaptic transmission. Nature *447*, 321-325.

Martin, S., Wilkinson, K.A., Nishimune, A., and Henley, J.M. (2007b). Emerging extranuclear roles of protein SUMOylation in neuronal function and dysfunction. Nat Rev Neurosci *8*, 948-959.

Matic, I., van Hagen, M., Schimmel, J., Macek, B., Ogg, S.C., Tatham, M.H., Hay, R.T., Lamond, A.I., Mann, M., and Vertegaal, A.C. (2008). In vivo identification of human small ubiquitin-like modifier polymerization sites by high accuracy mass spectrometry and an in vitro to in vivo strategy. Mol Cell Proteomics *7*, 132-144.

Matouschek, A., Pfanner, N., and Voos, W. (2000). Protein unfolding by mitochondria. The Hsp70 import motor. EMBO Rep *1*, 404-410.

Matsumoto, M.L., Wickliffe, K.E., Dong, K.C., Yu, C., Bosanac, I., Bustos, D., Phu, L., Kirkpatrick, D.S., Hymowitz, S.G., Rape, M.*, et al.* (2010). K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody. Mol Cell *39*, 477-484.

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

Mayer, M.P., and Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci *62*, 670-684.

McCarty, J.S., Rudiger, S., Schonfeld, H.J., Schneider-Mergener, J., Nakahigashi, K., Yura, T., and Bukau, B. (1996). Regulatory region C of the E. coli heat shock transcription factor, sigma32, constitutes a DnaK binding site and is conserved among eubacteria. J Mol Biol *256*, 829-837.

Meacham, G.C., Patterson, C., Zhang, W., Younger, J.M., and Cyr, D.M. (2001). The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. Nat Cell Biol *3*, 100-105.

Melchior, F., Schergaut, M., and Pichler, A. (2003). SUMO: ligases, isopeptidases and nuclear pores. Trends Biochem Sci *28*, 612-618.

Mesecke, N., Terziyska, N., Kozany, C., Baumann, F., Neupert, W., Hell, K., and Herrmann, J.M. (2005). A disulfide relay system in the intermembrane space of mitochondria that mediates protein import. Cell *121*, 1059-1069.

Metzger, M.B., Maurer, M.J., Dancy, B.M., and Michaelis, S. (2008). Degradation of a cytosolic protein requires endoplasmic reticulum-associated degradation machinery. J Biol Chem *283*, 32302-32316.

Mihara, K., and Omura, T. (1996a). Cytoplasmic chaperones in precursor targeting to mitochondria: the role of MSF and hsp 70. Trends Cell Biol *6*, 104-108.

Mihara, K., and Omura, T. (1996b). Cytosolic factors in mitochondrial protein import. Experientia *52*, 1063-1068.

Milenkovic, D., Gabriel, K., Guiard, B., Schulze-Specking, A., Pfanner, N., and Chacinska, A. (2007). Biogenesis of the essential Tim9-Tim10 chaperone complex of mitochondria: site-specific recognition of cysteine residues by the intermembrane space receptor Mia40. J Biol Chem *282*, 22472-22480.

Milenkovic, D., Ramming, T., Muller, J.M., Wenz, L.S., Gebert, N., Schulze-Specking, A., Stojanovski, D., Rospert, S., and Chacinska, A. (2009). Identification of the signal directing Tim9 and Tim10 into the intermembrane space of mitochondria. Mol Biol Cell *20*, 2530-2539.

Miller, B.R., and Cumsky, M.G. (1993). Intramitochondrial sorting of the precursor to yeast cytochrome c oxidase subunit Va. J Cell Biol *121*, 1021-1029.

Mingot, J.M., Kostka, S., Kraft, R., Hartmann, E., and Gorlich, D. (2001). Importin 13: a novel mediator of nuclear import and export. EMBO J *20*, 3685-3694.

Mishra, S.K., Ammon, T., Popowicz, G.M., Krajewski, M., Nagel, R.J., Ares, M., Jr., Holak, T.A., and Jentsch, S. (2011). Role of the ubiquitin-like protein Hub1 in splice-site usage and alternative splicing. Nature *474*, 173-178.

Miura, S., Mori, M., and Tatibana, M. (1983). Transport of ornithine carbamoyltransferase precursor into mitochondria. Stimulation by potassium ion, magnesium ion, and a reticulocyte cytosolic protein(s). J Biol Chem *258*, 6671-6674.

Miyauchi, Y., Yogosawa, S., Honda, R., Nishida, T., and Yasuda, H. (2002). Sumoylation of Mdm2 by protein inhibitor of activated STAT (PIAS) and RanBP2 enzymes. J Biol Chem *277*, 50131-50136.

Mizushima, N., Yoshimori, T., and Ohsumi, Y. (2011). The role of Atg proteins in autophagosome formation. Annu Rev Cell Dev Biol *27*, 107-132.

Model, K., Meisinger, C., and Kuhlbrandt, W. (2008). Cryo-electron microscopy structure of a yeast mitochondrial preprotein translocase. J Mol Biol *383*, 1049-1057.

Model, K., Prinz, T., Ruiz, T., Radermacher, M., Krimmer, T., Kuhlbrandt, W., Pfanner, N., and Meisinger, C. (2002). Protein translocase of the outer mitochondrial membrane: role of import receptors in the structural organization of the TOM complex. J Mol Biol *316*, 657-666.

Moldovan, G.L., Pfander, B., and Jentsch, S. (2006). PCNA controls establishment of sister chromatid cohesion during S phase. Mol Cell *23*, 723-732.

Mori, M., and Terada, K. (1998). Mitochondrial protein import in animals. Biochim Biophys Acta *1403*, 12-27.

