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A Tale of Two Proteins: The Budding Yeast STUbL Subunit Slx5 Functionally Interacts with the SUMO Ligase Siz1

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**A Tale of Two Proteins:
The Budding Yeast STUbL Subunit Slx5 Functionally Interacts
with the SUMO Ligase Siz1**

A thesis submitted in partial fulfillment of the requirement
for the degree of Bachelor of Science in Biology
from The College of William and Mary

by

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ABSTRACT

Eukaryotic cells utilize the dynamic addition and removal of post-translational modifications to modulate protein function. Two such modifiers are ubiquitin and SUMO (Small Ubiquitin-like MOdifier), which traditionally regulate their substrates in opposite ways. The discovery of SUMO-targeted ubiquitin ligases (STUbLs), E3 ligases that ubiquitylate sumoylated targets, offers an opportunity for cross-talk between the SUMO and ubiquitin pathways. STUbLs are crucial for the response to DNA damage and maintenance of genomic integrity, but currently only a few STUbL substrates are known.

Recently, we observed a novel interaction between the yeast STUbL subunit Slx5 and the SUMO ligase Siz1 both in a yeast two-hybrid system and *in vitro*. The goals of this study were to develop protein extraction and purification protocols for the purpose of determining if Slx5 and Siz1 also interact *in vivo*. This study additionally seeks to determine if Siz1 is a target for ubiquitylation by Slx5 *in vivo*. Here we report our finding that Slx5 and Siz1 Δ 440 co-affinity purify in *in vivo* pulldown experiments, and that Siz1 Δ 440 is ubiquitylated *in vivo* in an Slx5-dependent manner. We also describe the intrinsic binding ability of the RING domain present in E3 ligases for metal affinity purification.

INTRODUCTION

Post-translational Modification

After translation, individual amino acid residues of a protein can be covalently linked to a variety of chemical groups, including lipids, polysaccharides, or other small proteins (Walsh, 2006). The dynamic enzymatic addition and removal of these post-translational modifications modulates protein functions and activity within the cell, allowing for increased efficiency and timeliness in response to both internal factors and external cues. As an example, the most common post-translational modification of proteins is phosphorylation. The addition of a negatively-charged phosphate group changes the topology and therefore the functions or interactions of its target proteins, which are often involved in signal transduction pathways that modulate almost every cellular function, including transcriptional control, cell cycle progression and metabolism (Walsh, 2006; Karin and Hunter, 1995; Summers et al., 2011; Graves and Krebs, 1999). Of importance for maintaining cellular homeostasis and responding appropriately to changing conditions, phosphorylation is reversible – phosphate groups are added to their targets by a class of enzymes called kinases and are taken off by phosphatases (Walsh, 2006).

Ubiquitin

In contrast to phosphate, which is a chemical group, proteins can also be covalently modified by small proteins. In eukaryotes, one widely conserved small protein post-translational modifier is ubiquitin, which is part of the family of ubiquitin-like proteins (Ubls). Ubiquitin is a 76 amino acid protein (~8 kDa in molecular weight) that was named for its ubiquitous presence within cells and across eukaryotic species. It contains a C-terminal diglycine repeat, through which it is conjugated to a lysine residue on its

target protein, forming an isopeptide bond (Walsh, 2006; Hershko and Ciechanover, 1998). The mechanism of conjugation for the Ubl family consists of a three enzyme cascade that occurs in a stepwise fashion. The final carboxyl group of the Ubl is activated as a thioester by the E1 activating enzyme in an ATP-dependent manner, and consequently transferred to the E2 conjugating enzyme. Finally, an E3 ligating enzyme facilitates the transfer of ubiquitin to the ϵ -amino group of the lysine residue on its intended target protein, conferring high substrate specificity (Walsh, 2006; Kerscher et al., 2006). In the budding yeast *Saccharomyces cerevisiae*, there is only one E1 ubiquitin activating enzyme, Uba1 (McGrath et al., 1991). In contrast, there are several E2 ubiquitin conjugating enzymes: Ubc1-8, -10, -11, and -13. Two E2s of note are Ubc4 and Ubc6, both of which are implicated in the ubiquitylation of proteins that will subsequently be degraded (Hochstrasser, 1996). E3 Ubl ligases fall into two main categories defined by their catalytic domain. Those with a Homology to the E6AP Carboxyl Terminus (HECT) domain bind the Ubl directly, forming a thioester intermediate before transferring the Ubl to its target (Metzger et al., 2012). Other proteins have a Really Interesting New Gene (RING) domain: a zinc-finger variant that consists of a combination of cysteine and histidine residues which coordinate two zinc ions in a cross-brace structure to maintain the catalytic function of the protein (Saurin et al., 1996; Perry et al., 2008) (FIG 1). RING domain E3 ligases do not form an intermediate with the Ubl (e.g. ubiquitin), but instead facilitate its transfer from the E2 to the target protein (Metzger et al., 2012). Like phosphorylation and dephosphorylation, ubiquitylation is reversible; ubiquitin molecules can be cleaved off their targets by a family known as de-ubiquitylating (DUB) enzymes (Hochstrasser, 1996).

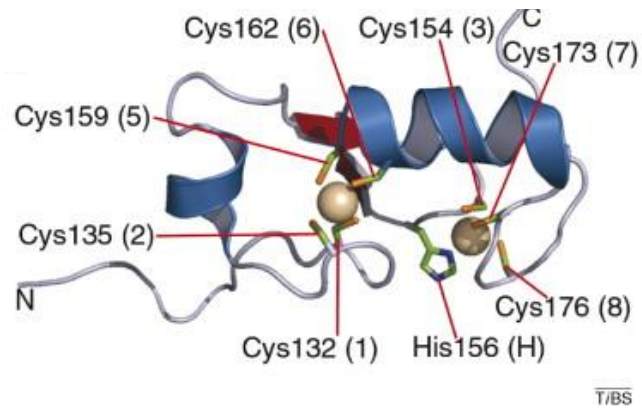


Figure 1: Three dimensional structure of a really-interesting new gene (RING) domain
 The solution structure of the core of RNF4, a SUMO-targeted Ubiquitin E3 ligase in humans that contains a RING domain necessary for its catalytic function. The light gray balls are Zn²⁺ atoms, coordinated in a cross-brace structure by conserved cysteine and histidine residues. PDB code: 2ea6. Figure from Perry, Tainer, and Boddy, 2008.

Since ubiquitin itself contains lysine residues, the above conjugation process can be repeated, forming chains. Ubiquitin has seven internal lysines – Lys 6, 11, 27, 29, 33, 48 and 63 – allowing multiple types of chains to form, each conferring different functions to the original target protein (Peng et al., 2003). Most prominently, chains of at least four ubiquitins linked through lysine-48 (K48) target the original substrate protein for degradation as a method of recycling proteins, which is crucial for cellular homeostasis (Johnson et al., 1995; Herrmann et al., 2007). K48 polyubiquitylated proteins are either escorted by chaperone proteins to or are directly recognized by the 26S proteasome, which unfolds proteins and feeds them into its barrel-shaped core with proteolytic activity (Herrmann et al., 2007).

Ubiquitin modification can also have non-proteolytic functions in multiple cellular processes. For example, polyubiquitylation has a role in the immune response: K63 chains have been implicated in protein kinase activation in the interleukin-1 and Toll-like receptor pathways, as well as multiple pathways that lead to the activation of NF- κ B, an

important transcription factor. Ubiquitylation is also involved in the coordination of DNA damage repair: K63 polyubiquitylation of histone proteins helps recruit the BRCA1 complex to double stranded DNA breaks so that they can be repaired by homologous recombination (Chen and Sun, 2009). Similarly, monoubiquitylated FANCD2 binds BRCA2 to participate in DNA damage repair, followed by deubiquitylation and cell cycle continuation, a process that is disrupted in patients with Fanconi anemia (Zhang et al., 2007). Monoubiquitination has other functions as well, including gene silencing and activation, endosomal trafficking, and internalization of receptors (Johnson, 2002).

