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SUMO-Dependent Substrate Targeting of the SUMO Protease Ulp1

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ABSTRACT PAGE

In the yeast Saccharomyces cerevisiae, the essential SUMO protease Ulp1 is responsible both for removing SUMO/Smt3 (small ubiquitin-like modifier) from specific target proteins and for processing precursor SUMO into its conjugation-competent form. Ulp1 localizes predominantly to nuclear pore complexes (NPCs) but has also been shown to deconjugate sumoylated septins at the bud neck of dividing cells. How Ulp1 is directed to bud-neck localized septins and other cytoplasmic deconjugation targets is not well understood. Using a structure/function approach, we set out to elucidate features of Ulp1 that are required for substrate targeting. To aid our studies, we took advantage of a catalytically inactive mutant of Ulp1 that is greatly enriched at the bud-neck of dividing yeast cells. We found that the localization of full-length Ulp1 to the bud-neck requires both SUMO and specific structural features of Ulp1's catalytic domain. In our analysis, we identified a 218 amino acid-long, substrate-trapping mutant of the catalytic domain of Ulp1, Ulp1(3)(C580S), that is necessary and sufficient for bud-neck localization. We used the targeting and SUMO-binding properties of Ulp1(3)(C580S) to purify Smt3-modified proteins from bacterial and yeast cell extracts. Furthermore, we find that the Ulp1(3)(C580S) interacts robustly with monomeric forms human SUMO1, SUMO2 as well as SUMO2 chains, making it a potentially useful tool for the analysis of sumoylated proteins. In summary, our study provides novel insights into how the Ulp1 SUMO protease is actively targeted to its substrates in vivo and in vitro.

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INTRODUCTION:

SUMO: A Post-translational modification

The human body consists of approximately 50 trillion cells. There are around 200 different types of cells in the body including epithelial cells of the skin, neurons in the brain, and contractile muscle cells to name a few. While these cells have very different functions in the body, they are all composed of 4 basic macromolecules including nucleic acids (DNA and RNA), lipids, carbohydrates (sugars), and proteins. Proteins are the drivers of many processes in the cell and have roles in metabolism, signaling, and cell structure. The human genome project has uncovered that the human genome encodes approximately 25,000 genes. However, although every cell contains the same genetic material, not every cell has the same characteristics and functions. Many differences between cells can be traced to differences in protein expression. More importantly, post-translational modifications of proteins, for example phosphorylation, acetylation, ubiquitination, or sumoylation, are utilized to increase the diversity and function of the proteome. Therefore, the total number of potential protein functions is far greater than the actual number of proteins encoded inside a cell. Here, we explore the functional consequences of SUMO-modification on sub-cellular protein targeting during cell division (Fig. 1)

Sumoylation is the attachment of a 98 residue (~14 kDa) protein called SUMO (small ubiquitin like modifier). SUMO is a conserved protein that shares approximately 18% identity with ubiquitin and both proteins become covalently

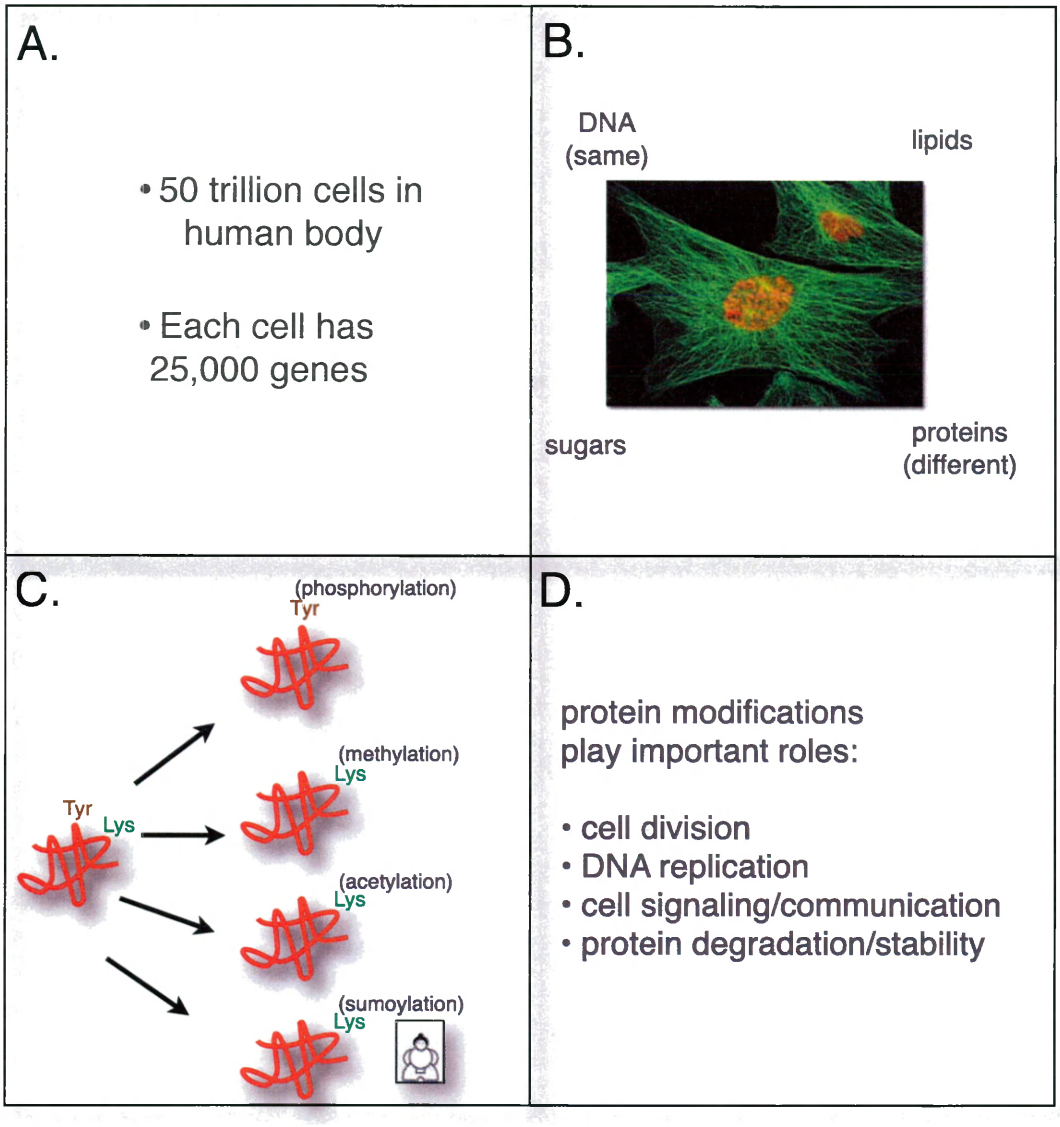


Figure 1. Post-translational modifications increase the functional diversity of the cellular proteome.
(A) The human body is composed of 50 trillion cells with each cell containing approximately 25,000 genes. (B) Cells are made of 4 basic macromolecules including nucleic acids (DNA and RNA), lipids, sugars, and proteins. All cells contain the same DNA but express different proteins. Picture from http://www.olympusfluoview.com. (C) Proteins (red) can be modified by different post-translational modifications that can alter the function of the protein and increase the diversity of the cellular proteome. These modifications include phosphorylation, methylation, acetylation, and sumoylation to name a few. (D) Roles of post-translational protein modifications in a cell.

attached to specific proteins in a reversible manner (Kerscher et al., 2006). Ubiquitin is best known for its role in the targeted destruction of proteins including key cell-cycle regulators but also holds many non-proteolytic functions (Chen and Sun, 2009). Sumoylation, on the other hand, does not directly target proteins for proteasomal degradation. Rather, modification of proteins with SUMO has been shown to modulate various cellular processes, including cell-cycle regulation, transcriptional activation, nucleocytoplasmic transport, DNA replication and repair, chromosome dynamics, apoptosis, ribosome biogenesis, and formation of nuclear bodies (Wang and Dasso, 2009). These functional distinctions between ubiquitin and SUMO have been further blurred by the recent discovery of SUMO-targeted Ubiquitin Ligases (STUbLs) that enable SUMO to play an indirect role in proteasome-mediated degradation (Perry et al., 2008).

SUMO proteins are highly conserved from yeast to humans. Yeast cells express one SUMO protein, Smt3, while vertebrates express three isoforms, SUMO1, SUMO2, and SUMO3 (Wilkinson and Henley, 2010). SUMO2, SUMO3 and yeast Smt3 can form SUMO chains. SUMO1, on the other hand, lacks the internal lysine required for polymerization and may function as a chain terminator for SUMO2 and 3 chains (Matic et al., 2008). SUMO modified proteins contain the sumoylation consensus motif Ψ-K-X-D/E where Ψ is a hydrophobic residue and X is any amino acid. All SUMO variants are conjugated to lysine residues of specific proteins, but only a fraction of these target proteins are modified with SUMO at any given time (Hannich et al., 2005; Wykoff and O'shea, 2005).

In metazoans, the dysregulation of sumoylation adversely affects developmental processes and has been implicated in the progression of neurodegeneration, cancer, and infectious diseases (Lamsoul et al., 2005; Sarge and Park-Sarge, 2009; Subramaniam et al., 2009). Over a thousand sumoylated proteins have been identified in yeast and humans, but only in a few cases has the role of sumoylation been studied in detail (Makhnevych et al., 2009). In the budding yeast, *Saccharomyces cerevisiae*, the ligation of SUMO to specific substrate proteins requires both an E1 heterodimer (Aos1 and Uba2) to activate SUMO, as well as E2 (Ubc9) and E3 (Siz1, Siz2, and Mms21) enzymes to catalyze the amide bond formation between the COOH-terminal carboxyl group of SUMO and the lysine side chain of acceptor proteins (Kerscher et al., 2006).

SUMO Interacting Motifs

Proteins can interact non-covalently with SUMO modified proteins through the use of SUMO interacting motifs (SIMs). SIMs are characterized by a hydrophobic core of amino acids (V/I-X-V/I-V/I) flanked by negatively charged acidic amino acids (Hannich et al., 2005). Interaction with SUMO requires the insertion of SIMs into the hydrophobic core of SUMO (Kerscher, 2007). The variability in the composition of the hydrophobic core of SUMO as well as the placement of charged amino acids allows SIMs to bind SUMO in either a parallel or anti-parallel orientation with respect to the β2 strand of SUMO (Kerscher, 2007). Basic residues of SUMO are proposed to have a role in electrostatic

interactions with negatively charged residues in SIM-containing proteins (Baba et al., 2005; Hecker et al., 2006; Song et al., 2005).

SUMO Proteases

Yeast contain two SUMO proteases while humans utilize six SUMO proteases termed sentrin proteases (SENPs) (Mukhopadhyay and Dasso, 2007). Yeast proteases are termed ubiquitin-like protease 1 and ubiquitin-like protease 2 (Ulp1 and Ulp2). Ulp1 is a 72 kDa protein encoded on the YPL020c open reading frame (ORF) on chromosome 16 (Li and Hochstrasser, 1999). Both Ulp1 and Ulp2 utilize a conserved cysteine protease domain that can remove the SUMO moiety from modified proteins. Ulp1 has two contrasting cellular functions. Ulp1 facilitates sumoylation in the context of processing the SUMO precursor by removing the amino acid residues ATY. The removal of ATY exposes a C-terminal di-glycine motif thus making SUMO competent for conjugation. Conversely, Ulp1 also facilitates desumoylation by removal of SUMO from nuclear and cytosolic proteins after conjugation (Li and Hochstrasser, 1999). SUMO deconjugation involves the cleavage of an amide bond between the C-terminus of SUMO and the epsilon amine group of the target lysine (Kerscher et al., 2006). Therefore, impairment of Ulp1 results in the accumulation of SUMO conjugates and the inability to carry out *de novo* sumoylation. The resulting lack of mature SUMO has been shown to adversely affect cellular DNA repair processes, the processing and nuclear export of the

60S pre-ribosomal particle, nuclear–cytoplasmic trafficking, and cell viability (Lewis et al., 2007; Palancade et al., 2007; Stade et al., 2002; Zhao and Blobel, 2005). Ulp2, on the other hand, does not contain SUMO processing activity and is not an essential gene, but it is required for normal chromosome stability and recovery from cell cycle arrest (Kroetz et al., 2009). Recent evidence suggests that Ulp2 and its mammalian orthologs Susp1/SENP6 and SENP7 play a role in the removal of SUMO and SUMO chains from nuclear proteins (Baldwin et al., 2009; Bylebyl et al., 2003; Kroetz et al., 2009; Mukhopadhyay et al., 2006; Uzunova et al., 2007).