Morita, Y., Kanei-Ishii, C., Nomura, T., and Ishii, S. (2005). TRAF7 sequesters c-Myb to the cytoplasm by stimulating its sumoylation. Mol Biol Cell *16*, 5433-5444.

Moutty, M.C., Sakin, V., and Melchior, F. (2011). Importin alpha/beta mediates nuclear import of individual SUMO E1 subunits and of the holo-enzyme. Mol Biol Cell *22*, 652-660.

Mukherjee, S., Thomas, M., Dadgar, N., Lieberman, A.P., and Iniguez-Lluhi, J.A. (2009). Small ubiquitin-like modifier (SUMO) modification of the androgen receptor attenuates polyglutamine-mediated aggregation. J Biol Chem *284*, 21296-21306.

Muller, J.M., Milenkovic, D., Guiard, B., Pfanner, N., and Chacinska, A. (2008). Precursor oxidation by Mia40 and Erv1 promotes vectorial transport of proteins into the mitochondrial intermembrane space. Mol Biol Cell *19*, 226-236.

Mumberg, D., Muller, R., and Funk, M. (1994). Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res *22*, 5767-5768.

Mumberg, D., Muller, R., and Funk, M. (1995). Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene *156*, 119-122.

Murakami, H., Pain, D., and Blobel, G. (1988). 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. J Cell Biol *107*, 2051-2057.

Murakami, K., and Mori, M. (1990). Purified presequence binding factor (PBF) forms an import-competent complex with a purified mitochondrial precursor protein. EMBO J *9*, 3201-3208.

Murakami, K., Tanase, S., Morino, Y., and Mori, M. (1992). Presequence binding factor-dependent and -independent import of proteins into mitochondria. J Biol Chem *267*, 13119-13122.

Murata, S., Minami, Y., Minami, M., Chiba, T., and Tanaka, K. (2001). CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep *2*, 1133-1138.

Naoe, M., Ohwa, Y., Ishikawa, D., Ohshima, C., Nishikawa, S., Yamamoto, H., and Endo, T. (2004). Identification of Tim40 that mediates protein sorting to the mitochondrial intermembrane space. J Biol Chem *279*, 47815-47821.

Needham, P.G., Patel, H.J., Chiosis, G., Thibodeau, P.H., and Brodsky, J.L. (2015). Mutations in the Yeast Hsp70, Ssa1, at P417 Alter ATP Cycling, Interdomain Coupling, and Specific Chaperone Functions. J Mol Biol *427*, 2948-2965.

Neupert, W. (1997). Protein import into mitochondria. Annu Rev Biochem *66*, 863-917.

Neupert, W., and Herrmann, J.M. (2007). Translocation of proteins into mitochondria. Annu Rev Biochem *76*, 723-749.

Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng *10*, 1-6.

Nillegoda, N.B., Kirstein, J., Szlachcic, A., Berynskyy, M., Stank, A., Stengel, F., Arnsburg, K., Gao, X., Scior, A., Aebersold, R.*, et al.* (2015). Crucial HSP70 co-chaperone complex unlocks metazoan protein disaggregation. Nature *524*, 247-251.

Nillegoda, N.B., Theodoraki, M.A., Mandal, A.K., Mayo, K.J., Ren, H.Y., Sultana, R., Wu, K., Johnson, J., Cyr, D.M., and Caplan, A.J. (2010). Ubr1 and Ubr2 function in a quality control pathway for degradation of unfolded cytosolic proteins. Mol Biol Cell *21*, 2102-2116.

Niskanen, E.A., Malinen, M., Sutinen, P., Toropainen, S., Paakinaho, V., Vihervaara, A., Joutsen, J., Kaikkonen, M.U., Sistonen, L., and Palvimo, J.J. (2015). Global SUMOylation on active chromatin is an acute heat stress response restricting transcription. Genome Biol *16*, 153.

Novoselova, T.V., Rose, R.S., Marks, H.M., and Sullivan, J.A. (2013). SUMOylation regulates the homologous to E6-AP carboxyl terminus (HECT) ubiquitin ligase Rsp5p. J Biol Chem *288*, 10308-10317.

O'Rourke, J.G., Gareau, J.R., Ochaba, J., Song, W., Rasko, T., Reverter, D., Lee, J., Monteys, A.M., Pallos, J., Mee, L.*, et al.* (2013). SUMO-2 and PIAS1 modulate insoluble mutant huntingtin protein accumulation. Cell Rep *4*, 362-375.

Ohta, S., and Schatz, G. (1984). A purified precursor polypeptide requires a cytosolic protein fraction for import into mitochondria. EMBO J *3*, 651-657.

Ono, H., and Tuboi, S. (1988). The cytosolic factor required for import of precursors of mitochondrial proteins into mitochondria. J Biol Chem *263*, 3188-3193.

Ono, H., and Tuboi, S. (1990a). Presence of the cytosolic factor stimulating the import of precursor of mitochondrial proteins in rabbit reticulocytes and rat liver cells. Arch Biochem Biophys *277*, 368-373.

Ono, H., and Tuboi, S. (1990b). Purification and identification of a cytosolic factor required for import of precursors of mitochondrial proteins into mitochondria. Arch Biochem Biophysics *280*, 299-304.

Owerbach, D., McKay, E.M., Yeh, E.T., Gabbay, K.H., and Bohren, K.M. (2005). A proline-90 residue unique to SUMO-4 prevents maturation and sumoylation. Biochem Biophys Res Commun *337*, 517-520.

Ozkaynak, E., Finley, D., Solomon, M.J., and Varshavsky, A. (1987). The yeast ubiquitin genes: a family of natural gene fusions. EMBO J *6*, 1429-1439.