SUMO

Another common member of the Ubl family is small ubiquitin-like modifier (SUMO), which is a 110-amino acid protein. SUMO is ~11kDa in size but appears to add ~20kDa to its target protein upon SDS-PAGE analysis (Johnson, 2004). Though SUMO and ubiquitin only have 18% sequence homology, they share a characteristic beta-grasp fold in their three-dimensional structure (Johnson, 2004; Perry et al., 2008). Similar to ubiquitin, SUMO is conjugated onto lysine residues in a three enzyme cascade which can be repeated, forming chains. However, unlike ubiquitin's classic role in proteasomal degradation, chains of SUMO typically alter the interactions, localization, activity and stability of its target protein.

The SUMO gene was first discovered in budding yeast *S. cerevisiae* as a high-copy suppressor of a mutation in the centromeric protein Mif2; thus budding yeast's one SUMO protein is known as Smt3 (Meluh and Koshland, 1995). There are three legitimate versions of the SUMO protein in humans: SUMO-2 and SUMO-3 are 97% homologous and both have internal lysines, allowing them to form chains. SUMO-1 shares only 50%

homology with SUMO-2/3 and does not have internal lysines, thereby terminating any chains into which it is incorporated (Geiss-Friedlander and Melchior, 2007).

SUMO is originally synthesized in a precursor form that is processed by the SUMO protease Ulp1, exposing a diglycine repeat at the C-terminus end of the protein (Hay, 2007). This conjugation-competent SUMO is activated by its E1 enzyme, the heterodimer Aos1-Uba2, and then passed on to its E2 conjugating enzyme, Ubc9. Ubc9 directly binds a SUMO attachment consensus sequence, ΨKXE, where Ψ is any hydrophobic amino acid, K is the lysine with which the isopeptide bond is formed, X is any amino acid residue, and E is glutamic acid (Johnson, 2004). *In vitro*, Ubc9 is actually capable of catalyzing the isopeptide bond between the lysine's ε-amino group and SUMO itself (Okuma et al., 1999).

Siz1 – a SUMO E3 ligase in budding yeast

In vivo, the transfer of SUMO to the target lysine is facilitated by an E3 ligase. There are four E3 SUMO ligases in *S. cerevisiae* – Siz1, Siz2, Mms21 and Zip3 (Johnson, 2004; Potts and Yu, 2005; Cheng et al., 2006). Siz1 and Siz2 carry out the majority of sumoylation in yeast and are homologous to the protein inhibitor of activated signal transducer and activator of transcription (PIAS) family of SUMO E3 ligases in humans. Siz1 acts in both the nucleus and cytoplasm, while Siz2 remains mostly nuclear (Takahashi et al., 2001; Ferreira et al., 2011; Pasupala et al., 2012). The PIAS/Siz proteins contain several domains, including an SP-RING domain, similar to that of the RING type ubiquitin E3 ligases, which is required for the protein's ligation activity (Takahashi et al., 2005; Palvimo, 2007). They also contain SUMO-interacting motifs (SIMs) to interact with sumoylated or SUMO-mimetic proteins. Interestingly,

phosphorylation adjacent to SIMs on PIAS1 is instrumental for the function of its SIM. This phosphate modification is not involved in the E3 ligase activity of the protein, but does affect its role as a transcriptional coregulator (Stehmeier and Muller, 2009). Additional domains in Siz1 contribute to substrate specificity: for example, the PINIT domain towards the N-terminal end of this E3 ligase is required for sumoylation of proliferating cell nuclear antigen (PCNA), a replication processivity factor, in the nucleus, whereas the C-terminal domain is required for *in vivo* sumoylation of the septin proteins at the bud neck of the dividing yeast cell (Reindle et al., 2006). In addition to those targets, Siz1's human homolog, PIAS1 also sumoylates p53, a crucial cell cycle regulator and tumor suppressor protein, and c-Jun, a similarly important transcription factor (Melchior et al., 2003). Siz1, like many E3 ligases, also has the capacity for auto-sumoylation (Kotaja et al., 2002).

Siz1 is 904 amino acids at full-length, but a C-terminal truncation of its last 440 amino acids, dubbed Siz1 Δ 440, is often used in this study. Without this C-terminal tail, Siz1 Δ 440 appears to be more stable, but still contains the major functional domains of the protein, including the DNA-binding SAP domain, the PINIT domain, and the RING domain for E3 ligase activity. Accordingly, it still promotes *in vitro* sumoylation of a target septin, Cdc3, and also possesses *in vitro* auto-sumoylation activity (Takahashi and Kikuchi, 2005, FIG 2).

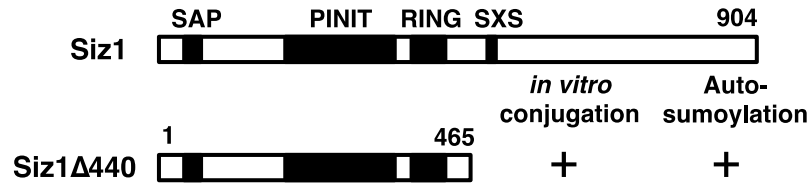


Figure 2: Domains and activity of Siz1 and Siz1Δ440

Full length Siz1 stretches 904 amino acids and contains a DNA binding SAP domain (amino acids 34-68), a PINIT motif (181-349), a catalytic SP-RING domain (352-409), and an SXS motif around amino acid 409. The truncation Siz1Δ440 still contains most major domains, with the SP-RING domain exposed at its C-terminal end. Accordingly, Siz1Δ440 maintains *in vitro* conjugation and auto-sumoylation abilities, making it appropriate to use in studies in place of full length Siz1. Figure modified from Takahashi and Kikuchi, 2005.

In contrast to ubiquitin's main role in targeting proteins for proteasomal degradation, SUMO modification tends to alter protein stability, localization, interactions, and activity. For example, the sumoylation of the septins during mitosis may help with the disassembly of the septin ring and completion of cytokinesis (Johnson and Blobel, 1999; Takahashi et al., 2008). Additionally, SUMO chains are formed in response to heat shock and conjugated to a wide range of proteins involved in cell cycle regulation, transcription, and DNA damage repair, among many other processes (Golebiowski et al., 2008).

Crosstalk between SUMO and Ubiquitin

Given the similarities between the mechanics of the ubiquitin and SUMO pathways, it is no surprise that there is interplay between the two modifications. Both Ubqs are conjugated to lysine residues, creating an opportunity for competition in binding sites. This occurs in the case of post-translation modification of IκBα, the inhibitor of NF-κB. When IκBα is polyubiquitylated on K21, it is degraded, thus activating NF-κB. However, if SUMO-1 is conjugated to K21, ubiquitin-targeted degradation is prevented, and NF-κB stays inactive. Phosphorylation also plays a role in these dynamics –

ubiquitylation requires phosphorylation of certain serine residues on I κ B α , while sumoylation is inhibited by phosphorylation. In this situation, SUMO and ubiquitin have antagonistic roles, designating their protein target to vastly different fates (Desterro et al., 1998).

Proliferating cell nuclear antigen (PCNA), a DNA-encircling protein that increases the processivity of DNA polymerases and is also a binding platform for various enzymes, also becomes both ubiquitylated and sumoylated. PCNA is polyubiquitylated on K164 in response to DNA damaging agents, allowing it to pass over lesions and continue replication of undamaged DNA. During S phase, independent of DNA damage, PCNA can also be sumoylated at the same lysine (Papouli et al., 2005). It was originally hypothesized that the two modifications compete for the binding site and have antagonistic roles (Hoegge et al., 2002). However, it appears that the two modifications actually cooperate in controlling various repair pathways at stalled replication forks (Papouli et al., 2005).