The substrate specificity of SUMO proteases is at least in part regulated through their localization (Mukhopadhyay and Dasso, 2007). For example, certain yeast (Ulp2) and vertebrate (SENP6 and SENP7) SUMO proteases localize within the nucleus. In contrast, both yeast (Ulp1) and vertebrate (SENP1 and SENP2) SUMO proteases reside at the nuclear envelope (NE) through their interactions with the NPC (Li and Hochstrasser, 2003; Panse et al., 2003; Strunnikov et al., 2001; Zhang et al., 2002). Distinct domains have been identified that are required for Ulp1 NPC localization (amino acid residues 1-403) and SUMO processing (amino acid residues 404-620) (Li and Hochstrasser, 2003; Makhnevych et al., 2007; Panse et al., 2003; Zhao et al., 2004). The Ulp1 localization domain promotes interaction with karyopherins, soluble proteins that mediate transport across the nuclear envelope, and help localize Ulp1 to the

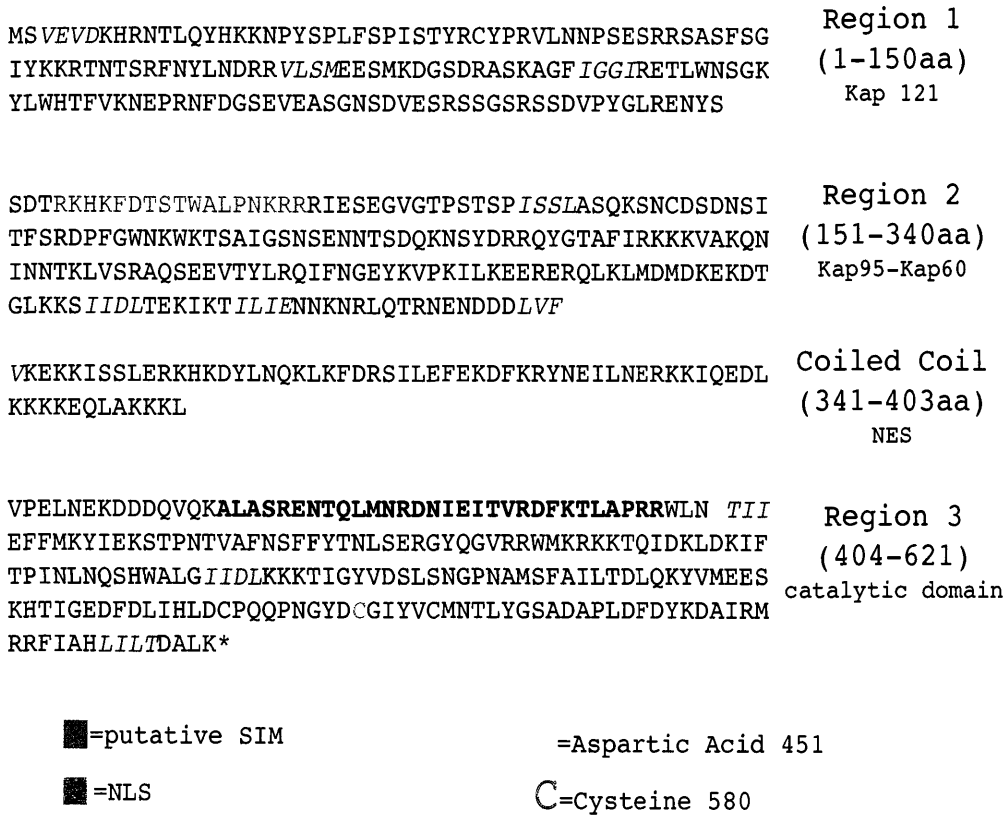


Figure 2. Ulp1 amino acid sequence and functional elements.
Ulp1 is a 621 amino acid protein that can be divided into 4 functional elements. Region 1 comprises residues 1-150 and is the binding site for the importin karyopherin 121 (Kap121). Region 2 composes residues 150-340 and is the binding site for karyopherin 95 and karyopherin 60 (Kap95-Kap60). Region 2 also contains a nuclear localization sequence (NLS) that is highlighted in green. Ulp1 contains a coiled coil domain in residues 341-403 that also may contain a putative nuclear export signal (NES). Region 3 composes residues 404-621 and is the catalytic domain of the protease. A SUMO-binding surface (SBS) critical for Ulp1's interaction with SUMO is highlighted in bold. Aspartic acid residue 451 (D) that forms a critical salt-bridge interaction with Smt3 is highlighted in yellow. The catalytic cysteine (C), residue 580, is highlighted in red. Putative SIMs are highlighted in blue italics.

nucleoplasmic side of the NPC.

The Ulp1 localization domain can be subdivided into region 1 (Kap121 binding domain) and region 2, (Kap60 and Kap95 binding domain) (Fig. 2). Juxtaposed to the NPC localization domain of Ulp1 is a coiled-coil (cc) domain with a putative nuclear export signal, and region 3, the catalytically active, conserved ubiquitin-like protease domain (UD) of Ulp1 (Li and Hochstrasser, 2003; Makhnevych et al., 2007; Panse et al., 2003). Only regions 1 and 2 are involved in Ulp1 localization to the NPC, and karyopherins seem to play a redundant role in NPC-anchoring. NPC-association of Ulp1 requires several proteins, including the nucleoporins Nup60 and Nup84, the silencing protein Esc1, and the myosin-like proteins Mlp1/2 (Lewis et al., 2007; Palancade et al., 2007; Zhao et al., 2004). Together these proteins may provide a scaffold for the functional regulation and substrate access of Ulp1 at the NPC.

The identification of NPC-localization domains in Ulp1 has done little to aid our understanding of how SUMO proteases are targeted to their respective substrates (Li and Hochstrasser, 2003; Zhang et al., 2002). One possibility is that SUMO proteases may contain structural features which allow for non-covalent interactions with SUMO and SUMO-modified proteins as they enter the nucleus. Indeed, conserved SUMO-interacting motifs, SIMs, have been predicted in the yeast SUMO protease Ulp2, as well as mammalian SENP1,2,6, and 7 (Kroetz et al., 2009; Matunis et al., 2006; Mukhopadhyay and Dasso, 2010). Even though SIMs have not been identified in Ulp1, the crystal structure

of the catalytic domain (region 3) bound to Smt3 reveals that both proteins interact through multiple residues that are distributed across a SUMO-binding surface (SBS) on the SUMO protease. Only the carboxy-terminus of bound Smt3 is inserted into a hydrophobic tunnel that leads towards Ulp1's active site. SUMO processing and deconjugation require an active-site cysteine residue that resides at the end of this tunnel. It has been suggested that this configuration may allow for the accommodation of many different sumoylated proteins, as well as SUMO precursors (Mossessova and Lima, 2000).

Ulp1 and several other SUMO proteases play important roles in mitosis (Dasso, 2008; Li and Hochstrasser, 1999). In budding yeast, loss of Ulp1-mediated desumoylation leads to cell cycle progression defects and cell death (Li and Hochstrasser, 1999). This observation suggests that Ulp1 plays a key role in the sumoylation dynamics of important cell cycle regulatory proteins. Though these cell cycle-specific targets have eluded identification, several nuclear and cytosolic proteins involved in DNA replication and mitosis have been identified as Ulp1 desumoylation substrates (Leisner et al., 2008; Stelter and Ulrich, 2003; Takahashi et al., 2000). How the NPC-localized Ulp1 is targeted to these mitotic substrates, especially those that are localized in the cytosol, is not entirely clear. In budding yeast the nuclear envelope does not break down during mitosis and access to cytosolic desumoylation targets is therefore not automatic. It has been reported that during mitosis, Kap121 blocks access of Ulp1 to its NPC-binding site, and thus promotes an interaction of Ulp1 with septins (Makhnevych et al.,

2007). A deletion mutant of Ulp1 lacking region 2 (Δ2), lacking the Kap60 and Kap95 binding domain, has previously been shown to localize to septins in a Kap121-dependent manner (Makhnevych et al., 2007). Curiously, it has recently been shown that region 2 also plays a role in nucleolar accumulation of Ulp1 after ethanol-induced stress (Sydorskyy et al., 2010).

Septins

One set of cytosolic substrates of the Ulp1 SUMO protease are the septins (Makhnevych et al., 2007; Takahashi et al., 2000). The septins in budding yeast comprise an evolutionarily conserved class of GTPases that are implicated in bud-site selection, bud emergence/growth, microtubule capture, and spindle positioning (Spiliotis, 2010). Members of the septin family in yeast include Cdc3, Cdc10, Cdc11, Cdc12, and Shs1/Sep7. These proteins are unique because they can form filaments that assemble into a ring structure and mark the site of new bud formation during cell division. At the end of mitosis, this ring separates and resembles a double collar residing at the junction between the mother and daughter cells.

The septins Cdc3, Cdc11, and Shs1 are subject to sumoylation. Sumoylation of the septins occurs very briefly from the onset of anaphase to cytokinesis, with SUMO being attached only to the mother-side of the double septin ring collar (Johnson and Blobel, 1999; Makhnevych et al., 2007; Takahashi et al., 1999). Cell cycle (G2/M) arrest with nocodazole, a microtubule

depolymerizing drug, greatly increases SUMO conjugation to septins (Takahashi et al., 2000). Septin sumoylation in budding yeast is mediated by the SUMO E3 ligase Siz1 (Johnson and Gupta, 2001; Takahashi et al., 2001). During most of the cell cycle, Siz1 resides in the nucleus. However, at M-phase, Siz1 exits the nucleus to sumoylate septin proteins and possibly other cytosolic substrates (Takahashi et al., 2008). Deletion of *SIZ1* from cells abolishes septin sumoylation while causing only mild growth and cell-cycle progression defects. At the end of mitosis, the septins are desumoylated by Ulp1 even though Ulp1 remains visibly enriched at the NPC (Johnson and Blobel, 1999; Makhnevych et al., 2007; Takahashi et al., 2000). During mitosis, the septins are the most highly sumoylated proteins in the cell (Johnson and Blobel, 1999). However, the role of septin sumoylation is not well understood. A mutant yeast strain lacking sumoylation sites in the septins Cdc3, Cdc11, and Shs1 showed a drastically decreased overall level of sumoylation in the cell (Johnson and Blobel, 1999). Furthermore, the triple mutant showed improper septin ring disassembly and retained unassembled septin rings from previous divisions. However, the triple mutant showed no growth defects and no sensitivity to stress conditions (Dasso, 2008; Johnson and Blobel, 1999). Notably, two other septins, Cdc10 and Cdc12, are expressed during vegetative growth and have been shown by proteomic analysis to be sumoylation targets (Dasso, 2008). In the triple mutant, it is possible that Cdc10 and Cdc12 compensate for the lack of septin sumoylation on Cdc3, Cdc11 and Shs1. This idea is supported by the finding that triple mutant is

synthetically lethal at 25°C in a Cdc12 temperature sensitive strain (Dasso, 2008).

Specific Aims of Thesis

Which mitotic sumoylation targets must be desumoylated to ensure proper cell-cycle progression, and how does Ulp1 target these proteins? To answer these questions, we sought to identify features of Ulp1 required for substrate-targeting *in vivo* and *in vitro*. Here we identify and analyze distinct mutations in Ulp1 that affect its targeting and retention to sumoylated target proteins at the bud-neck of dividing cells. We find that Smt3-interactions comprise an important aspect of the sub-cellular targeting of Ulp1 to its substrates. Our findings are confirmed by biochemical analyses that focus on the SUMO-binding properties of Ulp1(3)$^{(C580S)}$, a truncation mutant that interacts avidly with SUMO and sumoylated proteins *in vivo and in vitro*. Significantly, this study adds important new details to our understanding how Ulp1 interacts dynamically with its substrates and also provides potentially useful new directions to the study of Ulp1-interacting proteins.

MATERIALS AND METHODS:

Yeast strains, media and plasmids.

Yeast strains and plasmids used in this study are listed in Table 1. Yeast media preparation and manipulation of yeast cells was performed as previously published (Guthrie and Fink, 1991). Yeast strains were grown at 30°C unless otherwise indicated.

Table 1.

Name	Pertinent Genotypes or Parent Strain	Plasmids	Reference
MHY500	*Mata his3-Δ200 leu2-3,112 ura3-52 lys2-801trp1-1 gal2*		(Li and Hochstrasser, 2003)
BY4743	*MATa leu2Δ0 met15Δ0 ura3Δ0*		(Winzeler et al., 1999)
YOK 1611	MHY500	*ULP1-GFP/LEU2 (BOK 454)*	This study
YOK 1474	"	*ULP1$^{(C580S)}$-GFP/LEU2 (BOK 544)*	This study
YOK 1490	"	*ULP1(Reg1)-GFP/LEU2 (BOK 543)*	This study
YOK 1861	"	*UlLP1(Reg2)-GFP/LEU2 (BOK 677)*	This study
YOK 1479	"	*ULP1(Δ2)-GFP/LEU2 (BOK 536)*	This study
YOK 2016	"	*ULP1$^{(D451N\ C580S)}$-GFP/LEU2 (BOK 667)*	This study
YOK	"	*ULP1(Reg3)-*	This study