Pagliarini, D.J., Calvo, S.E., Chang, B., Sheth, S.A., Vafai, S.B., Ong, S.E., Walford, G.A., Sugiana, C., Boneh, A., Chen, W.K.*, et al.* (2008). A mitochondrial protein compendium elucidates complex I disease biology. Cell *134*, 112-123.

Pang, S.S., and Duggleby, R.G. (1999). Expression, purification, characterization, and reconstitution of the large and small subunits of yeast acetohydroxyacid synthase. Biochemistry *38*, 5222-5231.

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem *282*, 24131-24145.

Panse, V.G., Hardeland, U., Werner, T., Kuster, B., and Hurt, E. (2004). A proteome-wide approach identifies sumoylated substrate proteins in yeast. J Biol Chem *279*, 41346-41351.

Papouli, E., Chen, S., Davies, A.A., Huttner, D., Krejci, L., Sung, P., and Ulrich, H.D. (2005). Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p. Mol Cell *19*, 123-133.

Park, S.H., Bolender, N., Eisele, F., Kostova, Z., Takeuchi, J., Coffino, P., and Wolf, D.H. (2007). The cytoplasmic Hsp70 chaperone machinery subjects misfolded and endoplasmic reticulum import-incompetent proteins to degradation via the ubiquitin-proteasome system. Mol Biol Cell *18*, 153-165.

Park, S.H., Kukushkin, Y., Gupta, R., Chen, T., Konagai, A., Hipp, M.S., Hayer-Hartl, M., and Hartl, F.U. (2013). PolyQ proteins interfere with nuclear degradation of cytosolic proteins by sequestering the Sis1p chaperone. Cell *154*, 134-145.

Parker, J.L., and Ulrich, H.D. (2012). A SUMO-interacting motif activates budding yeast ubiquitin ligase Rad18 towards SUMO-modified PCNA. Nucleic Acids Res *40*, 11380-11388.

Paschen, S.A., Waizenegger, T., Stan, T., Preuss, M., Cyrklaff, M., Hell, K., Rapaport, D., and Neupert, W. (2003). Evolutionary conservation of biogenesis of beta-barrel membrane proteins. Nature *426*, 862-866.

Pearce, D.A., and Sherman, F. (1997). Differential ubiquitin-dependent degradation of the yeast apo-cytochrome c isozymes. J Biol Chem *272*, 31829-31836.

Pedrioli, P.G., Raught, B., Zhang, X.D., Rogers, R., Aitchison, J., Matunis, M., and Aebersold, R. (2006). Automated identification of SUMOylation sites using mass spectrometry and SUMmOn pattern recognition software. Nat Methods *3*, 533-539.

Peng, J., Schwartz, D., Elias, J.E., Thoreen, C.C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., and Gygi, S.P. (2003). A proteomics approach to understanding protein ubiquitination. Nat Biotechnol *21*, 921-926.

Perocchi, F., Jensen, L.J., Gagneur, J., Ahting, U., von Mering, C., Bork, P., Prokisch, H., and Steinmetz, L.M. (2006). Assessing systems properties of yeast mitochondria through an interaction map of the organelle. PLoS Genet *2*, e170.

Petrucelli, L., Dickson, D., Kehoe, K., Taylor, J., Snyder, H., Grover, A., De Lucia, M., McGowan, E., Lewis, J., Prihar, G.*, et al.* (2004). CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation. Hum Mol Genet *13*, 703-714.

Pfander, B., Moldovan, G.L., Sacher, M., Hoege, C., and Jentsch, S. (2005). SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. Nature *436*, 428-433.

Pfanner, N., and Neupert, W. (1987). Distinct steps in the import of ADP/ATP carrier into mitochondria. J Biol Chem *262*, 7528-7536.

Pfanner, N., and Neupert, W. (1990). The mitochondrial protein import apparatus. Annu Rev Biochem *59*, 331-353.

Pichler, A., Gast, A., Seeler, J.S., Dejean, A., and Melchior, F. (2002). The nucleoporin RanBP2 has SUMO1 E3 ligase activity. Cell *108*, 109-120.

Pichler, A., Knipscheer, P., Oberhofer, E., van Dijk, W.J., Korner, R., Olsen, J.V., Jentsch, S., Melchior, F., and Sixma, T.K. (2005). SUMO modification of the ubiquitin-conjugating enzyme E2-25K. Nat Struct Mol Biol *12*, 264-269.

Potts, P.R., and Yu, H. (2005). Human MMS21/NSE2 is a SUMO ligase required for DNA repair. Mol Cell Biol *25*, 7021-7032.

Praefcke, G.J., Hofmann, K., and Dohmen, R.J. (2012). SUMO playing tag with ubiquitin. Trends Biochem Sci *37*, 23-31.

Prasad, R., Kawaguchi, S., and Ng, D.T. (2010). A nucleus-based quality control mechanism for cytosolic proteins. Mol Biol Cell *21*, 2117-2127.

Psakhye, I., and Jentsch, S. (2012). Protein group modification and synergy in the SUMO pathway as exemplified in DNA repair. Cell *151*, 807-820.

Psakhye, I., and Jentsch, S. (2016). Identification of Substrates of Protein-Group SUMOylation. Methods Mol Biol *1475*, 219-231.

Rajan, S., Plant, L.D., Rabin, M.L., Butler, M.H., and Goldstein, S.A. (2005). Sumoylation silences the plasma membrane leak K+ channel K2P1. Cell *121*, 37-47.

Ramage, L., Junne, T., Hahne, K., Lithgow, T., and Schatz, G. (1993). Functional cooperation of mitochondrial protein import receptors in yeast. EMBO J *12*, 4115-4123.