SUMO-Targeted Ubiquitin Ligases

The discovery of a new class of SUMO-targeted ubiquitin ligases (STUbLs) offers a more direct connection between the SUMO and ubiquitin pathways and also implicates SUMO in proteasomal degradation. The formative member of the STUbL family was the budding yeast heterodimer Slx5/Slx8. The *SLX5* and *SLX8* genes were identified from a synthetic lethal screen as required for viability in the absence of Sgs1, a RecQ DNA helicase (Mullen et al., 2001). Slx5 was additionally identified as a high-copy suppressor of a SUMO protease mutant, *ulp1ts*, which is lethal when shifted to the non-permissible temperature, thus indicating a role for Slx5 in SUMO dynamics and cell health (Xie et al.,

2007). Slx5 and Slx8 co-immunoprecipitate from cell extracts, forming a heterodimer that can stimulate the ubiquitylation of substrates *in vitro*. (Mullen et al., 2001, Ii et al., 2007). Slx5 is the targeting subunit of this heterodimer, since it contains SIMs that non-covalently interact with sumoylated target proteins (Perry et al., 2008). However, sumoylation of a protein is not required for it to be a substrate of the STUbL, as exemplified by the yeast transcriptional factor *mat α 2* (Xie et al., 2010). Slx8 is the catalytic subunit of the heterodimer, as it displays RING-dependent auto-ubiquitylation activity in the absence of Slx5 (Xie et al., 2007).

Yeast cells lacking the *SLX5* gene are sensitive to genotoxic stress by UV radiation and hydroxyurea treatment, which stalls replication forks by depleting nucleotides (Mullen et al., 2001; Cook et al., 2009). Accordingly, both the yeast Slx5 and the human homolog of Slx5/Slx8, RNF4, reside at dsDNA breaks, implicating a role in repair (Cook et al., 2009; Galanty et al., 2012). A DNA repair and homologous recombination protein, Rad52, is ubiquitylated *in vitro* by Slx5/Slx8 in a manner enhanced by Rad52 sumoylation (Xie et al., 2007). Furthermore, it has recently been described that RNF4 creates mixed SUMO-ubiquitin chains that signal the recruitment of Rap80, followed by BRCA1, to resolve dsDNA breaks (Guzzo et al., 2012).

STUbLs have also been directly implicated in human cancers. RNF4 interacts with the HTLV-1 oncoprotein Tax, causing it to relocalize from the nucleus to the cytoplasm during DNA damage, releasing DNA damage repair proteins in the nucleus and activating NF- κ B pathways from the cytoplasm to induce an anti-apoptotic state in its host (Fryrear et al., 2012). Additionally, arsenic treatment of acute promyelocytic leukemia (APL) induces the sumoylation of the cancerous fusion protein PML-RAR α ,

followed by the recruitment of RNF4 to subsequently ubiquitylate and degrade the oncoprotein (Lallemand-Breitenbach et al., 2008). Interestingly, phosphorylation seems to be involved in this pathway: PML is phosphorylated through a mitogen-activated protein kinase pathways, which was associated with increased sumoylation and subsequent degradation of PML. Accordingly, a phosphorylation-defective PML mutant did not respond to arsenic treatment (Hayakawa and Privalsky, 2004). Phosphorylation may also help regulate RNF4 targeting to its substrates via an arginine rich region that is just downstream of its SIMs. This positively charged region strongly interacts with a negatively charged phosphate group, thus offering a mechanism for the interaction of RNF4 with phosphorylated (and sumoylated) proteins (Kuo et al., 2012).

Crosstalk between phosphorylation, sumoylation and ubiquitylation

As described earlier in examples with $\kappa B\alpha$ and PML, phosphorylation also seems to be involved in the regulation of sumoylation and ubiquitylation. In another key example, the human flap endonuclease, FEN1, which is crucial for cell cycle progression and genome maintenance, is phosphorylated. This stimulates its modification by SUMO-3, which in turn stimulates its ubiquitylation by a putative STUbL PRP19 (Guo et al., 2012). By contrast, phosphorylation of the transcription factor Sp1 stabilizes the protein by preventing the binding of and ubiquitylation by RNF4 (Ulrich, 2012). Thus, multiple types of crosstalk between these three modifications help tightly regulate the activity of their target proteins in dynamic situations, making them crucial for cell health.

Novel targets and functions of STUbLs

Though all three modifications discussed are of utmost importance, our lab focuses on the role of STUbLs to connect SUMO and ubiquitin. Our goal is to identify new targets of

the Slx5/Slx8 heterodimer to further unravel its specific role within the yeast cell, which can then be applied to other eukaryotes. In truncation studies of Slx5, we identified the regions required for interaction with Slx5, Slx8 and SUMO, as well as a novel yeast two-hybrid interactor, the SUMO ligase Siz1 (Westerbeck et al., manuscript in preparation, FIG 3). The regions of Slx5 that interact with SUMO and Siz1 are strikingly similar, indicating that the interaction with Siz1 may be SUMO-dependent or SUMO-enhanced.

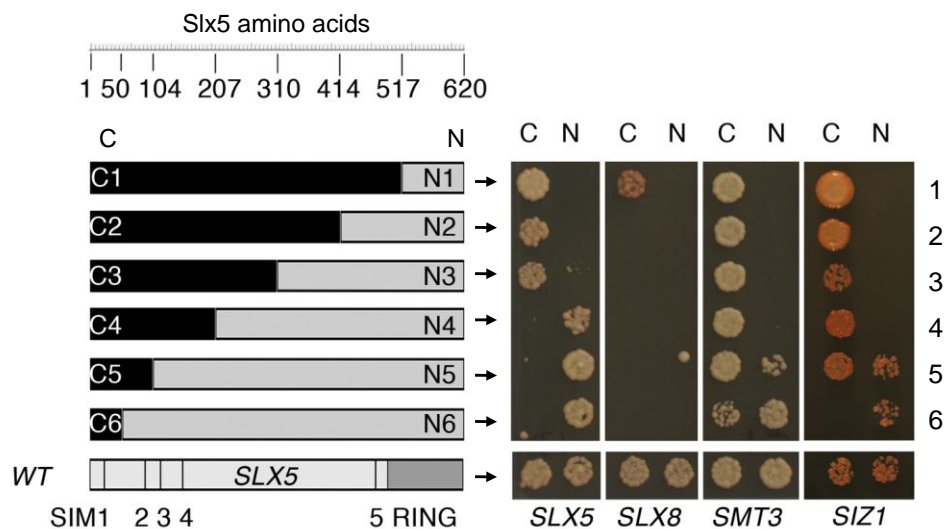


Figure 3: Slx5 structure function studies

Six C-terminal and N-terminal truncations of Slx5 were created, and along with full-length Slx5, were used in yeast two-hybrid assays against known (Slx5, Slx8, Smt3) and novel (Siz1) interactors. Growth indicates that the two proteins being tested interact with each other in the yeast two hybrid system. Note that the truncations of Slx5 that interact with Smt3 and Siz1 are strikingly similar, indicating that SUMO interacting motif (SIM)-containing regions of Slx5 are required for both interactions, and potentially that Slx5's interaction with Siz1 is SUMO-dependent. Figure by Jason Westerbeck.

Further experiments showed that Slx5 and Siz1 interact in an *in vitro* pulldown assay, and that Slx5 ubiquitylates Siz1 *in vitro*, indicating that Siz1 is in fact a target of Slx5 (Westerbeck, et al., manuscript in preparation; Matson, 2011 thesis; FIG 4, FIG 5).