1839		*GFP/LEU2 (BOK 633)*	
YOK 1907	"	*ULP1(Reg3$^{(C580S)}$)-GFP/LEU2 (BOK 662)*	This study
YOK 1903	"	*ULP1((Reg3ΔSBS$^{(C580S)}$)-GFP/LEU2 (BOK 687)*	This study
YOK 2203	"	*ULP1(SBS)-GFP/LEU2 (BOK 696)*	This study
YOK 1828	"	*ULP1((Reg3$^{(ts)}$)-GFP/LEU2 (BOK 635)*	This study
YOK 2157	"	*ULP1((Reg3$^{(ts\ C580S)}$)-GFP/LEU2 (BOK 776)*	This study
YOK 1857	"	*SMT3-GFP/LEU2 (BOK 642)*	(Panse et al., 2003)
YOK 2204	"	*Cdc3-CFP/LEU2 + ULP1$^{(C580S)}$-GFP/URA3 (BOK 789 + BOK 738)*	(Nishihama et al., 2009)
YOK 2205	"	*Cdc3-CFP/LEU2 + Ulp1(Reg3$^{(C580S)}$)-GFP/URA3 (BOK 789 + BOK 740)*	(Nishihama et al., 2009)
YOK 44	*smt3-331*		(Biggins et al., 2001)
YOK 1995	"	*ULP1$^{(C580S)}$-GFP/LEU2 (BOK 544)*	This study
YOK 847	*ubc9-1*		(Betting and Seufert, 1996)
YOK 2065	"	*ULP1$^{(C580S)}$-GFP/URA3 (BOK 738)*	This study
YOK 2144	"	*SMT3-GFP/URA3 (BOK 658)*	This study
GBY1	*MAT***a** *smt3 R11,15,19::TRP1*		(Bylebyl et al., 2003)
YOK 1910	GBY1	*ULP1$^{(C580S)}$-GFP/LEU2 (BOK 544)*	This study

yDS880	*MAT**a**-inc ade2-101 his3-200 leu2-1::GAL-HO-LEU2 lys2-801 RAD53::FLAG-HIS3 siz1::NAT siz2::HPH sml1::KAN trp1-63 ura3-52 VII-L::TRP-HO site-LYS2*		(Schwartz et al., 2007)
YOK 2067	"	*ULP1$^{(C580S)}$-GFP/URA3 (BOK 738)*	This study
YOK 2143	"	*SMT3-GFP/URA3 (BOK 658)*	This study
kap121ts	*kap121::ura3::HIS3 ura3-52 his3Δ200 trp1-1 leu2-3,112 lys2-801*	*pRS314-kap121-34*	(Leslie et al., 2002)
YOK 1487	*kap121ts*	*ULP1-GFP/LEU2 (BOK 454)*	This study
YOK 1488	*kap121ts*	*ULP1$^{(C580S)}$-GFP/LEU2 (BOK 544)*	This study
YOK 1944	*kap121ts*	*ULP1(Reg3$^{(C580S)}$)-GFP/LEU2 (BOK 662)*	This study
AH109	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200,gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3,GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UASMEL1TATA-lacZ, MEL1*		Clontech, CA Cat. # 630444
YOK 2173	AH109	*ULP1 (Reg3$^{(C580S)}$)-pOAD/LEU2 + SMT3-pOBD/TRP1 (BOK 641 + BOK 295)*	This study
YOK 2175	AH109	*ULP1 (Reg3$^{(D451N)}$)-pOAD/LEU2 + SMT3-pOBD/TRP1 (BOK 796 + BOK 295)*	This study

YOK 2177	AH109	*ULP1 (Reg3$^{(ts)}$)-pOAD/LEU2 + SMT3-pOBD/TRP1 (BOK 645 + BOK 295)*	This study
YOK 2181	AH109	*ULP1 (Reg3$^{(ts\ C580S)}$)-pOAD/LEU2 + SMT3-pOBD/TRP1 (BOK 775 + BOK 295)*	This study
YOK 2212	AH109	*ULP1 (Reg3$^{(D451N\ C580S)}$)-pOAD/LEU2 + SMT3-pOBD/TRP1 (BOK 799 + BOK 295*	This study
YOK 2183	AH109	*SMT3-pOAD/LEU2 + SLX5 pOBD/TRP1 (BOK 295 + BOK 289)*	This study
YOK 2185	AH109	*vector-pOAD/LEU2 + vector pOBD/TRP1 (BOK 312 + BOK 313)*	This study
YOK 428	*ulp1::KAN (segregant of heterozygous diploid ULP1/ulp1::KAN in BY4743 - (OpenBiosystems, Huntsville, AL. -- Cat.#* YSC1021-671376)	*ulp1ts/TRP/NAT GPD-FLAG-SMT3gg/pRS425*	This study
YOK 1398	Wildtype (YOK 821x*nup170Δ::G418 CDC3-YFP/HIS5)*		This study

DNA fragments containing Ulp1 under the control of its endogenous promotor were amplified from yeast genomic DNA and placed in-frame with a carboxy-terminal GFP tag in the CEN/LEU2 plasmid pAA3 (Sesaki and Jensen, 1999). Primer pairs used for full-length Ulp1 amplification were OOK2 (ULP1 (-310 to -294)) and OOK3 (ULP1 (+1842 to +1863)). To prepare truncated and mutated Ulp1-GFP constructs listed in Table 1, Quikchange XL Site-Directed Mutagenesis

(Stratagene) and Phusion Site-Directed Mutagenesis kits (Finnzyme) were used according to manufacturer's instruction. Primer sequence information for the construction of individual mutants and truncations are available upon request. All constructs were sequenced verified. Additionally, activity of tagged Ulp1 constructs was confirmed in complementation assays. For two-hybrid constructs, ORFs of the indicated genes were PCR-amplified and homologously recombined into gapped pOAD and pOBD2 vectors (Yeast Resource Center, WA). To overexpress and purify Ulp1 truncations from bacteria, the respective Ulp1 fragments were PCR-amplified and cloned into pMALc-HT (a gift from Sean Prigge, JHSOM), thereby adding an in-frame maltose-binding protein (MBP) module followed by a TEV protease cleavage site and a His_6 epitope tag. Ulp1 derivatives were expressed as MBP fusions in BL21 Star (DE3) cells containing a pRIL plasmid expressing several rare-codon tRNAs (a gift from Sean Prigge, JHSOM, MD). Cdc3-CFP/LEU2 plasmid YCp-111 (BOK 789) was a kind gift from Ryuichi Nishihama in John Pringle's lab.

Yeast Two-Hybrid Assays

Gal4-Activation-domain (AD) fusions of *ULP1* and the indicated *ULP1* mutants in pOAD were transformed into the AH109 reporter strain expressing a Gal4-DNA-binding-domain (BD) fusion of SMT3 in pOBD. Two-hybrid interactions were scored by streaking on dropout media lacking adenine.

Pulldown assays, affinity purification, and protein extracts:

Frozen bacterial cell pellets from 200ml of IPTG-induced BL21 Star (DE3) cells were thawed on ice and resuspended in 2ml 1x phosphate buffered saline (PBS) containing 1x Halt Protease Inhibitor Cocktail (Pierce Cat. # 78430). Ice-cold cells were sonicated using a Branson Sonifier and extracts were cleared by centrifugation at 15kRPM (21,000 RCF) for 8 minutes at 4°C. Cleared bacterial extracts were added to 15 mL conical tubes and diluted using 4ml 1x PBS containing the protease inhibitor cocktail. MBP-tagged proteins (MBP-Ulp1(3), Ulp1(3)$^{(C580S)}$, or Ulp1(3)$^{(C580S)}\Delta$SBS) were bound to 5ml columns containing 300μl amylose resin (New England Biolabs) and washed extensively with 1x PBS. Whole yeast cell extracts containing the indicated target proteins were passed over the amylose resin and proteins bound to MBP-Ulp1(3), Ulp1(3)$^{(C580S)}$, or Ulp1(3)$^{(C580S)}\Delta$SBS were eluted with 100mM maltose or SDS-PAGE sample buffer. For SUMO pulldown experiments, recombinant MBP-Ulp1(3)$^{(C580S)}$ or MBP-Ulp1(3) was incubated with SUMO-1 or SUMO-2 agarose (Boston Biochem) in 1 ml of 1xPBS with protease inhibitors (Thermo Scientific). Proteins bound to the agarose beads were washed in 1xPBS and eluted with 1x SDS-PAGE sample buffer. All protein extracts were run out on NOVEX 4-12% BIS-TRIS gradient gels (invitrogen #NP0321) using MOPS-SDS running buffer (Invitrogen #NP0001).

Fluorescent Microscopy

Unless otherwise noted, cells were grown in rich media, G2/M arrested using nocodazole (15 μg/ml/3h/30°C), washed in 2% dextrose, and harvested by centrifugation. Images of live cells were collected using a Zeiss Axioskop fitted with a Retiga SRV camera (Q-imaging), i-Vision software (BioVision Technologies), and a Uniblitz shutter assembly (Rochester, NY). Pertinent filter sets for the above applications include CZ909 (GFP), XF114-2 (CFP), XF104-2 (YFP) (Chroma Technology Group). Images were normalized using i-Vision software and pseudo-colored and adjusted using Adobe Photoshop software (Adobe Systems Inc.).

In vitro ubiquitylation reactions, recombinant proteins, and antibodies:

In vitro ubiquitylation assay – enzymes and substrates used in our *in vitro* ubiquitylation assays were quantified using a Protein 230 kit on the Agilent 2100 Bioanalyzer according to the manufacturer's instructions. 10x ubiquitylation buffer, E1 enzyme (Uba1), ATP, and 20x ubiquitin were provided in a commercial ubiquitylation kit (Enzo # BML-UW0400). Ubiquitylation buffer, IPP (100 U/ml), DTT (50 μM), E1 (Uba1), E2 (Ubc4), and E3 enzymes (RNF4) were combined with purified SUMO2 chains (#ULC-210 -- Boston Biochem, MA) and ubiquitin. Reactions totaled 27 μL and were incubated at 30°C for three hours. Reactions were stopped by adding an equal volume of SUTEB sample buffer (0.01% bromophenol blue, 10 mM EDTA, 1% SDS, 50 mM Tris at pH 6.8, 8 M Urea)

containing DTT (5 µL of 1 M DTT/1 mL SUTEB sample buffer). Protein products were boiled in a 65°C heat block for ten minutes and analyzed by western blot with anti human SUMO-2 antibody. Anti-human SUMO2 # BML-PW0510-0025 (ENZO Life sciences, PA), anti-GFP: JL8 # 632380 (Clontech, CA), anti-FLAG(M2) #F3165 (Sigma-Aldrich, MO), anti-PGK: 22C5 # 459250 (Invitrogen, CA).

Measurement of Ulp1 $3^{(C580S)}$-SUMO-1 binding affinity using surface plasmon resonance:

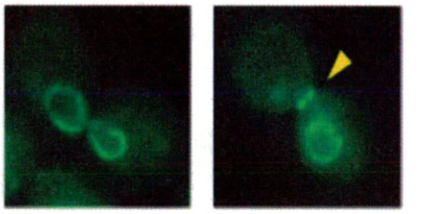
Affinity constants of Ulp1 $3^{(C580S)}$ and SUMO-1 were determined by Affina Biotechnologies (Stamford CT) using a Biacore 3000 instrument. Biotinylated SUMO-1 (Boston Biochem., Cat# UL-725) was immobilized on research grade streptavidin-coated sensor chips (Sensor Chip SA, Biacore Inc.) that were pretreated according to the manufacturers instructions. MBP-Ulp1 $3^{(C580S)}$ was injected at a flow rate of 20 µl/min in 20 mM sodium phosphate, 150 mM NaCl, 0.05% P-20, 1 mg/ml BSA, pH 7.4 (running buffer) for 3 min. Equillibrium binding data of MBP-Ulp1 $3^{(C580S)}$-SUMO-1 were calculated using the BIAevaluation software (Biacore Inc.).

RESULTS:

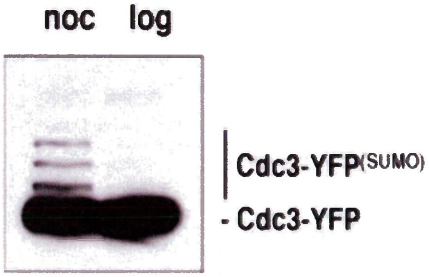
Ulp1 localization to the nuclear envelope and the septin ring.

As part of a larger study to identify how Ulp1 is targeted to its mitotic desumoylation substrates, we analyzed the localization of green fluorescent protein (GFP)-tagged versions of both the full-length wildtype Ulp1 (WT) and a catalytically inactive mutant of Ulp1 ($Ulp1^{(C580S)}$) in G2/M-arrested yeast cells. The C580S mutation replaces the catalytic cysteine with a serine residue, rendering the Ulp1 SUMO protease catalytically inactive (Li and Hochstrasser, 1999). Both fusion proteins were expressed under the control of the Ulp1 promotor on low-copy plasmids, and images were collected using a fluorescent microscope. Consistent with its localization to nuclear pore complexes, wildtype Ulp1 only stained the nuclear envelope of arrested yeast cells (Fig. 3 A - left). Unexpectedly, however, full-length $Ulp1^{(C580S)}$ was enriched both at the bud-neck and the nuclear envelope of G2/M arrested cells (Fig. 3 A - right). This bud-neck localization of full-length Ulp1 is reminiscent of the localization of the septin ring. Several sumoylated septins have been shown to be Ulp1 substrates and we show that the septin Cdc3 is higly sumoylated during G2/M arrest (Fig 3B). Indeed we found that the localization of $Ulp1^{(C580S)}$ at the bud-neck corresponds to the position of the septin ring (Fig 3C -- bottom). Specifically, cyan fluorescent protein (CFP)-tagged septin, Cdc3-CFP, colocalized with $Ulp1^{(C580S)}$ when septins were sumoylated during G2/M arrest (noc) (Fig. 3C). Therefore, $Ulp1^{(C580S)}$ resides at the bud-neck localized septin ring.

A.


Ulp1(WT)-GFP
Ulp1(C580S)-GFP

B.


noc
log
Cdc3-YFP(SUMO)
- Cdc3-YFP

C.