Rampelt, H., Kirstein-Miles, J., Nillegoda, N.B., Chi, K., Scholz, S.R., Morimoto, R.I., and Bukau, B. (2012). Metazoan Hsp70 machines use Hsp110 to power protein disaggregation. EMBO J *31*, 4221-4235.

Randall, S.K., and Shore, G.C. (1989). Import of a mutant mitochondrial precursor fails to respond to stimulation by a cytosolic factor. FEBS Lett *250*, 561-564.

Rao, H., and Sastry, A. (2002). Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the ubiquitin-associated domain proteins Dsk2 and Rad23. J Biol Chem *277*, 11691-11695.

Rassow, J., Guiard, B., Wienhues, U., Herzog, V., Hartl, F.U., and Neupert, W. (1989). Translocation arrest by reversible folding of a precursor protein imported into mitochondria. A means to quantitate translocation contact sites. J Cell Biol *109*, 1421-1428.

Rassow, J., Hartl, F.U., Guiard, B., Pfanner, N., and Neupert, W. (1990). Polypeptides traverse the mitochondrial envelope in an extended state. FEBS Lett *275*, 190-194.

Ravid, T., Kreft, S.G., and Hochstrasser, M. (2006). Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. EMBO J *25*, 533-543.

Reid, G.A., and Schatz, G. (1982). Import of proteins into mitochondria. Extramitochondrial pools and post-translational import of mitochondrial protein precursors in vivo. J Biol Chem *257*, 13062-13067.

Renatus, M., Parrado, S.G., D'Arcy, A., Eidhoff, U., Gerhartz, B., Hassiepen, U., Pierrat, B., Riedl, R., Vinzenz, D., Worpenberg, S.*, et al.* (2006). Structural basis of ubiquitin recognition by the deubiquitinating protease USP2. Structure *14*, 1293-1302.

Rodriguez, M.S., Dargemont, C., and Hay, R.T. (2001). SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. J Biol Chem *276*, 12654-12659.

Rogov, V., Dotsch, V., Johansen, T., and Kirkin, V. (2014). Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. Mol Cell *53*, 167-178.

Rojas-Fernandez, A., Plechanovova, A., Hattersley, N., Jaffray, E., Tatham, M.H., and Hay, R.T. (2014). SUMO chain-induced dimerization activates RNF4. Mol Cell *53*, 880-892.

Rojo, E.E., Guiard, B., Neupert, W., and Stuart, R.A. (1998). Sorting of D-lactate dehydrogenase to the inner membrane of mitochondria. Analysis of topogenic signal and energetic requirements. J Biol Chem *273*, 8040-8047.

Roscic, A., Moller, A., Calzado, M.A., Renner, F., Wimmer, V.C., Gresko, E., Ludi, K.S., and Schmitz, M.L. (2006). Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2. Mol Cell *24*, 77-89.

Rudiger, S., Germeroth, L., Schneider-Mergener, J., and Bukau, B. (1997). Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. EMBO J *16*, 1501-1507.

Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F., and Grosschedl, R. (2001). PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. Genes Dev *15*, 3088-3103.

Sacher, M., Pfander, B., Hoege, C., and Jentsch, S. (2006). Control of Rad52 recombination activity by double-strand break-induced SUMO modification. Nat Cell Biol *8*, 1284-1290.

Saeki, Y., Kudo, T., Sone, T., Kikuchi, Y., Yokosawa, H., Toh-e, A., and Tanaka, K. (2009). Lysine 63-linked polyubiquitin chain may serve as a targeting signal for the 26S proteasome. EMBO J *28*, 359-371.

Saitoh, H., and Hinchey, J. (2000). Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. J Biol Chem *275*, 6252-6258.

Saitoh, T., Igura, M., Obita, T., Ose, T., Kojima, R., Maenaka, K., Endo, T., and Kohda, D. (2007). Tom20 recognizes mitochondrial presequences through dynamic equilibrium among multiple bound states. EMBO J *26*, 4777-4787.

Sass, E., Blachinsky, E., Karniely, S., and Pines, O. (2001). Mitochondrial and cytosolic isoforms of yeast fumarase are derivatives of a single translation product and have identical amino termini. J Biol Chem *276*, 46111-46117.

Schatz, G., and Dobberstein, B. (1996). Common principles of protein translocation across membranes. Science *271*, 1519-1526.

Scheffner, M., Nuber, U., and Huibregtse, J.M. (1995). Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature *373*, 81-83.

Schimmel, J., Larsen, K.M., Matic, I., van Hagen, M., Cox, J., Mann, M., Andersen, J.S., and Vertegaal, A.C. (2008). The ubiquitin-proteasome system is a key component of the SUMO-2/3 cycle. Mol Cell Proteomics *7*, 2107-2122.

Schleyer, M., and Neupert, W. (1985). Transport of proteins into mitochondria: translocational intermediates spanning contact sites between outer and inner membranes. Cell *43*, 339-350.

Schulz, S., Chachami, G., Kozaczkiewicz, L., Winter, U., Stankovic-Valentin, N., Haas, P., Hofmann, K., Urlaub, H., Ovaa, H., Wittbrodt, J.*, et al.* (2012). Ubiquitin-specific protease-like 1 (USPL1) is a SUMO isopeptidase with essential, non-catalytic functions. EMBO Rep *13*, 930-938.

Schwaiger, M., Herzog, V., and Neupert, W. (1987). Characterization of translocation contact sites involved in the import of mitochondrial proteins. J Cell Biol *105*, 235-246.

Schwartz, M.P., Huang, S., and Matouschek, A. (1999). The structure of precursor proteins during import into mitochondria. J Biol Chem *274*, 12759-12764.