However, we had yet to show any *in vivo* interaction, a crucial piece of evidence for physiological relevance. Interestingly, there is some precedence for the coordination and

cooperation of SUMO E3 ligases and STUbLs, as the fission yeast SUMO ligase Nse2, STUbL and STUbL target Rad60 together have a role in suppressing DNA damage and genomic instability (Heideker et al., 2011). Describing a novel physical and functional interaction between a SUMO ligase and a STUbL in budding yeast could significantly add to our understanding of SUMO and ubiquitin dynamics and interplay between machinery of the two systems for a role in DNA damage or cell cycle progression.

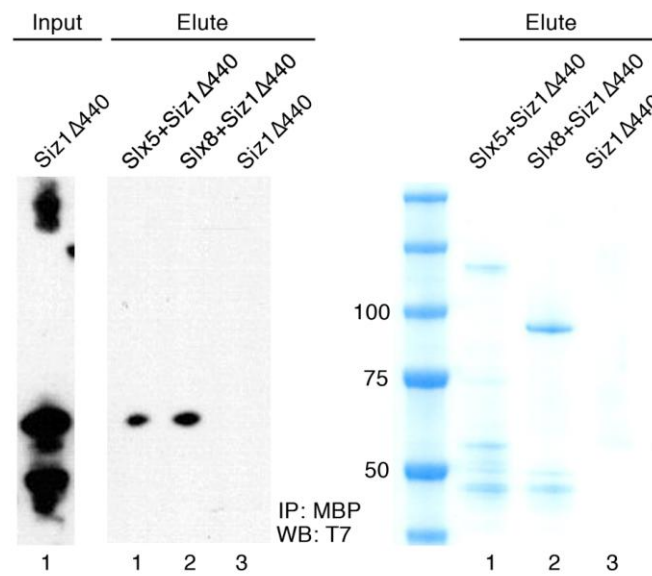


Figure 4: Siz1Δ440 interacts with Slx5 and Slx8 in an *in vitro* pulldown assay

Maltose binding protein (MBP)-Slx5, T7-Siz1Δ440, and MBP-Slx8 were expressed by IPTG induction in *E. coli*. Cultures were mixed together accordingly (Slx5 with Siz1Δ440, Slx8 with Siz1Δ440, or Siz1Δ440 alone) and subjected to immunoprecipitation with amylose resin to bind MBP. Elutions were western blotted with anti-T7 for Siz1Δ440, which was only detected when expressed in combination with MBP-Slx5 or MBP-Slx8, indicating that the two proteins physically interact *in vitro*. Figure by Brooke Matson.

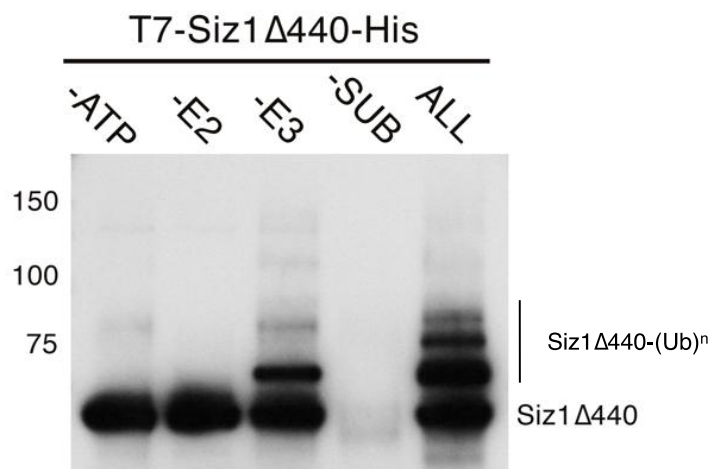


Figure 5: Slx5/Slx8 ubiquitylates Siz1 Δ 440 in an *in vitro* ubiquitylation assay

E1 (Uba1), E2 (Ubc4), E3 (Slx5/Slx8 STUbL), ATP, substrate (SUB, Siz1 Δ 440), and ubiquitin were added together in the above combinations at 30°C for 3 hours. Samples were separated by SDS-PAGE and probed with anti-T7 to visualize Siz1 Δ 440 and its modification status. Mono-ubiquitin was achieved without the E3 ligase, but Siz1 Δ 440 was poly-ubiquitylated only in the presence of all materials, indicating that it is an *in vitro* substrate of Slx5/Slx8. Figure by Brooke Matson.

Specific Aims

The specific aims of this thesis are as follows:

1. Develop reliable methods for extraction and purification of native proteins from budding yeast.

Our lab lacks a consistently successful technique for extracting and purifying proteins, which is especially necessary for *in vivo* studies involving ubiquitin, SUMO and phosphate modification.

2. Confirm an interaction between the STUbL subunit Slx5 and the SUMO ligase Siz1 *in vivo*.

We will add to the yeast two-hybrid and *in vitro* evidence of the novel interaction between Slx5 and Siz1 by performing an *in vivo* pulldown assay.

3. Determine if Siz1 is a target for STUbL-mediated ubiquitylation *in vivo*.

Slx5/Slx8 target Siz1 for ubiquitylation *in vitro*, which we aim to confirm *in vivo* using whole cell extracts and purified proteins.

MATERIALS AND METHODS

Yeast Growth Methods

Yeast media (YPD and SD) were prepared as described in Appendix A and were used for growth of all yeast strains, unless otherwise noted. Yeast strains were grown at 30°C.

Yeast Strain Constructions

All yeast strains used in this study are indicated by a "YOK" number and listed in Table 3 (page 40). Additionally, commonly used constructs are listed in Table 2. To create deletion strains of *SLX5* and *MSN5* in the JD52 background, deletion cassettes were PCR amplified from either an *slx5Δ::kanMX4* strain (YOK 747) with primer pairs corresponding to regions 280 base pairs upstream and 300 base pairs downstream from the ORF (OOK 275 and 276) or from an *msn5Δ::hygromycin* pAG32 plasmid with primers OOK 763 and 764. The *msn5Δ::hygromycin* cassette was subject to re-PCR to elongate its upstream and downstream overhangs (OOK 767 and 768). The resulting fragments of each were then transformed into wild-type JD52 (YOK 2062) and *siz1-13xmyc/HIS5* (YOK 2397) strains, generating *slx5Δ* (YOKs 2373 and 2376), *msn5Δ* (YOK 2505), *slx5Δ siz1-13xmyc/HIS5* (YOK 2591) and *msn5Δ siz1-13xmyc/HIS5* (YOK 2514). Double deletion *slx5Δ msn5Δ siz1-13xmyc/HIS5* strains were created by subsequently transforming both deletion cassettes into the *siz1-13xmyc/HIS5* parent. Deletion strains were confirmed by PCR and restriction digest (to confirm *msn5Δ::hygromycin*, primer pairs corresponding to 230 base pairs upstream and 180 basepairs downstream from the ORF were used – OOK 761 and 762). Additionally, *SIZ1* was tagged with 13xmyc in confirmed *slx5Δ* or *msn5Δ* deletion strains using protocols

from Gauss et al., 2005 (tagging done by M. Guillotte). While *msn5Δ* strains were not immediately used in this study, they are currently being used in further experiments.