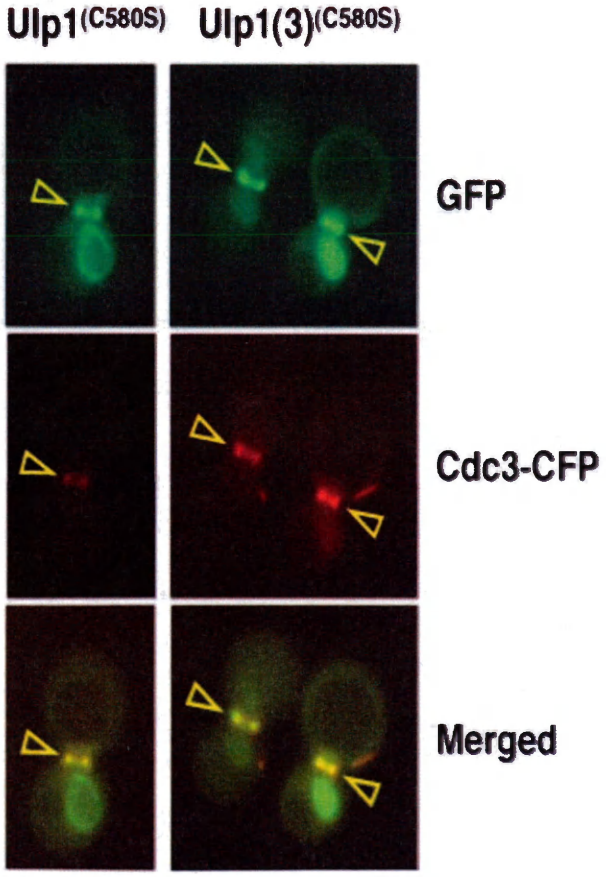

Ulp1(C580S)
Ulp1(3)(C580S)
GFP
Cdc3-CFP
Merged

Figure 3. Localization of Ulp1 and the catalytically inactive Ulp1$^{(C580S)}$ in dividing yeast cells. (A) Upper panel: Yeast cells (MHY500) were transformed either with a low-copy plasmid expressing GFP fusions of Ulp1 or the catalytically inactive Ulp1$^{(C580S)}$ mutant. Shown are representative images indicating the localization of GFP-tagged Ulp1 and Ulp1$^{(C580S)}$ after nocodazole-induced G2/M arrest (YOK 1611 and YOK 1474). Note that only the Ulp1$^{(C580S)}$ mutant can be visualized at the bud-neck of arrested cells. The arrowhead denotes the position of the bud-neck. (B) Confirmation of sumoylation of Cdc3. Whole cell extracts (WCEs) from yeast cells expressing the YFP-tagged septin Cdc3 (YOK 1398) were treated with nocodazole (noc) or grown logarithmically (log) prior to preparation of whole cell extracts. Extracted proteins were then separated on SDS-PAGE gels and probed with the JL-8 antibody (see materials and methods) to detect Cdc3-YFP and slower migrating sumoylated Cdc3-YFP adducts. Identity of sumoylated Cdc3-YFP bands was confirmed by comparing gel-shift assays with untagged and FLAG-tagged Smt3 (data not shown). (C) Colocalization of Cdc3 and Ulp1. A strain coexpressing full-length Ulp1$^{(C580S)}$-GFP (green) and Cdc3-CFP (red) (strain YOK 2204) was arrested in G2/M and then observed using a fluorescence microscope with the appropriate filter sets (left panel). Indicated (arrow heads) are septin-localized pseudo-colored Ulp1-GFP (green) and Cdc3-CFP (red) and the merged image (overlay). Also shown, for comparison (right panel), is the colocalization of the Ulp1(3)$^{(C580S)}$-GFP truncation and Cdc3-CFP (strain YOK 2205). Ulp1(3)$^{(C580S)}$-GFP is further described in Fig. 5.

Our data suggest that introducing the C580S mutation into the catalytic domain of Ulp1 somehow alters the subcellular distribution of this SUMO protease mutant to associate with a bud-neck associated substrate, possibly a sumoylated septin protein. Localization changes have also been reported for catalytically inactive, substrate-trapping mutants of phosphatases that form stable complexes with their substrates *in vivo* (Flint et al., 1997).

SUMO is required for Ulp1 localization to the septin-ring.

Next, we tested whether the C580S mutation that visually increased the ability of full-length Ulp1 to associate with the septin ring *in vivo* was, in fact,

SUMO-dependent. For this purpose, the Ulp1$^{(C580S)}$ construct was expressed in two Smt3 mutants (*smt3-331* and *smt3-R11,15,19*) or two SUMO pathway mutants (*ubc9-1, siz1Δ siz2Δ*) (Betting and Seufert, 1996; Biggins et al., 2001; Bylebyl et al., 2003; Schwartz et al., 2007; Takahashi et al., 1999; Takahashi et al., 2001). Logarithmically growing cells of each mutant were arrested in G2/M, and images were collected to assess the septin ring localization of Ulp1$^{(C580S)}$ in comparison to a *SMT3 wildtype* strain. In our analyses, we found that in both the absence of SUMO chains (in the R11,15,19 mutant) and improperly formed SUMO chains (in the *smt3-331* mutant), the localization of Ulp1$^{(C580S)}$ to the septin ring was reduced but not abolished (Fig. 4A). However, we obtained different results in the *ubc9-1* strain, a mutant of the SUMO E2 conjugating enzyme which impairs SUMO conjugation, and the *siz1Δ siz2Δ* strain, a SUMO E3 ligase double mutant that lacks sumoylation of septins and many other proteins (Betting and Seufert, 1996; Johnson and Gupta, 2001; Takahashi et al., 2001). Consistent with a role for Smt3 in the localization of Ulp1$^{(C580S)}$, we were unable to detect septin ring localization of Ulp1$^{(C580S)}$ in *ubc9-1* and *siz1Δ siz2Δ* strains. However, Ulp1$^{(C580S)}$ was retained at the nuclear envelope (Fig. 4A). As an additional control, the septin ring localization of GFP-tagged Smt3 was undetectable in both *ubc9-1* and *siz1Δsiz2Δ* strains (Fig. 4B).

In summary, Smt3 is required for Ulp1 localization to the bud-neck that comprises the septin ring. Therefore, Ulp1 is targeted to the septin ring of dividing cells in a SUMO-dependent fashion. Our data also suggest that the

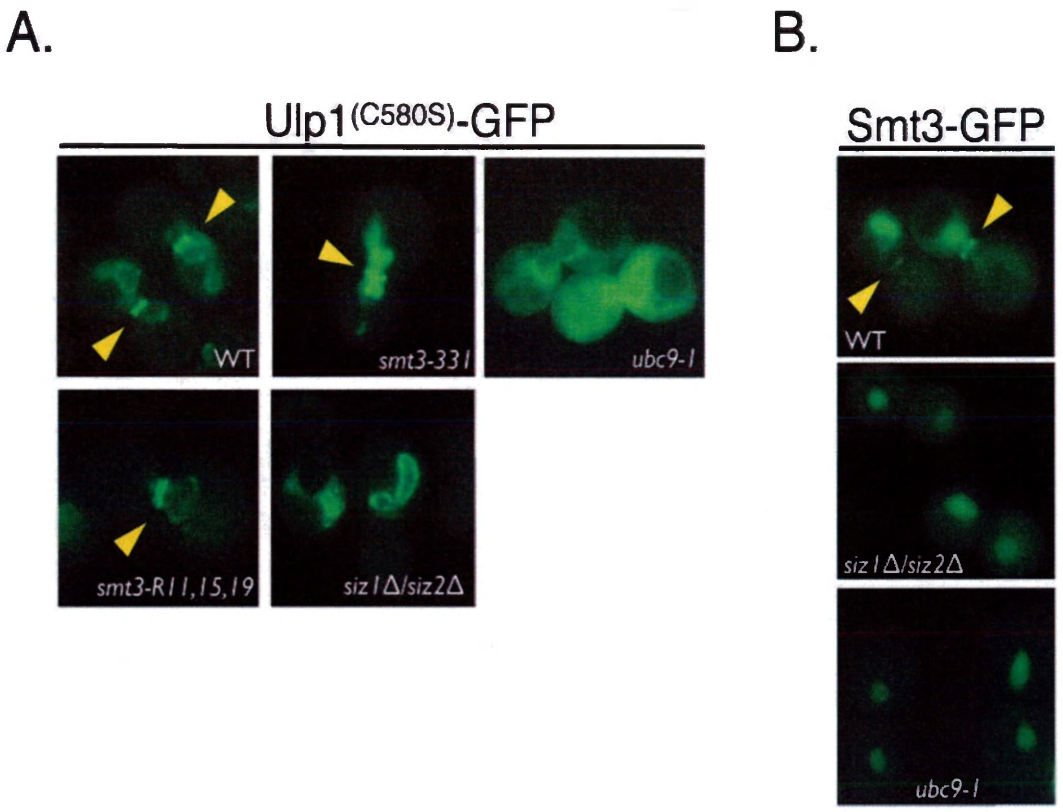


Figure 4. SUMO is required for the localization of Ulp1$^{(C580S)}$ to the septin ring. (A) The indicated mutants (*smt3-331, ubc9-1, smt3-R11,15,19, siz1Δ siz2Δ*) and a *wildtype* control strain (WT) were transformed with a plasmid expressing GFP-tagged Ulp1$^{(C580S)}$. Shown are representative images indicating the localization of GFP-tagged Ulp1$^{(C580S)}$ after G2/M arrest. The septin ring localization of Ulp1$^{(C580S)}$ is indicated when present (arrowheads). Note that Ulp1$^{(C580S)}$ fails to localize to the septins in SUMO-conjugating and -ligating enzyme mutants (*ubc9-1* and *siz1Δ siz2Δ*, respectively). (B) Septin localization of Smt3-GFP is absent in *ubc9-1* and *siz1Δ siz2Δ* strains. Localization of SUMO-GFP was visualized in G2/M-arrested *wildtype* (WT), *ubc9-1*, and *siz1Δ siz2Δ* strains using fluorescence microscopy. Position of the septin ring is indicated (arrow heads).

formation of SUMO chains on substrates may enhance this targeting of Ulp1.

Distinct and separate Ulp1 domains are required for localization to the septin ring.

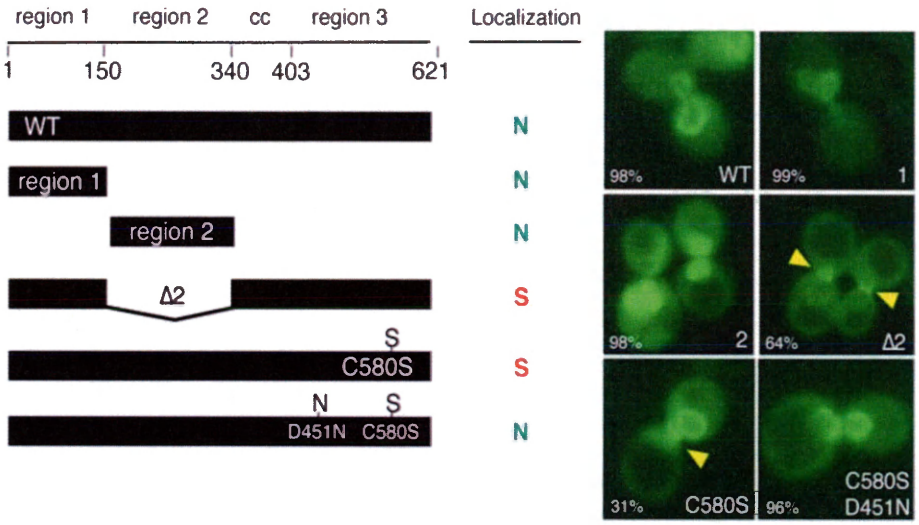
Our finding that a single point mutation in Ulp1, C580S, dramatically enhanced the localization of full-length Ulp1 to the septin ring in a SUMO-dependent fashion warranted a more detailed analysis of the targeting domains in Ulp1. Therefore, we generated a collection of GFP-tagged Ulp1 truncations and domains that were expressed under control of the Ulp1 promotor. We reasoned that the truncations and domains of Ulp1 that retained substrate-targeting information would also localize to the septin ring in G2/M-arrested cells. In all, we assessed the localization of ten GFP-tagged constructs in comparison to full-length wildtype Ulp1 (WT) and full-length Ulp1$^{(C580S)}$ (C580S). Our choice of individual constructs was guided by previous findings that Ulp1 consists of functionally separate domains. These domains include a Kap121-binding domain with a role in septin localization (region 1), a Kap95-Kap60-binding domain with a role in NPC anchoring (region 2), a coiled-coil domain harboring a nuclear export signal (CC), and the catalytic UD domain (region 3) (Li and Hochstrasser, 2003; Makhnevych et al., 2007; Panse et al., 2003). Representative images of these domains and their subcellular localizations are shown in Figure 5A and B. As previously reported, we found that the Ulp1 protein lacking region 2, (Δ2), localized to the septin ring in the majority of large-budded, arrested cells

(Makhnevych et al., 2007). Therefore, region 2 of Ulp1 normally antagonizes localization and/or retention at the septin ring. This result is complemented by our novel finding that the full-length Ulp1$^{(C580S)}$ localized to the septin ring in 31% of all arrested, large-budded cells (n>100) (Fig 3A and 5A).