Schwartz, M.P., and Matouschek, A. (1999). The dimensions of the protein import channels in the outer and inner mitochondrial membranes. Proc Natl Acad Sci U S A *96*, 13086-13090.

Seeler, J.S., and Dejean, A. (2003). Nuclear and unclear functions of SUMO. Nat Rev Mol Cell Biol *4*, 690-699.

Seifert, A., Schofield, P., Barton, G.J., and Hay, R.T. (2015). Proteotoxic stress reprograms the chromatin landscape of SUMO modification. Sci Signal *8*, rs7.

Shalizi, A., Gaudilliere, B., Yuan, Z., Stegmuller, J., Shirogane, T., Ge, Q., Tan, Y., Schulman, B., Harper, J.W., and Bonni, A. (2006). A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. Science *311*, 1012-1017.

Sheffield, W.P., Nguyen, M., and Shore, G.C. (1986). Expression in Escherichia coli of functional precursor to the rat liver mitochondrial enzyme, ornithine carbamyl transferase. Precursor import and processing in vitro. Biochem Biophys Res Commun *134*, 21-28.

Sheffield, W.P., Shore, G.C., and Randall, S.K. (1990). Mitochondrial precursor protein. Effects of 70-kilodalton heat shock protein on polypeptide folding, aggregation, and import competence. J Biol Chem *265*, 11069-11076.

Shimizu, Y., Okuda-Shimizu, Y., and Hendershot, L.M. (2010). Ubiquitylation of an ERAD substrate occurs on multiple types of amino acids. Mol Cell *40*, 917-926.

Shin, E.J., Shin, H.M., Nam, E., Kim, W.S., Kim, J.H., Oh, B.H., and Yun, Y. (2012). DeSUMOylating isopeptidase: a second class of SUMO protease. EMBO Rep *13*, 339-346.

Sickmann, A., Reinders, J., Wagner, Y., Joppich, C., Zahedi, R., Meyer, H.E., Schonfisch, B., Perschil, I., Chacinska, A., Guiard, B.*, et al.* (2003). The proteome of Saccharomyces cerevisiae mitochondria. Proc Natl Acad Sci U S A *100*, 13207-13212.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics *122*, 19-27.

Sollner, T., Griffiths, G., Pfaller, R., Pfanner, N., and Neupert, W. (1989). MOM19, an import receptor for mitochondrial precursor proteins. Cell *59*, 1061-1070.

Sollner, T., Pfaller, R., Griffiths, G., Pfanner, N., and Neupert, W. (1990). A mitochondrial import receptor for the ADP/ATP carrier. Cell *62*, 107-115.

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

Steffan, J.S., Agrawal, N., Pallos, J., Rockabrand, E., Trotman, L.C., Slepko, N., Illes, K., Lukacsovich, T., Zhu, Y.Z., Cattaneo, E.*, et al.* (2004). SUMO modification of Huntingtin and Huntington's disease pathology. Science *304*, 100-104.

Stehmeier, P., and Muller, S. (2009). Phospho-regulated SUMO interaction modules connect the SUMO system to CK2 signaling. Mol Cell *33*, 400-409.

Stein, I., Peleg, Y., Even-Ram, S., and Pines, O. (1994). The single translation product of the FUM1 gene (fumarase) is processed in mitochondria before being distributed between the cytosol and mitochondria in Saccharomyces cerevisiae. Mol Cell Biol *14*, 4770-4778.

Steinacher, R., and Schar, P. (2005). Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation. Curr Biol *15*, 616-623.

Sternsdorf, T., Jensen, K., Reich, B., and Will, H. (1999). The nuclear dot protein sp100, characterization of domains necessary for dimerization, subcellular localization, and modification by small ubiquitin-like modifiers. J Biol Chem *274*, 12555-12566.

Stojanovski, D., Guiard, B., Kozjak-Pavlovic, V., Pfanner, N., and Meisinger, C. (2007). Alternative function for the mitochondrial SAM complex in biogenesis of alpha-helical TOM proteins. J Cell Biol *179*, 881-893.

Sugar, J., Schimpfessel, L., Rozen, E., and Crokaert, R. (1970). The mitochondrial alcohol dehydrogenase of the yeast "Saccharomyces cerevisiae". Arch Int Physiol Biochim *78*, 1009-1010.

Suissa, M., and Schatz, G. (1982). Import of proteins into mitochondria. Translatable mRNAs for imported mitochondrial proteins are present in free as well as mitochondria-bound cytoplasmic polysomes. J Biol Chem *257*, 13048-13055.

Swanson, R., Locher, M., and Hochstrasser, M. (2001). A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. Genes Dev *15*, 2660-2674.

Taipale, M., Jarosz, D.F., and Lindquist, S. (2010). HSP90 at the hub of protein homeostasis: emerging mechanistic insights. Nat Rev Mol Cell Biol *11*, 515-528.

Takahashi, Y., Iwase, M., Strunnikov, A.V., and Kikuchi, Y. (2008). Cytoplasmic sumoylation by PIAS-type Siz1-SUMO ligase. Cell Cycle *7*, 1738-1744.

Takahashi, Y., and Kikuchi, Y. (2005). Yeast PIAS-type Ull1/Siz1 is composed of SUMO ligase and regulatory domains. J Biol Chem *280*, 35822-35828.

Takahashi, Y., Mizoi, J., Toh, E.A., and Kikuchi, Y. (2000). Yeast Ulp1, an Smt3-specific protease, associates with nucleoporins. J Biochem *128*, 723-725.