Large Scale Protein Induction, Extraction and Purification

(see also Appendix A; Szymanski and Kerscher, accepted manuscript)

Yeast strains were grown in 33ml of the appropriate selective media with 2% sucrose until they reached log phase ($OD_{600} = 0.5-1.5$). At this point, 17 ml of 3x YEP with 6% galactose was added to each culture, for a final concentration of 1x YEP and 2% galactose in a final volume of 50ml. Cells were grown for another 6 hours before harvesting. A 100-200 OD cell pellet was washed with 1x ice cold PBS plus 1x protease inhibitor cocktail (Thermo Scientific, #1860932), snap frozen in liquid nitrogen and stored at -80°C until further use. To extract proteins, frozen cell pellets were lysed in 500 μl of desired cell lysis buffer (see Table 1 for a list of buffers) with appropriate protease inhibitors and 200 μl of acid washed beads (425-600 μm , Sigma-Aldrich) in an Omni Bead Ruptor 24 (six 20 second pulses with 1 minute on slushy ice in between each pulse). The lysate was clarified by centrifugation at 15,000 rpm for 15 minutes at 4°C . 4ODs of clarified lysate were prepared as whole cell extract by the addition of five volumes of 20% TCA, followed by a wash with 2% TCA and resuspension in 200 μl TCA sample buffer with BME by extensive vortexing and a 2-5 minute incubation at 110°C . To purify select proteins, 100 μl of clarified lysate (corresponding to 20 ODs) was added to 50-100 μl of affinity resin that had been washed 5 times with desired wash buffer, and the final volume was increased to 1ml with cell lysis buffer plus protease inhibitors. Extracts were nutated top over bottom with the resin for 2-5 hours at 4°C . The resin was then washed five times with wash buffer. Affinity resin-bound proteins were eluted by

top over bottom nutation with 100 µl of elution buffer for five minutes. Two to three elutions were performed and subsequently pooled, followed by preparation in LDS-sample buffer.

RING Domain Protein Purification

To determine if RING domain proteins intrinsically bind metal affinity resin, pAG425-GAL1-ccdB-Siz1 Δ 440-HA (BOK 795) and GAL1/10-GST-Slx5 (BOK 629, OpenBiosystems Yeast GST collection YSC4515-202484078) were transformed into JD52 wild type background (YOK 2062), creating YOKs 2353 and 2071, respectively. YOK 2397, with endogenously tagged Siz1-13xmyc, was also used. As a positive control, pYES2.1-GAL-Slx5-V5/His₆-TOPO (BOK 390) was transformed into a JD52 background, creating YOK 2096. Transformants were grown by large scale induction as described above, with endogenously expressing strains receiving 3xYEP/6% dextrose at the galactose induction step. The cell lysis buffer used was Buffer C (see Table 1 for this and all further buffers). To test the purification of native proteins, 100µl of clarified lysate was added to 100 µl of TALON metal affinity resin (Clontech, 635502) or 100µl of uncharged amylose resin that had been washed five times with Buffer G, and the final volume was increased to 1ml with Buffer C with a different brand of protease inhibitor cocktail (Thermo Scientific, #1860932). Denatured proteins were prepared by the same method, except that guanidinium hydrochloride was added to 6M before extracts were added to the resin. Extracts were rotated top over bottom with the TALON resin for 3 hours at 4°C. The resin was then washed five times with Buffer G. TALON-bound proteins were eluted by incubation with 150 µl of Buffer F for five minutes. Two elutions were performed and subsequently pooled together. Western Blotting to analyze

protein extraction and purification of V5/His₆-, GST-, myc- and HA-tagged proteins was performed as described below.

Co-immunoprecipitation assay

To determine if Slx5 and Siz1 interacted *in vivo*, GAL1/10-GST-Slx5 (BOK 629, OpenBiosystems Yeast GST collection YSC4515-202484078), pAG425-GAL1-ccdB-Siz1 Δ 440-HA (BOK 795) or both GST-Slx5 and Siz1 Δ 440-HA were transformed into *ubc4 Δ ubc6 Δ mata Δ* cells (YOK 2501, from Xie et al., 2010), and saved as YOKs 2507, 2508, and 2509, respectively. Transformants were grown in 33ml of SD –uracil, –leucine or –leucine–uracil, respectively, induced and proteins were extracted and purified as described above. The cell lysis buffer used was Buffer H. To purify GST-tagged and co-purifying proteins, 100 μ l of clarified lysate was added to 100 μ l of immobilized glutathione agarose (Thermo Scientific, #15160) that had been washed five times with Buffer I, and the final volume was increased to 1ml with Buffer H containing a different protease inhibitor cocktail (Thermo Scientific, #1860932). Extracts were nutated top over bottom with the glutathione agarose for 2.25 hours at 4°C. The agarose was then washed five times with Buffer I. 2ODs of the flow through were saved, and proteins were precipitated by TCA as described above for the whole cell extract. Glutathione agarose-bound proteins were eluted with 100 μ l of Buffer J for five minutes. Three elutions were performed and subsequently pooled. Western Blotting to analyze protein extraction and purification of GST- and HA-tagged proteins was performed as described below.

SUMO-FLAG shift assay

To confirm the SUMO modification on Siz1 Δ 440, a pYES2.1-GAL1-V5/His6 vector containing the Siz1 Δ 440 ORF (BOK 794) was transformed into *ulp1 Δ ulp1ts* yeast cells also expressing FLAG-Smt3gg (YOK 428) or Smt3gg (YOK 430). Transformants were grown overnight, spinning at 30°C in SD-uracil-leucine media with 2% sucrose. At log phase (OD₆₀₀ = 1.0-1.6), each culture was equally divided, spun down, and the cells were resuspended in either 4ml of SD-uracil-leucine with 2% dextrose or 4ml of SD-uracil-leucine with 2% galactose and 2% sucrose. After a 5 hour induction, 4ODs of cells were harvested from each culture and proteins were extracted by TCA protein precipitation. V5- and FLAG-tagged proteins were visualized by Western Blotting as described below.

Determining the Ubiquitylation Status of Siz1 Δ 440

To determine if Siz1 Δ 440 was ubiquitylated and to test whether that ubiquitylation was Slx5 dependent, a pYES2.1-GAL1-V5/His6 vector containing the Siz1 Δ 440 ORF (BOK 794) was transformed into JD52 wildtype (YOK 2062) and JD52 Slx5::kan (YOK2376) along with either the empty 2 μ TRP vector pRS424 (BOK342) or pRS314-CUP1-Ubiquitin(G76A)-myc-Cyc1 (BOK309), creating YOKs 2377-2382. Transformants were grown in SD-tryptophan-uracil as described in the large scale induction protocol above. After 2 hours of galactose induction, cultures were copper-induced with the addition of 100 μ M CuSO₄ and allowed to grow for 3 more hours. 4ODs of cells were removed and proteins were extracted by TCA protein precipitation, while the remaining cells were washed in 1xPBS with 1x protease inhibitor cocktail and snap-frozen with liquid nitrogen. V5- and myc-tagged TCA-extracted proteins were analyzed by Western Blotting as described below. The blot was also reprobbed for PGK as a loading control.

Western Blot







Proteins were separated by SDS-PAGE on either a pre-cast NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen, NP0321) for 50 minutes at 200V in 1x MOPS buffer. Proteins were transferred to PVDF membrane (Millipore, IPVH00010) by semi-dry transfer in 1x semi-dry transfer buffer (10x: 58g Tris, 29.3 Glycine, 18.75ml 20% SDS in 1L ddH₂O; 1x: 100ml 10x semi-dry, 200ml methanol, 700ml ddH₂O) for 25 minutes at 19V. Blots were blocked in 1x TBST containing 4% milk (10x TBS: 50ml 1M Tris-HCl pH 8.0, 150ml 5M NaCl, 300ml ddH₂O; 1x TBST: 100ml 10xTBS, 900ml ddH₂O, 1ml TWEEN-20) for an hour at room temperature and subsequently incubated with 4% milk/1x TBST containing primary antibody overnight at 4°C. antibodies used. After three five minute washes with 1x TBST, blots were incubated with secondary antibodies for 1-3 hours at room temperature and then washed with 1x TBST three times for 20 minutes. Blots were incubated with chemiluminescent substrate (Millipore, WBKLS0100), wrapped in plastic wrap, exposed to x-ray film and subsequently developed. GST-tagged proteins were detected with anti-GST antibody (1:5000 dilution; Abcam, ab6613) and an anti-goat secondary (1:10000; Santa Cruz Biotechnology sc-2020). HA-tagged proteins were detected with an anti-HA antibody (1:10000; Abcam, ab9110) and an anti-rabbit secondary (1:10000; Abcam, ab6721). V5-tagged proteins were detected with an anti-V5 antibody (1:10000; Invitrogen, R960-25) and an anti-mouse secondary (1:15000; Abcam, ab97040). Myc-tagged proteins were detected with a 9E10 anti-myc antibody (1:5000; Covance, MMS-150R) and an anti-mouse secondary (1:10000). FLAG-tagged proteins were detected with an anti-FLAG antibody (1:10000; Sigma, F3165) and an anti-mouse secondary (1:10000). PGK served as a loading control and was detected using anti-PGK

(1:300000; Invitrogen, A6457) and secondary anti-mouse (1:30000). Ubiquitin was detected with an anti-Ubi antibody (1:4000; Covance, MMS-258R) and an anti-mouse secondary (1:10000). Additionally, proteins were visualized on the gel after a 10 minute wash in water with SimplyBlue SafeStain (Invitrogen, LC6060).