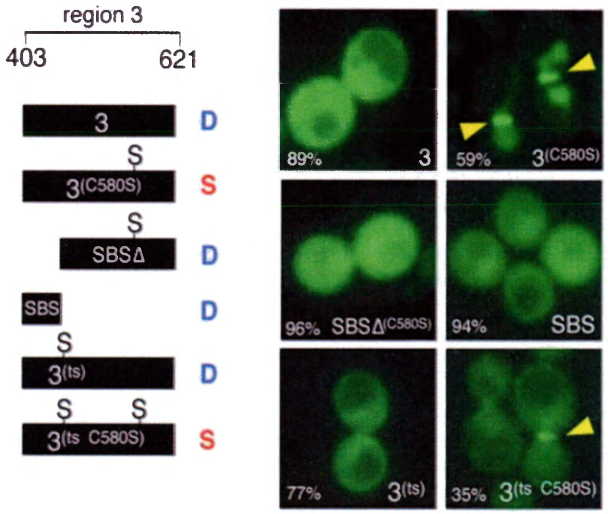
Next, we investigated other residues of Ulp1 that could affect the septin ring localization of the Ulp1$^{(C580S)}$ mutant, possibly by interfering with its targeting to sumoylated substrates. Aspartate 451 (D451) in Ulp1 is required to form an essential salt-bridge with arginine 64 of Smt3 (Ihara et al., 2007; Mossessova and Lima, 2000). Therefore, we introduced a D451N mutation into Ulp1$^{(C580S)}$ and found that it abolished the accumulation of the full-length Ulp1 double mutant (D451N, C580S) at the septin ring (Fig. 5A). This finding underscores the importance of Smt3 in targeting full length Ulp1 to the septin ring shown in Figure 4. Additionally, it may indicate that aspartate 451 is required for targeting of sumoylated proteins while the C580S mutation is required for retention of Ulp1 at the septin ring.

Most intriguingly, we found that a truncation consisting only of region 3 with the C580S mutation (Ulp1(3)$^{(C580S)}$), displayed septin ring localization in 59% of cells (Fig 3C (right panel) and Fig 5B). In stark contrast, regions 1, 2, and wildtype region 3, lacking the C580S mutation, failed to localize to the septin ring (Fig. 5A and B). However, where Ulp1 is displaced, the septin ring stays intact (data not shown). Therefore, necessary and sufficient SUMO-dependent targeting information is contained in region 3 of Ulp1 but not region 1 and 2. The

A.


region 1
region 2
cc
region 3
Localization
1
150
340
403
621
WT
region 1
region 2
Δ2
C580S
D451N C580S
N
N
N
S
S
N
98% WT
99% 1
98% 2
64% Δ2
31% C580S
96% C580S D451N

B.


region 3
403
621
3
3(C580S)
SBSΔ
SBS
3(ts)
3(ts C580S)
D
S
D
D
D
S
89% 3
59% 3(C580S)
96% SBSΔ(C580S)
94% SBS
77% 3(ts)
35% 3(ts C580S)

C.

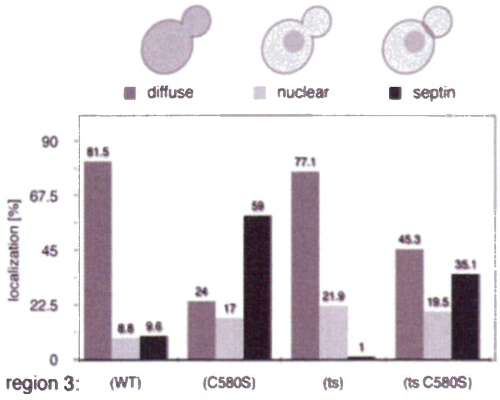

diffuse
nuclear
septin
localization [%]
90
67.5
45
22.5
0
61.5
8.8
9.6
24
17
59
77.1
21.9
1
45.3
19.5
35.1
region 3:
(WT)
(C580S)
(ts)
(ts C580S)

Figure 5. Distinct and separate Ulp1 domains are required for localization to the septin ring. (A and B) Left: Schematic representation of Ulp1 deletion and truncation mutants used in this study. The length of each construct (amino acid scale: 1-621), individual domains of Ulp1, and pertinent amino acid changes are shown. WT: full-length Ulp1, region 1: Ulp1(1-150) , region 2: Ulp1(151-340), region 3: Ulp1(341-621), Δ2: Ulp1 lacking region 2, C580S: catalytically inactivating mutation, D451N: deleted salt-bridge with SUMO. Colored letters N, S, D summarize the observed nuclear, septin or diffuse localization of the indicated constructs, respectively. SBS corresponds to a shallow SUMO-binding surface on Ulp1 (28,51,52). Right: Representative images of G2/M arrested cells expressing the GFP-tagged Ulp1 constructs shown on the left. The fraction of cells (%) with nuclear, septin or diffuse localization and the presence and position of bud-neck localized Ulp1 constructs is indicated (arrow heads). (C) Quantification of distinct subcellular localization of wildtype and mutant Ulp1 region 3 constructs. Large-budded G2/M arrested cells were imaged to assess either diffuse, nuclear, or bud-neck localization (n>100).

latter conclusion is also confirmed by two-hybrid assays with Smt3 (Fig. 9).

The previously published co-crystal structure of Ulp1 with Smt3 (MMDB database # 13315) reveals that amino acids 418-447 of region 3 make extensive contact with Smt3 and constitute an exposed SUMO-binding surface (SBS - see also Fig. 6) (Mossessova and Lima, 2000). The SBS is situated next to, but does not include, the critical D451 residue that contacts Smt3 (Ihara et al., 2007). Additionally, deletion of this SBS in region 3 of Ulp1 abolishes the complementation of a *ulp1Δ* deletion mutant (Li and Hochstrasser, 2003). In an attempt to identify critical residues in the evolutionary conserved SBS domain, we used psi-blast to compare the protein sequence of the yeast Ulp1 catalytic domain to all non-redundant protein sequences in the NCBI database for seven iterations and limited the output to the top 250 matches. While there was an obvious bias toward model organisms for which more sequences were available,

our results contained 81 different species; 61% of the sequences were identified as verified or predicted sentrin/SUMO protease/Ulp1 genes, 24% were identified as unnamed protein products or hypothetical genes and 15% were "other" (crystal structures, unanalyzed sequence, etc.). The alignment of these sequences allowed us to identify areas of strong conservation (see figure 7A and 7B). Using this approach we identified several highly conserved residues in the SBS. However, these amino-acids did not contact Smt3 in the published co-crystal structure and likely play structural roles in Ulp1 folding (Mossessova and Lima, 2000).

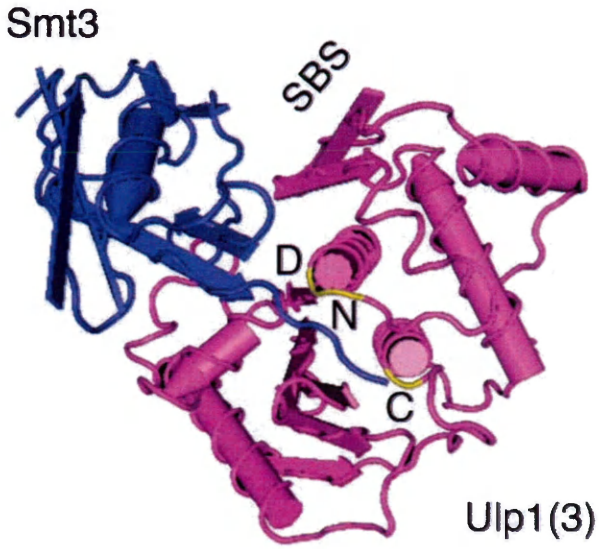


Figure 6. 3D co-crystal structure of Ulp1 Reg 3 and Smt3. Three dimensional representation of the co-crystal structure of the catalytic domain of Ulp1 (Ulp1(3) -- magenta) with yeast SUMO (Smt3 -- blue). Indicated in yellow and labeled with the appropriate amino acids are N450, D451, and C580. Also shown is the SUMO-binding surface (SBS).

We also investigated the effect of deleting the entire SBS domain on the localization of Ulp1(3)$^{(C580S)}$. A Ulp1(3)$^{(C580S)}$ΔSBS construct does not localize to the septin ring in the majority of cells (96%). The results match those obtained by Li and Hochstrasser using a wildtype Ulp1(3)ΔSBS construct (C173) (Li and Hochstrasser, 2003). We also cloned and expressed the SBS domain as a fusion with the green fluorescent protein (SBS-GFP). This construct distributed diffusely throughout the cell and failed to localize to the septin ring (Fig. 5 middle). These data suggest that the SBS domain of region 3 may be required for the initial interaction with sumoylated substrates but additional features of Ulp1 are required for targeting (D451) and retention (C580S) of this SUMO protease at the septins.

Next, we directed our attention to the temperature-sensitive *ulp1ts-333* allele. This mutant allele causes cells to arrest in mitosis and accumulate unprocessed SUMO precursor and sumoylated proteins (Li and Hochstrasser, 1999). Our *ulp1ts* construct of region 3, Ulp1(3)^{ts}, contains three mutations (I435V, N450S, I504T), and introduction of C580S into Ulp1(3)^{ts} showed a greatly reduced incident and intensity of septin ring localization (compare panels in Fig. 5B and 5C). We noted that the (N450S) mutation in the ts construct was located next to the salt-bridge forming residue D451 described above and that both residues are highly conserved in the consensus sequence of Ulp1-like molecules (Fig. 7A and 7B). This suggested that residues altered in *ulp1ts-333*, specifically N450, may contribute to Smt3 interaction and possibly substrate targeting. It is

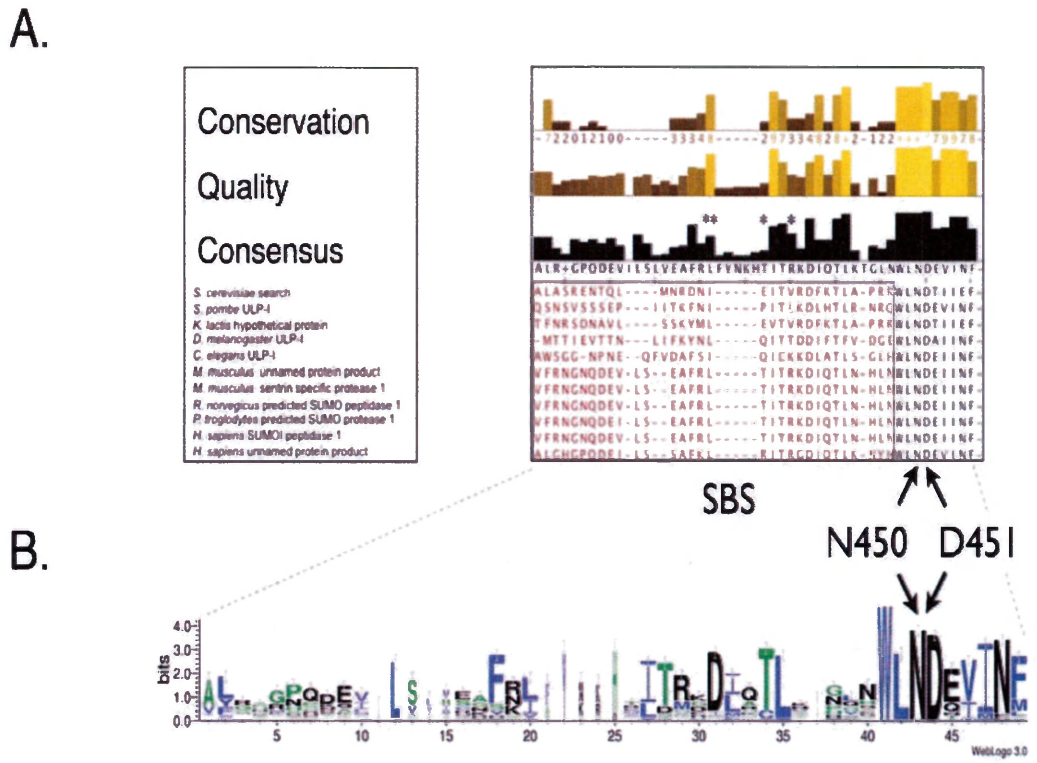


Figure 7. Identification of important features required for Ulp1 targeting and SUMO-binding. (A) The yeast Ulp1 catalytic domain was blasted against all non-redundant protein sequences in the NCBI database using psi-blast. After 7 iterations, the top 100 query sequences (only 11 are shown) were aligned, which included a variety of animal, plant and fungal species. Shown in red are residues that constitute the SUMO-binding surface (SBS). Also indicated are the salt-bridge forming D451 and one of the residues mutated in the *ulp1ts* allele, N450. *Conservation*: conservation of amino acid properties. *Quality*: alignment quality based on Blosum 62 scores; high values suggest no or conservative mutations. *Consensus*: percent identity. Calculated using Jalview. **(B)**: Consensus SBS based on the alignment of 250 sequences from 81 species. The height of the letters corresponds to the frequency of the amino acid in the alignment; width is based on the proportion of sequences that contain a character (many gaps lead to narrow letters). Also indicated are the salt-bridge forming D451 and one of the residues mutated in the *ulp1ts* allele, N450 (weblogo.threeplusone.com)(Crooks et al., 2004).

possible that N450S perturbs the salt-bridge interaction formed between D451 of Ulp1 and R64 of Smt3 therefore reducing the interaction with Smt3 and contributing to the temperature sensitive phenotype. In support of this, correction of the N450S mutation in Ulp1(3)$^{ts\ (S450N)}$ partially rescued the slow growth defect of a *ulp1Δ* strain at 30° and 37°C (data not shown). The effect of the ulp1ts mutation on Ulp1's ability to interact with Smt3 is explored in more detail below (Fig. 9).