Tang, Z., El Far, O., Betz, H., and Scheschonka, A. (2005). Pias1 interaction and sumoylation of metabotropic glutamate receptor 8. J Biol Chem *280*, 38153-38159.

Tatham, M.H., Geoffroy, M.C., Shen, L., Plechanovova, A., Hattersley, N., Jaffray, E.G., Palvimo, J.J., and Hay, R.T. (2008). RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. Nat Cell Biol *10*, 538-546.

Tatham, M.H., Jaffray, E., Vaughan, O.A., Desterro, J.M., Botting, C.H., Naismith, J.H., and Hay, R.T. (2001). Polymeric chains of SUMO-2 and SUMO-3 are

conjugated to protein substrates by SAE1/SAE2 and Ubc9. J Biol Chem *276*, 35368-35374.

Tatham, M.H., Matic, I., Mann, M., and Hay, R.T. (2011). Comparative proteomic analysis identifies a role for SUMO in protein quality control. Sci Signal *4*, rs4.

Terada, K., and Mori, M. (2000). Human DnaJ homologs dj2 and dj3, and bag-1 are positive cochaperones of hsc70. J Biol Chem *275*, 24728-24734.

Terada, K., Ohtsuka, K., Imamoto, N., Yoneda, Y., and Mori, M. (1995). Role of heat shock cognate 70 protein in import of ornithine transcarbamylase precursor into mammalian mitochondria. Mol Cell Biol *15*, 3708-3713.

Terziyska, N., Grumbt, B., Kozany, C., and Hell, K. (2009). Structural and functional roles of the conserved cysteine residues of the redox-regulated import receptor Mia40 in the intermembrane space of mitochondria. J Biol Chem *284*, 1353-1363.

Truscott, K.N., Kovermann, P., Geissler, A., Merlin, A., Meijer, M., Driessen, A.J., Rassow, J., Pfanner, N., and Wagner, R. (2001). A presequence- and voltage-sensitive channel of the mitochondrial preprotein translocase formed by Tim23. Nat Struct Biol *8*, 1074-1082.

Ungermann, C., Guiard, B., Neupert, W., and Cyr, D.M. (1996). The delta psi- and Hsp70/MIM44-dependent reaction cycle driving early steps of protein import into mitochondria. EMBO J *15*, 735-744.

Uzunova, K., Gottsche, K., Miteva, M., Weisshaar, S.R., Glanemann, C., Schnellhardt, M., Niessen, M., Scheel, H., Hofmann, K., Johnson, E.S.*, et al.* (2007). Ubiquitin-dependent proteolytic control of SUMO conjugates. J Biol Chem *282*, 34167-34175.

van der Veen, A.G., and Ploegh, H.L. (2012). Ubiquitin-like proteins. Annu Rev Biochem *81*, 323-357.

van Nocker, S., Sadis, S., Rubin, D.M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D., and Vierstra, R.D. (1996). The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in Saccharomyces cerevisiae and plays a nonessential, substrate-specific role in protein turnover. Mol Cell Biol *16*, 6020-6028.

van Wilpe, S., Ryan, M.T., Hill, K., Maarse, A.C., Meisinger, C., Brix, J., Dekker, P.J., Moczko, M., Wagner, R., Meijer, M.*, et al.* (1999). Tom22 is a multifunctional organizer of the mitochondrial preprotein translocase. Nature *401*, 485-489.

Varshavsky, A. (1997). The ubiquitin system. Trends Biochem Sci *22*, 383-387.

Vasiljev, A., Ahting, U., Nargang, F.E., Go, N.E., Habib, S.J., Kozany, C., Panneels, V., Sinning, I., Prokisch, H., Neupert, W.*, et al.* (2004). Reconstituted TOM core complex and Tim9/Tim10 complex of mitochondria are sufficient for translocation of the ADP/ATP carrier across membranes. Mol Biol Cell *15*, 1445-1458.

Verhoef, L.G., Lindsten, K., Masucci, M.G., and Dantuma, N.P. (2002). Aggregate formation inhibits proteasomal degradation of polyglutamine proteins. Hum Mol Genet *11*, 2689-2700.

Verma, R., Aravind, L., Oania, R., McDonald, W.H., Yates, J.R., 3rd, Koonin, E.V., and Deshaies, R.J. (2002). Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. Science *298*, 611-615.

Vestweber, D., and Schatz, G. (1988). A chimeric mitochondrial precursor protein with internal disulfide bridges blocks import of authentic precursors into mitochondria and allows quantitation of import sites. J Cell Biol *107*, 2037-2043.

Wang, C., and Youle, R.J. (2009). The role of mitochondria in apoptosis*. Annu Rev Genet *43*, 95-118.

Wang, Z., and Prelich, G. (2009). Quality control of a transcriptional regulator by SUMO-targeted degradation. Mol Cell Biol *29*, 1694-1706.

Weinberg, S.E., Sena, L.A., and Chandel, N.S. (2015). Mitochondria in the regulation of innate and adaptive immunity. Immunity *42*, 406-417.

Wiedemann, N., Kozjak, V., Chacinska, A., Schonfisch, B., Rospert, S., Ryan, M.T., Pfanner, N., and Meisinger, C. (2003). Machinery for protein sorting and assembly in the mitochondrial outer membrane. Nature *424*, 565-571.

Wienhues, U., Becker, K., Schleyer, M., Guiard, B., Tropschug, M., Horwich, A.L., Pfanner, N., and Neupert, W. (1991). Protein folding causes an arrest of preprotein translocation into mitochondria in vivo. J Cell Biol *115*, 1601-1609.

Wilkinson, K.A., Nishimune, A., and Henley, J.M. (2008). Analysis of SUMO-1 modification of neuronal proteins containing consensus SUMOylation motifs. Neurosci Lett *436*, 239-244.