Table 1: Buffers for protein extraction and purification

Buffer	Composition	Notes
A	50mM HEPES pH 7.3, 500mM NaCl, 10% glycerol, 10mM imidazole, 1x protease inhibitor cocktail (Thermo Scientific, #1860932), 25mM N-ethylmaleimide (NEM)	Lysis buffer, used in FIG6a, FIG7a to extract Slx5
B	50mM HEPES pH 7.3, 150mM NaCl, 10% glycerol	Wash buffer, used in FIG 8
C	50mM HEPES at pH 7.3, 200mM NaCl, 1% Triton X-100, 10mM imidazole, 1x protease inhibitor cocktail, 25mM NEM	Lysis buffer, used to extract Siz1 proteins in FIG 9, FIG 10b
D	50mM Tris HCl, 150mM NaCl, 1% Triton X-100, 0.1% deoxycholate, 1x protease inhibitor cocktail and 25mM NEM	Deoxycholate lysis buffer, used in FIG10a to extract Siz1 Δ 440
E	200mM imidazole, 50mM Tris-HCl, 200mM NaCl	Elution buffer, used to elute Siz1 Δ 440-V5/His from TALON resin in FIG 10a
F	200mM imidazole, 50mM Hepes, 200mM NaCl	Elution buffer, used to elute Siz1 Δ 440-HA from TALON resin in FIG 10b, FIG 11
G	50mM HEPES at pH 7.3, 200mM NaCl, 1% Triton X-100, 20mM imidazole	Wash Buffer, used to wash TALON resin in FIG 11
H	50mM HEPES at pH 7.3, 200mM NaCl, 1% Triton X-100, 1x protease inhibitor cocktail (Promega, G6521), 25mM NEM, 1mM sodium orthovanadate	Lysis buffer, used for Slx5-Siz1 co-purification in FIG12
I	50mM HEPES at pH 7.3, 200mM NaCl, 1% Triton X-100	Wash buffer, used for Slx5-Siz1 co-purification in FIG12
J	50mM HEPES at pH 7.3, 200mM NaCl, 10mM reduced glutathione [Acros Organics, 120000010]	Elution buffer, used for Slx5-Siz1 co-purification in FIG12
K	Promega Mammalian Cell Lysis Buffer	Lysis buffer, used to extract Siz1 in FIG 9

Table 2: Summary of Slx5 and Siz1 constructs used

Construct	Graphic representation	BOK #	Promoter	High copy? (2μ origin)	Gene	Epitope tag
A		794	GAL1	yes	Siz1Δ440	V5/6xHIS
B		795	GAL1	yes	Siz1Δ440	HA
C		898	GAL1	yes	Siz1	V5/6xHIS
D		--	endogenous	--	Siz1	13xmyc
E		390	GAL1	yes	Slx5	V5/6xHIS
F		629	GAL1/10	yes	Slx5	GST

RESULTS

Testing existing methods for protein extraction from Saccharomyces cerevisiae

In order to successfully complete *in vivo* protein interaction and functional studies with Slx5, it is necessary to extract full-length, non-degraded native proteins. This can be quite difficult to achieve due to the mechanical strength and elasticity of the yeast cell wall and the low abundance of some target proteins such as Slx5. Previous attempts in the lab at *in vivo* co-purification experiments had been largely unsuccessful, prompting us to develop and optimize protein extraction protocols.

A yeast strain containing both Slx5-V5/His₆ and Siz1Δ440-HA (YOK 2354) (constructs E and B in Table 2) was grown and the cell pellet saved as described in Materials and Methods. Cell pellets were added with 200μl of glass beads and 500μl of Buffer A and subjected to bead beating for 10 cycles of 20 seconds at 5.5m/s followed by 1 minute on slushy ice. Proteins were clarified and WCE prepared in LDS-sample buffer, and the sample was separated by SDS-PAGE. Upon western blotting and probing with anti-V5, some full length Slx5 was detected, but there were many degradation products (FIG 6a). Additionally, staining the gel for overall proteins revealed a smear of proteins with no discrete bands, indicating a low-quality protein preparation (FIG 6b).

Extraction of full length Slx5

While the above bead-beating methods showed promise, further optimization was required to consistently extract and visualize full-length proteins to be used in subsequent experiments. Therefore, the procedure was modified as follows: Slx5-V5/His₆ and Siz1Δ440-HA (YOK 2354) (constructs E and B in Table 2) were co-expressed by galactose induction, and cell pellets were washed in 1x PBS with protease inhibitors

before being snap frozen in liquid nitrogen. 500µl of Buffer A was added to each pellet, which was subjected to bead beating 10 times for 20 seconds each with 1.5 minutes on slushy ice in between each cycle. Cell lysate was clarified by centrifugation for 10 minutes at 4°C, followed by centrifugation in a SpinX filter tube at 15,000 rpm for 5 minutes at 4°C, and WCE was prepared for SDS-PAGE by TCA precipitation rather than sample buffer. 4ODs of WCE was added to 800µl of 20% TCA, washed in 800µl 2% TCA, and resuspended in 200µl of TCA sample buffer with BME followed by a 2 minute incubation at 100°C (see Appendix A for full method). Preparation of whole cell extracts in this manner was much more successful, leading to the visualization of full-length Slx5 upon western blotting with anti-V5 (FIG 6c). Additionally, staining a gel of WCE samples prepared in TCA reveals many discrete bands, indicating a high quality protein extraction (FIG 6d).