In conclusion we find that several features, most importantly D451 and C580S, in region 3 of Ulp1, beyond the previously identified SBS domain, are required for targeting and retention at the septin ring (see Fig. 5, 6 and 7).

Kap121-independent SUMO-targeting information resides in Ulp1(3)$^{(C580S)}$.

Above, we describe our identification of necessary and sufficient substrate-targeting information in the catalytic domain (region 3) of Ulp1. However, region 3 of Ulp1 may not be the only domain involved in targeting to the septins. Region 1 of Ulp1, the Kap121-binding domain, has previously been implicated in septin-targeting. Specifically, it has been reported that Kap121 is required for targeting Ulp1 to the septin ring during mitosis (Makhnevych et al., 2007). Therefore, we decided to assess the role of Kap121 in the substrate-targeting of Ulp1(3)$^{(C580S)}$. Specifically, we used a *kap121ts* mutant (Leslie et al., 2002) to assess the septin ring targeting of wildtype Ulp1, full-length Ulp1$^{(C580S)}$, and Ulp1(3)$^{(C580S)}$. In our analysis, we found that full-length Ulp1$^{(C580S)}$ required Kap121 function for

targeting to the septin ring. At the permissive temperature (30°C), Ulp1$^{(C580S)}$ demarcated the nuclear envelope and septin ring of G2/M arrested cells. After a shift to the non-permissive temperature, however, Ulp1$^{(C580S)}$ could no longer be detected at the septin ring (Fig. 8 - middle panel). Surprisingly, Ulp1(3)$^{(C580S)}$, was localized to the bud-neck at the permissive and non-permissive temperature for *kap121ts*. As shown here at 30°C and 37°C, Ulp1(3)$^{(C580S)}$ resided both inside the nucleus and at the septin ring (Fig. 8 - right panel).

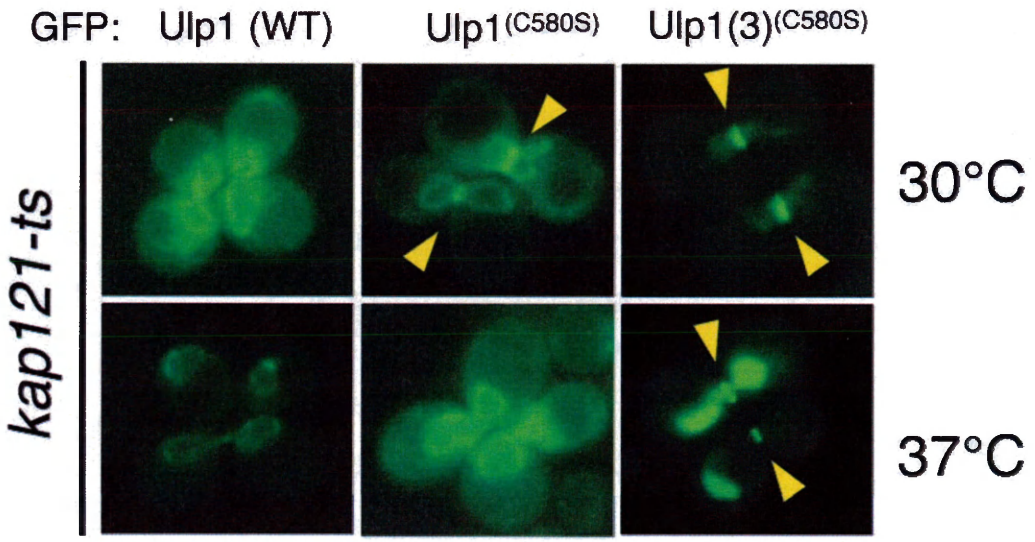


Figure 8. Kap121-independent SUMO-targeting information resides in region 3 of Ulp1 (A) *kap121ts* cells were transformed with plasmids expressing GFP-tagged wildtype (WT) Ulp1, Ulp1$^{(C580S)}$, and Ulp1(3)$^{(C580S)}$ under the control of the Ulp1 promotor. Shown are representative images indicating the localization of GFP-tagged Ulp1 constructs in large-budded cells at 30°C and 37°C, the non-permissive temperature for *kap121-ts*. The position of bud-neck localized Ulp1 constructs is indicated (arrowheads).

Our data suggest that Ulp1 contains both Kap121-dependent and independent bud-neck targeting information. The only requirement to detect full-

length Ulp1 and Ulp1(3) at the bud-neck is the C580S mutation and functional Kap121 (Fig. 3, 4, 5, and 8). In contrast Ulp1(3)$^{(C580S)}$, which lacks all domains required for NPC interaction through Kap121, Kap60, and Kap95, localizes to the budneck and inside the nucleus. In summary, this finding provides strong evidence that Kap121-independent bud-neck-targeting information resides in the catalytic domain (region 3) of Ulp1.

Multiple features in the catalytic domain of Ulp1 affect SUMO interactions.

Our finding that a single amino-acid change in the catalytic domain of Ulp1 results in greatly enhanced, SUMO-dependent localization to the bud-neck also prompted us to investigate the two-hybrid interactions of Ulp1(3)$^{(C580S)}$ with budding yeast SUMO (Smt3-BD). Full-length wildtype Ulp1, the full-length catalytically inactive Ulp1$^{(C580S)}$ mutant, the Ulp1 Kap121-interacting domain (region 1), the Ulp1 Kap60/Kap95-interacting domain (region 2), and the catalytic domain (region 3) all failed to interact with Smt3 fused to the Gal4 DNA-binding domain (data not shown). However, the catalytically inactive Ulp1(3)$^{(C580S)}$ truncation interacted weakly, but reproducibly and above background, with Smt3 (see Fig. 9)

Next, we focused on the important residues near the SBS domain of Ulp1 region 3 (see Fig. 5B and 6). First, we investigated the D451N mutant of Ulp1 that prevents the interaction of Ulp1 with SUMO (Ihara et al., 2007; Mossessova and Lima, 2000)

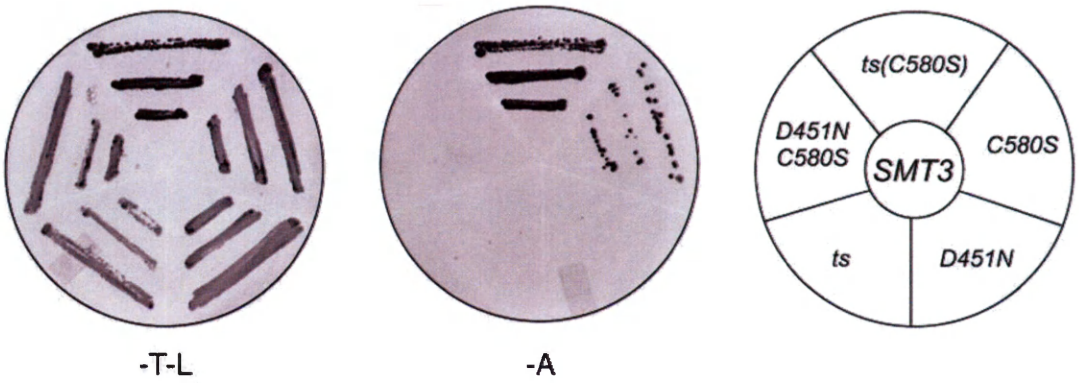


Figure 9. Distinct and separate Ulp1 domains are required for interaction with SUMO. (A) Two-hybrid analysis of various Ulp1 region 3 truncations with SUMO (C580S: catalytically inactive; D451N: deleted salt-bridge with SUMO; ts: mutations including S450N in *ulp1ts-333*). The presence of both Smt3 (pOBD2/TRP1) and Ulp1 constructs (pOAD/LEU2) was confirmed by growth on medium lacking tryptophan and leucine (-T-L). The interaction between Ulp1 constructs and Smt3 is shown as duplicate streaks on media lacking adenine (-A). See Fig. 5A and B for a graphic representation of individual constructs

As shown above, D451N, when introduced into Ulp1$^{(C580S)}$, prevented localization to the septin ring (Fig 5A). Accordingly, we found that introduction of the D451N mutation into Ulp1(3)$^{(C580S)}$ destroyed its interaction with Smt3 (see Fig. 9). We reasoned that Ulp1(3)$^{(C580S)}$ alone fails to score strongly with Smt3 because it is avidly interacting with free Smt3 or is sequestered by sumoylated proteins in the cell and, therefore, failed to interact with the BD-Smt3 fusion. We therefore proposed that weakening of the interaction between Ulp1(3)$^{(C580S)}$ and Smt3 could allow for a two-hybrid interaction to be scored. In support of this hypothesis, we found that introducing the *ulp1ts* mutations in the Ulp1(3)$^{(C580S)}$ construct, forming Ulp1(3)$^{ts\ (C580S)}$, also enhanced interaction with Smt3. These observations provide evidence that the targeting of Ulp1 to sumoylated substrates is a closely balanced act involving both Smt3 targeting and retention.

The Ulp1(3)$^{(C580S)}$ truncation binds SUMO and SUMO-modified proteins.

We hypothesized that if Ulp1(3)$^{(C580S)}$ were to interact avidly with Smt3, this mutated moiety of Ulp1 could efficiently interact with SUMO adducts *in vitro*. Therefore, to test the direct interaction of Ulp1(3)$^{(C580S)}$ with SUMO, we fused this domain to the carboxy-terminus of the maltose binding protein (MBP) and expressed large amounts of the recombinant fusion protein in bacteria. Subsequently, the MBP-Ulp1(3)$^{(C580S)}$ fusion protein was purified from bacterial extracts and bound to amylose resin. As a control to assess the ability of the MBP-Ulp1(3)$^{(C580S)}$ to interact with sumoylated proteins, we also purified a second MBP-fused Ulp1(3)$^{(C580S)}$ construct lacking the SBS domain (3$^{(C580S)}$ΔSBS).

First, we determined the ability of MBP-Ulp1(3)$^{(C580S)}$ to affinity-purify sumoylated proteins from crude yeast cell extracts. *ulp1ts-333* cells expressing FLAG-tagged-SMT3 (YOK428) were grown to log-phase prior to preparation of yeast cell extracts (see material and methods). These extracts were then incubated with resin-bound MBP-Ulp1(3)$^{(C580S)}$, MBP-Ulp1(3)$^{(C580S)}$-ΔSBS, or unbound amylose resin. After washing, bound yeast proteins were eluted, separated on SDS-PAGE gels, and analyzed by western blotting with an anti-FLAG antibody. Flag-SMT3-modified proteins present in the whole cell extracts (WCE) (Fig. 10A lane 2) could clearly be detected bound to MBP-Ulp1(3)$^{(C580S)}$ (lane 5) but not the MBP-Ulp1(3)$^{(C580S)}$-ΔSBS control protein (lane 4). We identified both unconjugated Flag-Smt3 protein as well as several higher

molecular weight adducts. These data suggest that Ulp1(3)$^{(C580S)}$ can efficiently bind and enrich sumoylated proteins from crude yeast cell extracts. To demonstrate the versatility of Ulp1(3)$^{(C580S)}$ -aided Smt3 purification, we also purified monomeric and conjugated GFP-Smt3 from yeast cells (Fig. 10B). Additionally, we probed extracts and eluted proteins shown in figure 10B with an anti Cdc11 antibody, revealing the specific co-purification of Cdc11 with immobilized Ulp1(3)$^{(C580S)}$ (Fig. 10C).

In the reciprocal experiment, we tested whether a GFP-tagged Ulp1(3)$^{(C580S)}$ construct expressed in yeast cells could bind immobilized SUMO2 which is highly conserved to yeast Smt3. In this experiment, yeast cells expressing CEN-plasmid levels of the GFP-tagged Ulp1(3), Ulp1(3)$^{(C580S)}$, or the Ulp1(3)$^{(C580S)}$-ΔSBS (see Fig. 5) were grown to log-phase prior to preparation of yeast cell extracts. Individual extracts were then incubated with SUMO2 immobilized on agarose beads (see material and methods). After washing, bound yeast proteins were eluted, separated on SDS-PAGE gels, and analyzed by western blotting with an anti GFP antibody. This time, the GFP-tagged Ulp1(3)$^{(C580S)}$ could be detected in the WCE and bound to the SUMO2 agarose (Fig. 10D). In contrast, neither the wildtype catalytic domain of Ulp1 (Ulp1(3)) nor Ulp1(3)$^{(C580S)}$ΔSBS bound to SUMO2-agarose. Similarly, the Ulp1(3)$^{(C580S)}$ could also be purified on SUMO-1 agarose (data not shown).

We also analyzed if immobilized Ulp1(3)$^{(C580S)}$ could be used to purify SUMO chains. For this experiment, we incubated purified SUMO2 chains with

our immobilized Ulp1(3)$^{(C580S)}$ or the unbound amylose resin. After washing, bound SUMO2 chains were eluted, separated on SDS-PAGE gels, and analyzed by western blotting with an anti- SUMO2 antibody. SUMO2 chains could clearly be detected in the input (Fig. 10E-lane 2) and bound to the MBP-Ulp1(3)$^{(C580S)}$ (lane 4), but not the resin-only control (Fig. 10E-lane 3). Both lower and higher molecular weight adducts of SUMO2 were purified with preference for higher molecular weight chains (5-7mers). These data suggest that the Ulp1(3)$^{(C580S)}$ can efficiently bind and enrich SUMO2 chains *in vitro* and that the MBP fusion of Ulp1(3)$^{(C580S)}$ may also be useful for the purification of sumoylated proteins from mammalian cells.