Williams, C.C., Jan, C.H., and Weissman, J.S. (2014). Targeting and plasticity of mitochondrial proteins revealed by proximity-specific ribosome profiling. Science *346*, 748-751.

Williamson, A., Wickliffe, K.E., Mellone, B.G., Song, L., Karpen, G.H., and Rape, M. (2009). Identification of a physiological E2 module for the human anaphase-promoting complex. Proc Natl Acad Sci U S A *106*, 18213-18218.

Wohlschlegel, J.A., Johnson, E.S., Reed, S.I., and Yates, J.R., 3rd (2004). Global analysis of protein sumoylation in Saccharomyces cerevisiae. J Biol Chem *279*, 45662-45668.

Wrobel, L., Topf, U., Bragoszewski, P., Wiese, S., Sztolsztener, M.E., Oeljeklaus, S., Varabyova, A., Lirski, M., Chroscicki, P., Mroczek, S.*, et al.* (2015). Mistargeted mitochondrial proteins activate a proteostatic response in the cytosol. Nature *524*, 485-488.

Wu, Y., and Sha, B. (2006). Crystal structure of yeast mitochondrial outer membrane translocon member Tom70p. Nat Struct Mol Biol *13*, 589-593.

Wykoff, D.D., and O'Shea, E.K. (2005). Identification of sumoylated proteins by systematic immunoprecipitation of the budding yeast proteome. Mol Cell Proteomics *4*, 73-83.

Xu, P., Duong, D.M., Seyfried, N.T., Cheng, D., Xie, Y., Robert, J., Rush, J., Hochstrasser, M., Finley, D., and Peng, J. (2009). Quantitative proteomics reveals

the function of unconventional ubiquitin chains in proteasomal degradation. Cell *137*, 133-145.

Xu, W., Marcu, M., Yuan, X., Mimnaugh, E., Patterson, C., and Neckers, L. (2002). Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. Proc Natl Acad Sci U S A *99*, 12847-12852.

Yau, R., and Rape, M. (2016). The increasing complexity of the ubiquitin code. Nat Cell Biol *18*, 579-586.

Yin, Y., Seifert, A., Chua, J.S., Maure, J.F., Golebiowski, F., and Hay, R.T. (2012). SUMO-targeted ubiquitin E3 ligase RNF4 is required for the response of human cells to DNA damage. Genes Dev *26*, 1196-1208.

Young, J.C., Hoogenraad, N.J., and Hartl, F.U. (2003). Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70. Cell *112*, 41-50.

Zhang, H., Saitoh, H., and Matunis, M.J. (2002). Enzymes of the SUMO modification pathway localize to filaments of the nuclear pore complex. Mol Cell Biol *22*, 6498-6508.

Zhang, Y.Q., and Sarge, K.D. (2008). Sumoylation of amyloid precursor protein negatively regulates Abeta aggregate levels. Biochem Biophys Res Commun *374*, 673-678.

Zhao, Q., Xie, Y., Zheng, Y., Jiang, S., Liu, W., Mu, W., Liu, Z., Zhao, Y., Xue, Y., and Ren, J. (2014). GPS-SUMO: a tool for the prediction of sumoylation sites and SUMO-interaction motifs. Nucleic Acids Res *42*, W325-330.

Zhao, X., and Blobel, G. (2005). A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization. Proc Natl Acad Sci U S A *102*, 4777-4782.

Zhou, W., Ryan, J.J., and Zhou, H. (2004). Global analyses of sumoylated proteins in Saccharomyces cerevisiae. Induction of protein sumoylation by cellular stresses. J Biol Chem *279*, 32262-32268.

Zhu, X., Zhao, X., Burkholder, W.F., Gragerov, A., Ogata, C.M., Gottesman, M.E., and Hendrickson, W.A. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. Science *272*, 1606-1614.

Zunino, R., Schauss, A., Rippstein, P., Andrade-Navarro, M., and McBride, H.M. (2007). The SUMO protease SENP5 is required to maintain mitochondrial morphology and function. J Cell Sci *120*, 1178-1188.

7 INDEX OF ABBREVIATIONS

7.1 Abbreviations

A_x	absorbance at x nm
Ac	acetyl group
ACT	aspartate kinase, chorismate mutase, TyrA
ADP	adenosine 5'-diphosphate
AIM	Atg8-interacting motif
ALS_ss_C	acetolactate synthase small subunit C-terminus
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
bp	base pair
CHX	cycloheximide
CPC	cysteine-proline-cysteine
CSM	complete supplement mixture
dNTP	2'-deoxyribonucleoside-5'-triphosphate
DTT	dithiothreitol
CCD	charge-coupled device
CHIP	carboxy terminus of HSC70-interacting protein
cl.	clone
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Drp1	dynamin-related protein 1
ds	double stranded
DUB	deubiquitylating enzyme
E1	activating enzyme
E2	conjugating enzyme
E3	ligase
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia* (for example)
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FAD	flavin adenine dinucleotide
FAT10	human leukocyte antigen F-associated transcript 10
FUB1	Fau ubiquitin-like protein 1
FUS	fused in sarcoma
G418	geneticin sulfate
GABARAP	gamma-aminobutyric acid receptor-associated protein
Gal	galactose
GAP	GTPase-activating protein
GFP	green fluorescent protein
GLUT	glucose transporter
GST	glutathione S-transferase
HA	influenza hemagglutinine epitope
HCS	high content screening