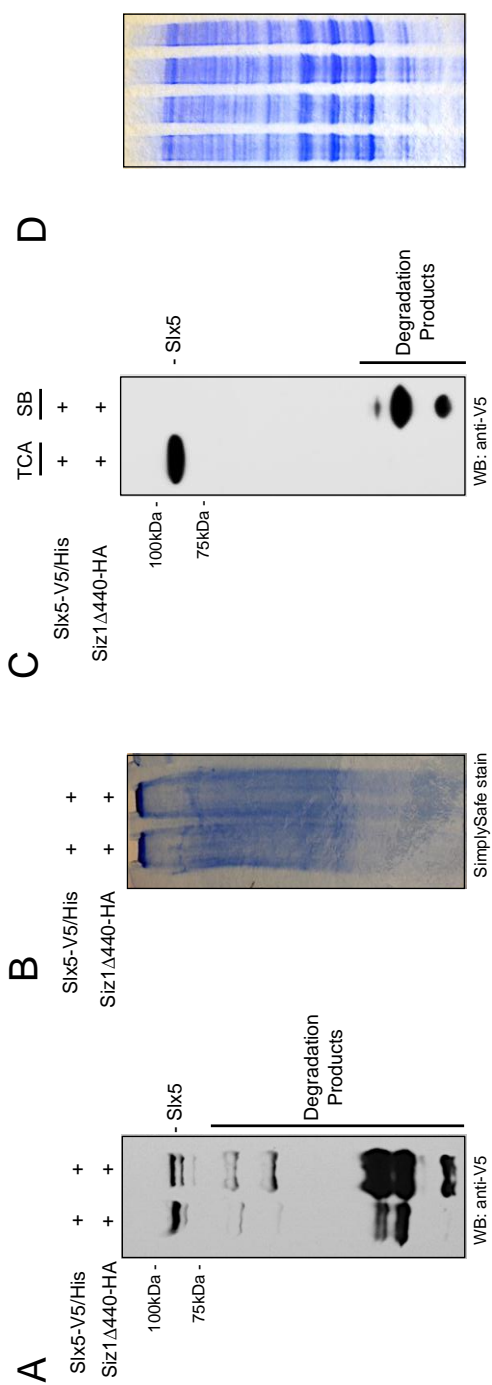


Figure 6: Existing methods for protein extraction, purification, and visualization need to be further optimized
Six5-V5/His and Siz1Δ440-HA were co-overexpressed in yeast (YOK 2354). Proteins were extracted in Buffer A with ten cycles of bead beating. (A) Whole cell extracts were prepared in sample buffer before separation by SDS-PAGE and subsequent western blotting (WB) with anti-V5 antibody. While some full length protein was extracted, there were also many degradation products. (B) These proteins were also visualized with a gel stain, which revealed a smear of proteins, rather than discrete bands, indicating a low-quality protein preparation. (C) In contrast, whole cell extracts were prepared by TCA precipitation (TCA) or sample buffer (SB) before separation by SDS-PAGE and . The sample prepared in TCA revealed full-length Six5, while the sample prepared in SB only showed degradation products. (D) Representative samples of TCA-precipitated WCE (from YOK 2514 and 2592) were visualized by staining with a gel stain. This stain shows discrete bands, indicating a high quality protein preparation.

Purification of Slx5-V5/His₆ from TALON metal affinity resin

With the successful extraction of full-length Slx5, we needed to confirm that we could purify the protein as well. Slx5-V5/His₆ and Siz1Δ440-HA (YOK 2354) or just Siz1Δ440-HA (YOK 2353) (constructs E and B in Table 2) were galactose-expressed and proteins were extracted as described in the previous section. Clarified lysate was added to 100μl of TALON metal affinity resin, and the samples were nutated for 2 hours at 4°C. The resin was washed with Buffer B and bound proteins were eluted by addition of an equal volume of 2xLDS-sample buffer followed by a 5 minute incubation at 100°C. Western blot analysis with anti-V5 revealed that Slx5-V5/His successfully purified from TALON resin (FIG 7).

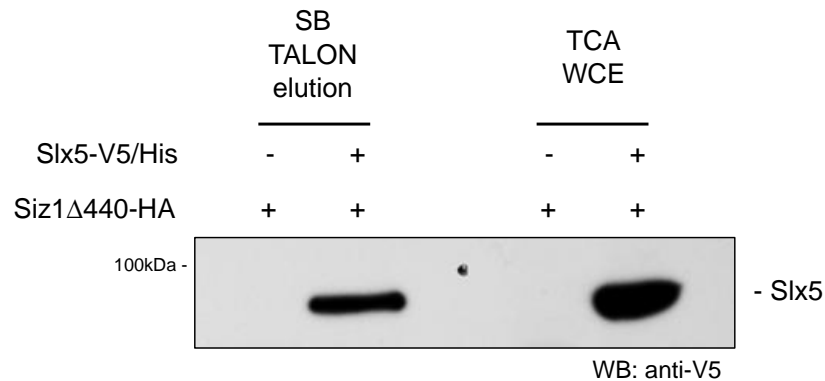


Figure 7: Successful purification of full-length Slx5 from TALON metal affinity resin

Siz1Δ440-HA was expressed in yeast alone or with Slx5-V5/His (YOK 2353 and 2354). 4ODs of cells were prepared by TCA extraction as whole cell extract (WCE). Proteins were extracted from the remaining cells by bead beating in Buffer A. Clarified lysate was incubated with TALON metal affinity resin. After washing the resin with Buffer B, bound proteins were eluted with sample buffer (SB). Separation by SDS-PAGE and subsequent Western Blotting (WB) with anti-V5 antibody reveals Slx5 in both the WCE and the SB elution from TALON resin, indicating that Slx5 can be successfully purified with our protocols.

Extraction of full length and truncated Siz1

Since protocols had now been developed that could successfully extract and purify Slx5-V5/His₆ from TALON metal affinity resin, the ability to extract truncated and full-length versions of Siz1 needed to be confirmed as well, since we were interested in it as Slx5's potential binding partner and in the domains involved in this interaction.

We first expressed the truncated version, Siz1 Δ 440-HA, both alone and with Slx5-V5/His₆ (YOKs 2353 and 2354) (constructs B and E in Table 2). Strains were galactose induced, and proteins were extracted with Buffer C as described in the Materials and Methods, similar to the protocol used to extract full-length Slx5 above. WCEs were prepared in TCA and visualized by western blotting with the anti-HA antibody (FIG 8a). Several high molecular weight adducts above the main Siz1 Δ 440 were observed, indicating that modified forms of the protein exist and were preserved in our preparation. We were also able to extract full-length Siz1-13xmyc (YOK 2397) (construct D in Table 2) from an endogenous promoter in Buffer K (FIG 8c). Again note how modified forms of this protein were preserved with this extraction. We then expressed full length Siz1-V5/His₆ (YOK 2510) (construct C in Table 2) as above, and were able to visualize the full-length protein by western blotting with anti-V5 (FIG 8b). However, this preparation did not yield modified adducts on Siz1. Finally, we expressed Siz1-V5/His₆ (construct C in Table 2) out of multiple backgrounds (YOKs 2510, 2720, and 2721). Cultures were treated with MG132 to inhibit the proteasome (methods in Appendix A), and extracted as above. In this case, modified versions of Siz1 were apparent (FIG 8d).

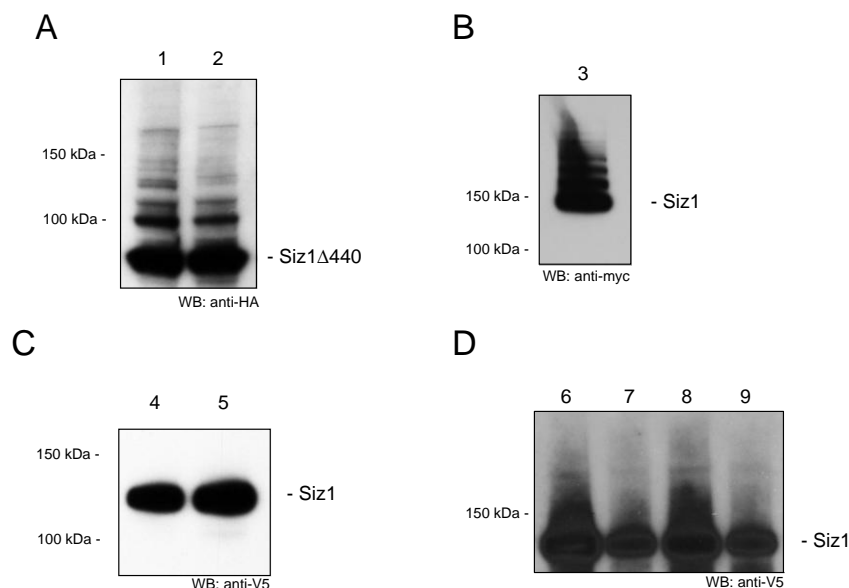


Figure 8: Successful extraction of truncated and full-length Siz1

Whole cell extracts (WCE) of strains expressing various Siz1 constructs were extracted by bead beating with Buffer C and proteins precipitated by TCA. Proteins were separated by SDS-PAGE followed by Western Blotting (WB) with antibodies to HA, V5, or myc epitope tags. (A) Lane 1 and 2: WCE from YOK 2508 and 2509 containing galactose overexpressed Siz1 Δ 440-HA. Note modified versions of Siz1 Δ 440. (B) Lane 3: WCE from YOK 2397 containing endogenous levels of Siz1-13xmyc. Clarified lysate was prepared in Promega lysis buffer. Note sumoylated adducts of Siz1. (C) Lane 4 and 5: WCE from YOK 2510 and 2512 containing galactose overexpressed Siz1-V5/His. Note the absence of sumoylated adducts. (D) Lane 6, 7, 8, and 9: WCE from YOK 2510, 2720, 2721 and 2510, respectively, containing galactose overexpressed Siz1-V5/His. These cells were also subject to proteasome-inhibition by MG132. Note modifications on Siz1.