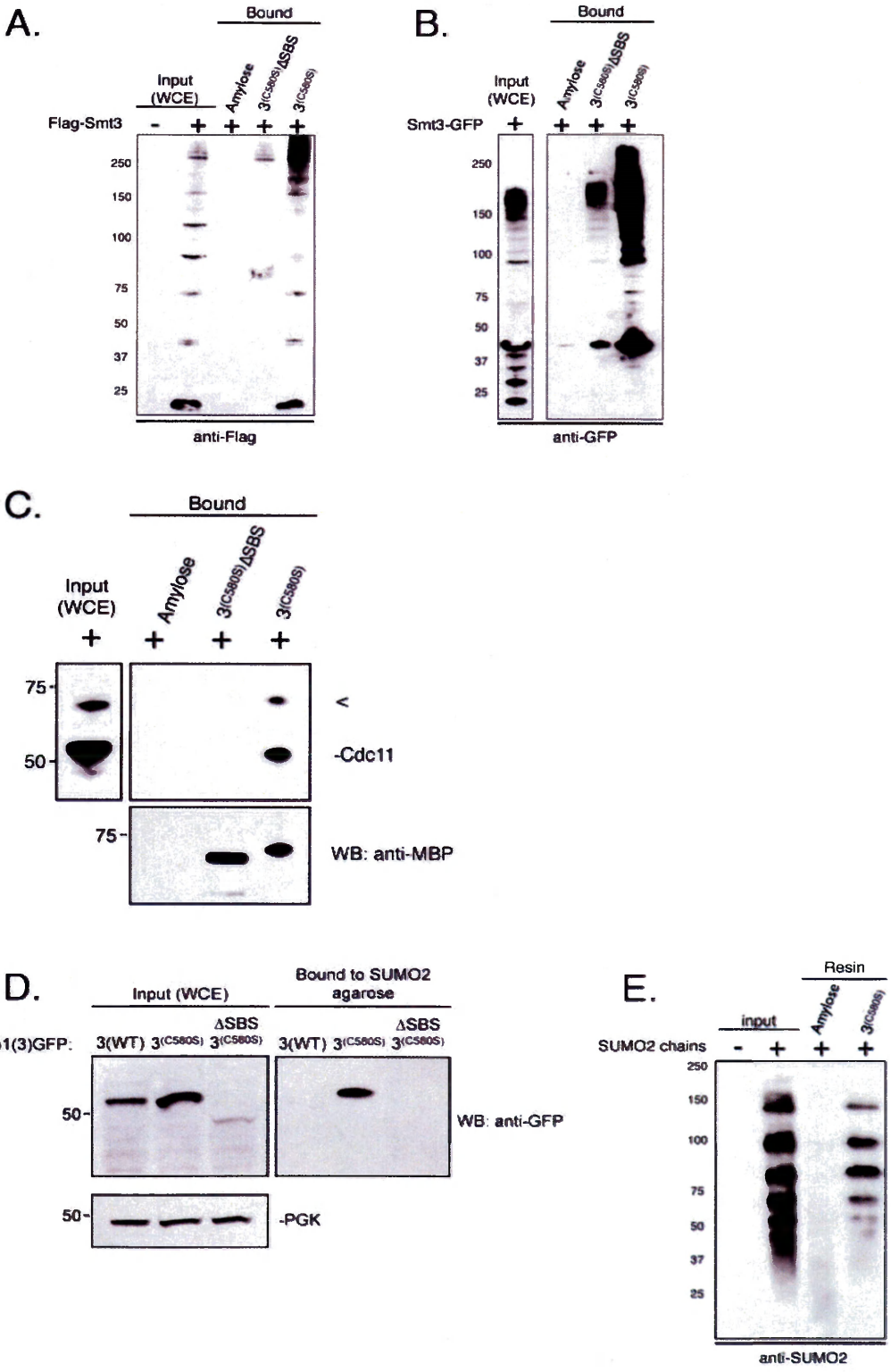

A.
Bound
Input (WCE)
Amylose
3(C580S)ΔSBS
3(C580S)
Flag-Smt3
- + + + +
250
150
100
75
50
37
25
anti-Flag
B.
Bound
Input (WCE)
Amylose
3(C580S)ΔSBS
3(C580S)
Smt3-GFP
+ + + +
250
150
100
75
50
37
25
anti-GFP
C.
Bound
Input (WCE)
Amylose
3(C580S)ΔSBS
3(C580S)
+ + + +
75
50
-Cdc11
75
WB: anti-MBP
D.
Input (WCE)
Bound to SUMO2 agarose
Ulp1(3)GFP:
3(WT) 3(C580S) ΔSBS 3(C580S)
3(WT) 3(C580S) ΔSBS 3(C580S)
50
WB: anti-GFP
50
-PGK
E.
Resin
input
Amylose
3(C580S)
SUMO2 chains
- + + +
250
150
100
75
50
37
25
anti-SUMO2

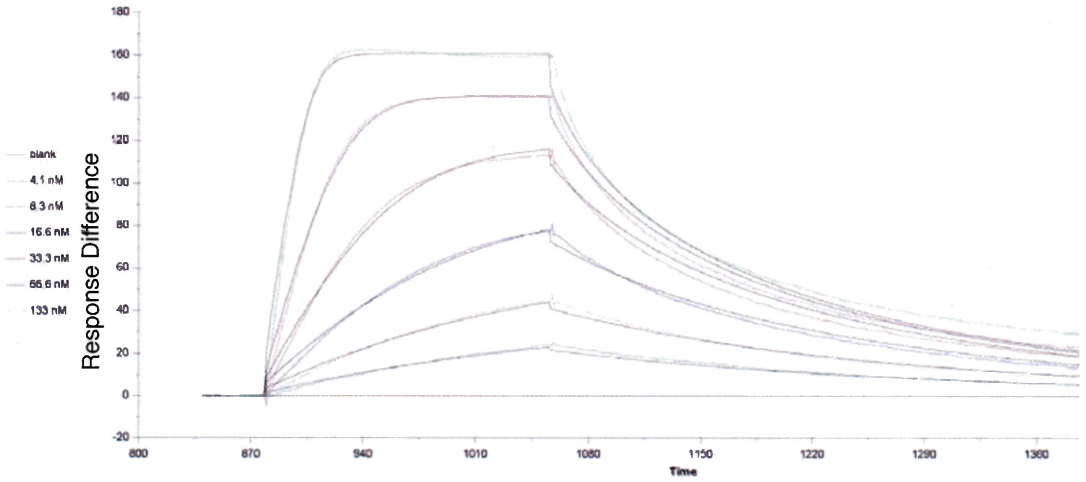
Figure 10. The Ulp1(3)$^{(C580S)}$ truncation binds SUMO and SUMO-modified proteins. (A and B) Immobilized Ulp1(3)$^{(C580S)}$ was analyzed for its ability to affinity-purify Smt3 from yeast whole cell extracts (WCE). WCEs containing FLAG-tagged-Smt3 (left) or GFP-Smt3 (right) (input) were prepared under non-denaturing conditions and incubated with immobilized MBP-Ulp1(3)$^{(C580S)}$ (3$^{(C580S)}$), MBP-Ulp1(3)$^{(C580S)}$ lacking the SUMO-binding surface (3$^{(C580S)}$ΔSBS) or unbound resin (amylose). After washing and elution, bound Smt3 and Smt3 conjugates were detected using either anti-Flag or anti-GFP antibodies. (C) Immobilized Ulp1(3)$^{(C580S)}$ was analyzed for its ability to affinity-purify Cdc11 from yeast WCEs. WCE containing GFP-Smt3 (YOK 1857) was prepared under non-denaturing conditions and incubated with immobilized MBP-Ulp1(3)$^{(C580S)}$, MBP-Ulp1(3)$^{(C580S)}$ lacking the SUMO-binding surface (3$^{(C580S)}$ΔSBS) or unbound resin (amylose). After washing and elution, bound Cdc11 was detected using an anti-Cdc11 antibody. Arrowhead indicates modified Cdc11. (D) WCEs from logarithmically growing yeast cells expressing GFP-tagged Ulp1(3), Ulp1(3)$^{(C580S)}$, Ulp1(3)$^{(C580S)}$ΔSBS (input) were prepared under non-denaturing conditions. Extracts were then incubated with SUMO2 immobilized on agarose beads. After washing and elution with sample buffer, bound proteins were detected using an anti-GFP antibody. (E) SUMO2 chains were incubated with resin-bound MBP-Ulp1(3)$^{(C580S)}$ or unbound resin (amylose). After washing and elution with sample buffer, bound proteins were detected using an anti-SUMO2 antibody. SUMO2 chains loading control (input).

Determination of Ulp1 3$^{(C580S)}$ binding affinity to immobilized SUMO-1

The ability of Ulp1 3$^{(C580S)}$ to bind SUMO and SUMO modified proteins led us to further investigate the binding affinity between Ulp1 3$^{(C580S)}$ and SUMO-1. Affinity constants of Ulp1 3$^{(C580S)}$ with SUMO-1 were determined using a Biacore 3000 surface plasmon resonance (SPR) instrument (Affina Biotechnologies, Stamford CT) (Fig. 11A). The equilibrium dissociation constant between Ulp1 3$^{(C580S)}$ and SUMO-1 was determined to be 1.29 x 10^{-8} M or 12.9 nM (Fig. 11B). Previously, it has been demonstrated that the affinity of SIMs for SUMO is in the range of 2-3 μM (Hecker et al 2006). Thus, the interaction between Ulp1 3$^{(C580S)}$

and SUMO-1 is 150-230 times stronger than any previously described SIM and SUMO interaction.

A.



B.

Molecules	K_a (1/Ms)	K_d (1/Ms)	K_D (M)
MBP-Ulp1 $3^{(C580S)}$ – Biotinylated SUMO-1	4.11×10^5	5.31×10^3	1.29×10^{-8}

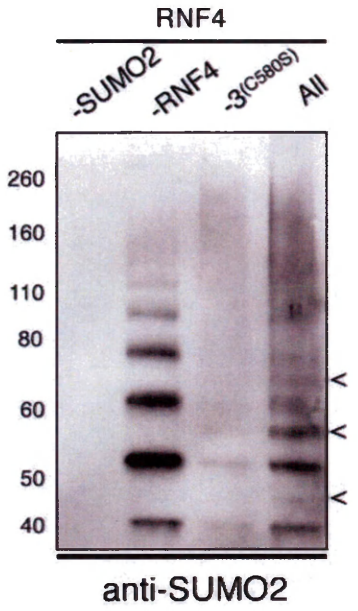
Figure 11. Determination of affinity constants between Ulp1(3)$^{(C580S)}$ and SUMO-1. (A) Surface plasmon resonance (SPR) technique was used to study the binding kinetics between MBP-Ulp1(3)$^{(C580S)}$ and SUMO-1. Biotinylated SUMO-1 was immobilized on research grade streptavidin-coated sensor chips. Purified MBP-Ulp1(3)$^{(C580S)}$ was injected as the soluble analyte at a flow rate of 20 μl/min in running buffer (see materials and methods). The respective graph indicates the interaction between Ulp1 $3^{(C580S)}$ and SUMO-1 at various concentrations of MBP-Ulp1(3)$^{(C580S)}$. (B) Affinity constants between MBP-Ulp1(3)$^{(C580S)}$ and biotinylated-SUMO-1 as determined using BIAevaluation software. K_a-association rate constant, K_d-dissociation rate constant, K_D equillibrium dissociation constant

A SUMO2-binding platform for substrate ubiquitylation.

Sumo-targeted ubiquitin ligase proteins (STUbLs) like the yeast Slx5/Slx8 heterodimer and the human RNF4 protein efficiently ubiquitylate proteins modified with SUMO chains (Geoffroy and Hay, 2009; Tatham et al., 2008). These proteins interact with their respective sumoylated ubiquitylation targets through SIMs. STUbL reactions have been reconstituted *in vitro*, but the purification of target proteins modified with SUMO chains has been both technically difficult or prohibitively expensive. The ability of Ulp1(3)$^{(C580S)}$ to interact with SUMO may, therefore, provide a simple way to purify a SUMO-chain modified STUbL target of choice.

To test if Ulp1(3)$^{(C580S)}$ can serve as a platform to modify a purified protein with SUMO-2 chains, we incubated the immobilized MBP-Ulp1(3)$^{(C580S)}$ with SUMO-2 chains and removed unbound SUMO-2 chains by washing with buffer. The MBP-Ulp1(3)$^{(C580S)}$ SUMO-2 chain complex was then eluted and added into a STUbL *in vitro* ubiquitylation reaction containing recombinant RNF4. Proteins in the STUbL-mediated ubiquitylation assay were separated on SDS-PAGE gels, and analyzed by western blotting with an anti-SUMO antibody. Consistent with previous observations, we were able to detect ubiquitylated SUMO-2 chains after the STUbL reaction (Fig. 12A). This ubiquitylation was dependent on RNF4 and SUMO2 chains. Based on these results, we propose that the Ulp1(3)$^{(C580S)}$ may provide a useful, widely applicable tool for the study of sumoylated proteins and STUbL targets (Fig. 12B).

A.



B.