HDAC	histone deacetylase
HECT	homologous to the E6-AP carboxy terminus
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
hphNT1	gene conferring resistance to hygromycin B
HRP	horseradish peroxidase
HS	heat shock
HSC	heat shock cognate
HSP	heat shock protein
IκB-α	nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor-α
IM	(mitochondrial) inner membrane
IMP	(mitochondrial) inner membrane peptidase
IMS	(mitochondrial) intermembrane space
IPTG	isopropyl β-D-1-thiogalactopyranosid
ISG15	interferon-stimulated gene 15
kanMX4	gene conferring resistance to G418
LB	lysogeny broth
LC	liquid chromatography
LC3	microtubule-associated protein 1A/1B light chain 3
LIR	LC3-interacting region
MAPL	mitochondria-anchored protein ligase
MEF2A	myocyte-specific enhancer factor 2A
MIA	mitochondrial intermembrane space assembly
MOPS	3-(*N*-morpholino)propanesulfonic acid
MPIB	Max Planck Institute of Biochemistry
MPP	mitochondrial processing peptidase
mRNA	messenger RNA
MS	mass spectrometry
MSF	mitochondrial import stimulation factor
mt	mitochondrial
MTS	mitochondrial targeting sequence
MUL1	mitochondrial ubiquitin ligase activator of NF-κB 1
NAT	nourseotricin
natNT2	gene conferring resistance to nourseotricin
NBR1	next to BRCA1 gene 1 protein
NEDD8	neuronal-precursor-cell expressed developmentally downregulated protein 8
NEF	nucleotide exchange factor
NEM	*N*-ethylmaleimide
Ni-NTA	Ni^{2+}-charged nitrilotriacetic acid
NLS	nuclear localization signal
NPC	nuclear pore complex
NSE2	non-structural maintenance of chromosomes element 2 homolog
OD_x	optical density at x nm
OM	outer (mitochondrial) membrane
ORF	open reading frame
OXA	cytochrome oxidase activity

PAGE	polyacrylamide gel electrophoresis
PAM	presequence translocase-associated motor
PBF	presequence binding factor
PBS	phosphate-buffered saline
Pc2	polycomb 2 homolog
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PD	pull-down
PEG	polyethylene glycol
P_i	phosphate
PIAS	protein inhibitor of activated STAT
PIP	PCNA-interacting protein
PP_i	pyrophosphate
PTM	posttranslational modification
PVDF	polyvinylidene fluoride
Ran	Ras-related nuclear protein
RanBP2	Ran-binding protein 2
RanGAP1	Ran GTPase-activating protein 1
RING	really interesting new gene
RNA	ribonucleic acid
Rpl	ribosomal protein of the large (60S) subunit
rpm	rounds per minute
Rps	ribosomal protein of the small (40S) subunit
RSUME	RWD-containing SUMOylation enhancer
SAE	SUMO-activating enzyme
SAF-A/B	scaffold attachment protein A/B
SAM	sorting and assembly machinery
SAP	SAF-A/B, Acinus, PIAS
SC	synthetic complete
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
SENP	sentrin-specific protease
sHSP	small HSP
SILAC	stable isotope labeling by amino acids in cell culture
SIM	SUMO-interacting motif
SP-RING	Siz/PIAS-RING
SQSTM1	sequestosome-1
STUbL	SUMO-targeted ubiquitin ligase
SUMO	small ubiquitin-like modifier
TBE	Tris, boric acid, EDTA
TBS	Tris-buffered saline
TBST	TBS containing 0.1 % Tween-20
TCA	trichloroacetic acid
TDG	thymine DNA glycosylase
TIM	translocase of the (mitochondrial) inner membrane
TOM	translocase of the (mitochondrial) outer membrane
TOPORS	topoisomerase I-binding arginine/serine-rich protein
TRAF7	tumor necrosis factor receptor-associated factor 7

Tris	Tris(hydroxymethyl)aminomethane
Triton X-100	4-(1’,1’,3’,3’-tetramethylbutyl)phenyl polyethyleneglycol ether
Tween-20	Polyethylene glycol sorbitan monolaurate
Ub	ubiquitin
UBA	ubiquitin-associated
UBD	ubiquitin-binding domain
UBL	ubiquitin-like
UCH	ubiquitin C-terminal hydrolase
UFM1	ubiquitin-fold modifier 1
UPS	ubiquitin-proteasome system
UV	ultraviolet
v/v	volume per volume
WB	western blot
WT	wild type
w/v	weight per volume
YPD	yeast extract, peptone, dextrose
ZMIZ1	zinc finger MIZ domain-containing protein 1

7.2 Amino acids

1-letter code	3-letter code	Amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartate
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	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	Tryptophan
Y	Tyr	Tyrosine

7.3 Prefixes and units

k	kilo (10^3)
m	milli (10^{-3})
µ	micro (10^{-6})
n	nano (10^{-9})

°C	degree Celsius
Da	Dalton
g	gram
g	acceleration of gravity
h	hour(s)
Hz	Hertz
l	liter
min	minute(s)
M	molar
s	second(s)
S	Svedberg
V	Volt

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Manns, J., Daubrawa, M., Driessen, S., **Paasch, F.**, Hoffmann, N., Loffler, A., Lauber, K., Dieterle, A., Alers, S., Iftner, T., *et al.* (2011). Triggering of a novel intrinsic apoptosis pathway by the kinase inhibitor staurosporine: activation of caspase-9 in the absence of Apaf-1. FASEB J *25*, 3250-3261.

Alers, S., Loffler, A.S., **Paasch, F.**, Dieterle, A.M., Keppeler, H., Lauber, K., Campbell, D.G. Fehrenbacher, B., Schaller, M., Wesselborg, S., *et al.* (2011). Atg13 and FIP200 act independently of Ulk1 and Ulk2 in autophagy induction. Autophagy *7*, 1423-1433.