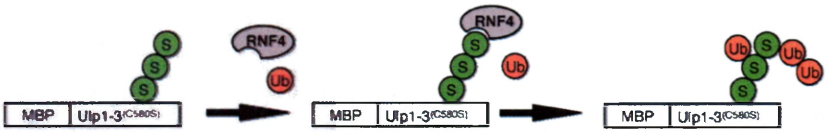


Figure 12. MBP-Ulp1(3)$^{(C580S)}$ can serve as a SUMO2 binding platform for STUbL-mediated substrate ubiquitylation. (A) SUMO-2 chains (Boston Biochem) were incubated with resin-bound MBP-Ulp1(3)$^{(C580S)}$. The complex of MBP-Ulp1(3)$^{(C580S)}$ with SUMO-2 chains was then eluted and added into an *in vitro* ubiquitylation reaction with the STUbL E3 RNF4. Proteins in the STUbL reactions were separated by SDS-PAGE and analyzed by western blotting with an anti-SUMO2 antibody. Arrows indicate modified SUMO-2 chains. Lane1: no SUMO chains; lane 2: no Rnf4; lane 3: no Ulp1(3)$^{(C580S)}$; lane 4: all reagents (B) Proposed model for using MBP-Ulp1(3)$^{(C580S)}$ as a SUMO-2 binding platform for substrate ubiquitylation. SUMO-2 (spheres labeled S), ubiquitin (spheres labeled Ub). RNF4 (gray oval labeled RNF4).

DISCUSSION:

In this study we demonstrated that region 3 of Ulp1, the catalytic domain, contains critical information for the subcellular targeting to sumoylated substrates, including the septin Cdc3. To determine how Ulp1 is targeted to its substrates, we took advantage of a catalytically inactive Ulp1 mutant (C580S) that exhibited a partial redistribution from the nuclear envelope to the bud-neck of dividing yeast cells. The re-localization of Ulp1 depended on functional Smt3 and sumoylated proteins at the bud-neck of dividing cells.

Importantly, using this novel Ulp1 in vivo septin-ring localization assay, we traced the critical targeting information to two features in region 3 of Ulp1, a previously identified SUMO-binding surface (SBS) (amino acids 418-447) and a SUMO contacting residue (D451) that reside near the carboxy terminus of Smt3 (see Fig. 5). D451 of Ulp1 has previously been shown to contact Smt3 through a salt bridge interaction (Ihara et al., 2007; Mossessova and Lima, 2000). Therefore, it is possible that perturbation of the D451 salt-bridge results in the reduced ability to dock Smt3 in place once it has contacted the SBS domain. Indeed, in our analysis, we provide evidence that the salt-bridge forming D451N mutation abolishes the targeting of Ulp1 to septins and weakens the interaction of the catalytic domain of Ulp1 with Smt3 (Fig 5A and 9).

The sole requirement for the enrichment of full-length Ulp1 at the septin ring was the inactivating C580S mutation in the catalytic domain of Ulp1. This finding has important implications for the Ulp1-targeting role played by the amino-

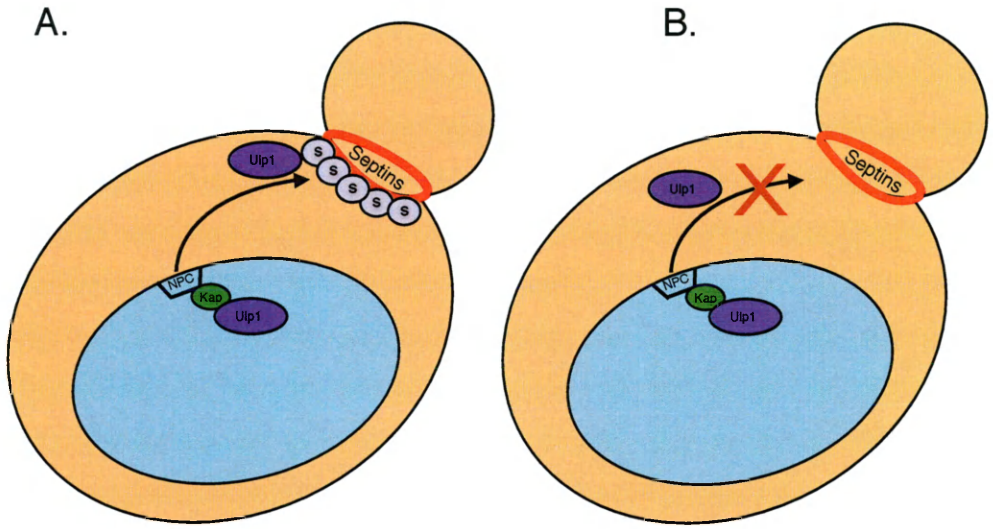


Figure 13. Model. (A) Ulp1 is anchored to the nuclear pore complex (NPC) by karyopherins (Kap) including Kap121 and Kap95-Kap60. During mitosis, Ulp1 is released from the NPC and allowed access to the cytoplasm. Ulp1 is targeted to cytoplasmic substrates (septins) via non-covalent interactions between its catalytic domain and SUMO. (B) SUMO is required for proper septin ring localization of Ulp1. Yeast mutant strains where sumoylation of the septins is abolished no longer have proper Ulp1 localization at the bud-neck.

terminal karyopherin binding domains of Ulp1 (discussed below). Additionally, catalysis of Smt3 appears to be required for substrate release. The catalytically inactive Ulp1(3)$^{(C580S)}$ mutant is prominently localized to the bud-neck and nucleus of dividing yeast cells while the catalytically active wildtype Ulp1(3) shows merely a diffuse staining throughout the cell (compare Fig. 5B top). This is not due to different stabilities of either protein because both proteins are expressed at equivalent levels (see Fig. 10D). We propose, and show in our biochemical analysis below, that the C580S mutation may trap a bound Smt3

protein in place, allowing us to observe it in association with cellular desumoylation substrates. In support of this assessment, combining the D451N with the C580S mutation abolishes all visible bud-neck localization (Fig. 5A). Therefore, we propose that Ulp1$^{(C580S)}$ first targets and docks Smt3 through the SBS domain and the salt-bridge forming D451 residue prior to trapping it in place due to its inability to cleave after the di-glycine motif of Smt3. We can assume that a trapped substrate prevents further catalysis or interactions with other Smt3 molecules, an assessment that is borne out by our finding that despite its septin-targeting and SUMO-binding properties, Ulp1(3)$^{(C580S)}$ interacts only weakly with Smt3 in a two-hybrid assay (Fig. 9). A better understanding of how the sumoylated substrate is trapped by Ulp1(3)$^{(C580S)}$ may have important implications for the rational design of inhibitors for Ulp1-like SUMO proteases but may have to wait for elucidation of the co-crystal structure with a trapped substrate.

The interaction of budding yeast Ulp1 with Smt3 relies on multiple hydrophobic and salt bridge interactions between the catalytic domain (region 3) of Ulp1 and the carboxy-terminal extension of Smt3. Making multiple interactions with Smt3, Ulp1 is particularly well suited to interact with a wide variety of sumoylated substrates (Mossessova and Lima, 2000; Mukhopadhyay and Dasso, 2007). Other SUMO proteases, Ulp2 and several SENP proteins (Senp1,2,6,7), are believed to interact non-covalently with their sumoylated substrates through dedicated SIMs (Kroetz et al., 2009; Mukhopadhyay and

Dasso, 2007). From our structure-function analysis of region 3, Ulp1 seems to employ a unique mode of interaction with Smt3 and sumoylated substrates. Ulp1 does not appear to contain bona fide canonical SIMs and neither of the amino-terminal domains of Ulp1 (region 1 and 2) interact with Smt3 or become enriched at the septin ring. This assessment is also underscored by the arrangement of Smt3 and Ulp1 in the co-crystal structure (Mossessova and Lima, 2000). The hydrophobic groove of Smt3 that would interact with a SIM-containing protein is turned away from the domains of Ulp1 that interact with Smt3. Interestingly, this may suggest that Ulp1 can be recruited to proteins that are covalently or non-covalently modified with SUMO and SUMO chains.

Our research demonstrates for the first time that non-covalent interactions between Ulp1 and SUMO are not only important for SUMO binding, but also for the cytosolic targeting of this SUMO protease to the bud-neck and potentially sumoylated septins (Fig. 13). Septins are not the only cytosolic substrates of Ulp1, but arguably the most prevalent (Johnson and Blobel, 1999; Takahashi et al., 2000) and, therefore, may be readily scored in our bud-neck targeting assay (Fig. 5A and 5B). We predict that Ulp1 is also targeted to other cytosolic and septin bound sumoylated substrate proteins, for example the karyogamy protein Kar9 (Leisner et al., 2008). However, due to the low local concentrations in comparison to sumoylated septins, these proteins may be hard to detect. We propose, however, that sumoylated proteins that accumulate or aggregate in the cytosol of yeast cells may be readily detectable by Ulp1(3)$^{(C580S)}$. As detailed

below, Ulp1(3)$^{(C580S)}$ also provides a useful tool to purify these sumoylated proteins (Fig. 10) and these studies are underway (Kerscher and Elmore, unpublished results). In conclusion, our findings provide strong evidence that SUMO, at least in the case of sumoylated proteins at the septin ring, is a required signal for the cytoplasmic targeting of Ulp1. Our alignments of the SBS domain and the juxtaposed salt-bridge forming D451 residue reveals that this mode of targeting may also be conserved in other metazoan Ulp1-like SUMO-proteases (Fig. 7A and 7B).

Though we clearly show that Ulp1 becomes enriched at the bud-neck, we do not yet fully understand how Ulp1 arrives at this subcellular localization. Our findings support the previous observation that Kap121 plays an important role in promoting Ulp1 targeting to the septin ring. Similar to a previously described Ulp1 mutant that lacks the Kap60/Kap95 binding domain (region 2) (Makhnevych et al., 2007), the septin ring localization of the full-length Ulp1$^{(C580S)}$ protein described here is dependent on functional Kap121. It is unlikely that the association with Kap121 shuttles Ulp1 to the septins. Rather, as previously reported, in mitosis Kap121 becomes associated with a transport inhibitory nucleoporin, Nup53, and may thus exclude Ulp1 access to the inner phase of the nuclear pore complex (Makhnevych et al., 2003). This suggests that in the absence of Kap121-binding, a fraction of Ulp1 is free to associate with sumoylated septins. Our studies confirm that Ulp1 lacking the Kap60/Kap95 binding domain (region 2) are enriched at the NPC and the septin ring (Fig. 5A).

We extend these observations by showing that the ability to target sumoylated septins resides in the catalytic domain (region 3) of Ulp1 (Fig. 5B). We find that a Ulp1(3)$^{(C580S)}$ mutant, but not wildtype Ulp1(3), is enriched at the septin ring in the absence of Kap121.

Taken together, these data suggest that both the Ulp1(3)$^{(C580S)}$ mutant and WT Ulp1(3) can interact with sumoylated septins but, unlike the subtrate-trapping Ulp1(3)$^{(C580S)}$ mutant, the catalytically active WT Ulp1(3) may be quickly released after desumoylation of the target protein giving it a diffuse appearance in the cell. How Kap121 helps Ulp1-Δ2 to be retained at the septin ring in the absence of the C580S mutation is currently not clear. Kap121 may theoretically promote the interaction with a bud-neck-localized protein. However, the localization of Kap121 to septins has not previously been reported.

One intriguing aspect of our study is the analysis of the substrate-trapping Ulp1(3)$^{(C580S)}$ construct. Detailed binding studies on the avidity of the Ulp1(3)$^{(C580S)}$ protein with Smt3 are currently underway but three lines of evidence reveal the avid interaction of Ulp1(3)$^{(C580S)}$ with SUMO proteins and sumoylated substrates. First, this Ulp1-derived construct shows a pronounced interaction with the bud-neck comprised of sumoylated septins in vivo. Second, the reduced interaction of Ulp1(3)$^{(C580S)}$ with Smt3 in a two-hybrid assay can be re-established by introduction of mutations that weaken the interaction with Smt3. And third, the purified, recombinant Ulp1(3)$^{(C580S)}$ protein is a potent affinity-tag for the purification of Smt3 conjugates and SUMO-modified proteins. A related study

involving the C603S mutant of the human SENP1 protease confirms our assessment of the substrate-trapping feature. The authors observe re-localization of their SENP1(C603S) mutant in vivo to promyelocytic leukemia (PML) nuclear bodies and domains of the HDAC4 protein, suggesting that SUMO-dependent-targeting may be a conserved feature of Ulp1-like SUMO proteases (Bailey and O'hare, 2004). The latter may also provide a useful strategy for the identification of mitotically important desumoylation substrates. Indeed, two-hybrid screens with Ulp1(3)$^{(C580S)}$ in the lab have already identified several novel cytosolic desumoylation targets (Donaher and Kerscher unpublished observations). Finally, we are also exploring the ability of Ulp1(3)$^{(C580S)}$ to act as a SUMO-chain binding-tag that can be used to promote the interaction of putative STUbL target proteins with RNF4 and other STUbLs (Fig. 12).

How Ulp1 and other SUMO proteases target specific mitotic substrates for desumoylation remains unknown. Our analysis of SUMO-dependent Ulp1-targeting to the septin ring provides important evidence that Ulp1-like SUMO proteases do not passively await their desumoylation substrates but rather dynamically localize to them in a cell cycle specific manner. Future experiments that take advantage of the SUMO-binding properties of the substrate-trapping Ulp1(3)$^{(C580S)}$ construct may prove useful for the identification of clinically relevant targets of conserved Ulp1-like SUMO proteases in yeast and human cells.

ACKNOWLEDGEMENTS

I dedicate this thesis to my family, especially my mother who always encouraged me in my studies and helped me develop a love of learning that continues to this day.

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