Purification of Siz1 Δ 440 with and without a 6xHIS tag from TALON metal affinity resin

After successful extraction of truncated and full-length Siz1 protein, we attempted to purify these proteins. This ability would be useful to identify proteins that co-purify with Siz1, for example Slx5. Purified protein could also be used for other downstream assays, such as sumoylation reactions. We expressed Siz1 Δ 440-V5/His₆ (YOK 2377) (construct A in Table 2) by galactose induction and extracted proteins as described previously for Slx5, except here we used Buffer D. Clarified lysates were subjected to purification as described in Materials and Methods using TALON metal affinity resin. The protein bound resin was washed in 50mM Tris-HCl with 0M, 200mM, 500mM or 1M of NaCl to determine the stringency of washes that would still maintain the purified protein.

Proteins were eluted with Buffer E and separated by SDS-PAGE. Subsequent western blots were probed with anti-V5, which revealed that Siz1 Δ 440 was successfully purified in every wash condition, indicating a strong interaction with the resin (FIG 9a).

Additionally, proteins from the previously described strains that contained either Siz1 Δ 440-HA alone or with Slx5-V5/His₆ (YOKs 2353 and 2354) (constructs B and E in Table 2) were extracted and purified on TALON resin as described in Materials and Methods with Buffer C for lysis, Buffer G for wash and Buffer F for elution with the goal of purifying Slx5-V5/His₆ and probing for co-purifying Siz1 Δ 440-HA. However, Siz1 Δ 440-HA consistently purified from the TALON resin in the absence of Slx5-V5/His₆ (FIG 9b and other unpublished observations). We hypothesized that this ability was due to the exposed, native metal-coordinating C-terminal SP-RING domain of the Siz1 truncation, which might have the ability to bind the cobalt ion present on the TALON resin. This finding would indicate that we were actually purifying proteins in their native conformations and warranted further investigation.

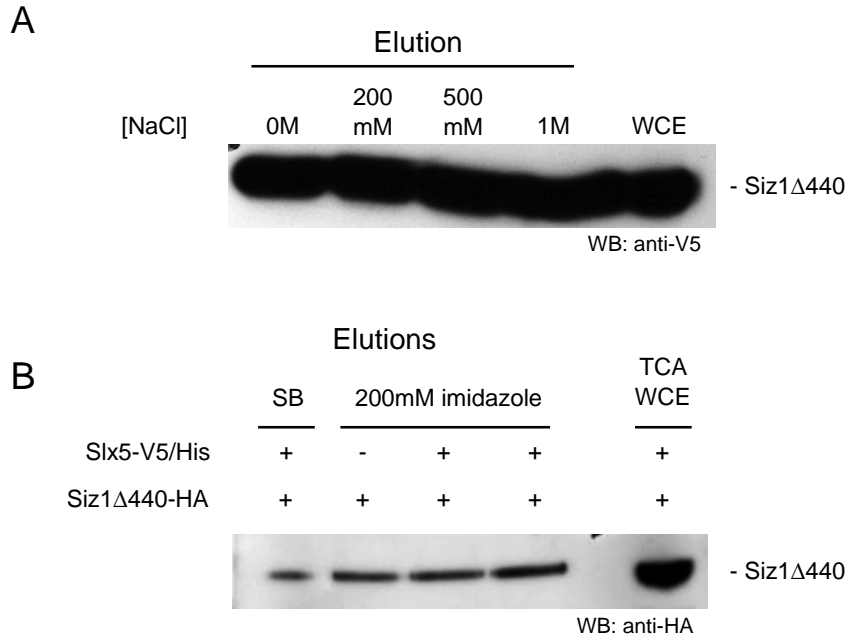


Figure 9: Successful purification of Siz1Δ440 with and without a His tag from TALON metal affinity resin
 Proteins were overexpressed by galactose induction and extracted by bead beating. Whole cell extracts (WCE) were prepared in TCA while clarified lysates were incubated with TALON metal affinity resin. Eluted proteins were separated by SDS-PAGE and after Western Blotting (WB) were probed with antibodies to the V5 or HA epitope tags. (A) Siz1Δ440-V5/His (from YOK 2377) was extracted in Buffer D. Resin was washed with increasing concentrations of NaCl. TALON bound proteins were eluted with Buffer E, showing that Siz1Δ440-V5/His was purified under every wash condition. (B) Siz1Δ440-HA and Slx5-V5/His (from YOK 2353 and 2354) were galactose overexpressed in various combinations as indicated. Proteins were extracted in Buffer C. TALON resin bound proteins were eluted with sample buffer (SB) or Buffer F. Siz1Δ440 purified from TALON resin even in the absence of a His tag.

RING domain proteins intrinsically bind TALON metal affinity resin

As detailed above, we serendipitously observed that Siz1Δ440-HA purifies from TALON metal affinity resin, which normally binds 6xHIS tagged-proteins. We confirmed by sequencing that Siz1Δ440-HA does not contain a 6xHis epitope tag, but it does contain an exposed SP-RING domain at its C-terminal end, which naturally coordinates metal ions (FIG S1). We hypothesized that the natively folded RING domain in Siz1Δ440 could be coordinating with the metal ion on TALON resin independently of a 6xHis tag. A precedence for intrinsic protein binding to metal affinity resins exists, as it has been

described that the cholera toxin B subunit binds Ni^{2+} resin in a manner mediated by its native histidine residues (Dertzbaugh and Cox, 1998).

To test this possibility for RING domain-containing proteins, we expressed several proteins that contain RING domains – Siz1 Δ 440-HA (YOK 2353), Slx5-GST (YOK 2071), Siz1-myc (YOK 2397), and Slx5-V5/His₆ (YOK 2096) (constructs B, F, D and E in Table 2) as a positive control for TALON binding. We then extracted the proteins by bead beating with Buffer C in both native and denaturing conditions, incubated samples with TALON metal affinity resin, followed by washes with Buffer G and elution of bound proteins with Buffer F. Slx5-V5/His₆ purified from TALON resin in both native and denaturing conditions, indicating that 6xHIS-tagged proteins purify as expected (FIG 10a). Siz1 Δ 440-HA, Slx5-GST and Siz1-myc purified from TALON resin in native, but not denaturing conditions, indicating that a properly folded RING domain facilitates binding of non-His₆-tagged proteins to TALON metal affinity resin (FIG 10b,c,d). These proteins were eluted from the TALON resin with imidazole, which competes for histidine binding sites with the Co^{2+} ions, indicating that the binding of the RING domain proteins was specific to the metal ion. None of the proteins were purified with uncharged amylose resin, again indicating that the RING domain purification is dependent on the metal ion in TALON resin. Overall it appears that natively folded RING domain proteins can be intrinsically purified with TALON metal affinity resin, which could be useful in many applications.

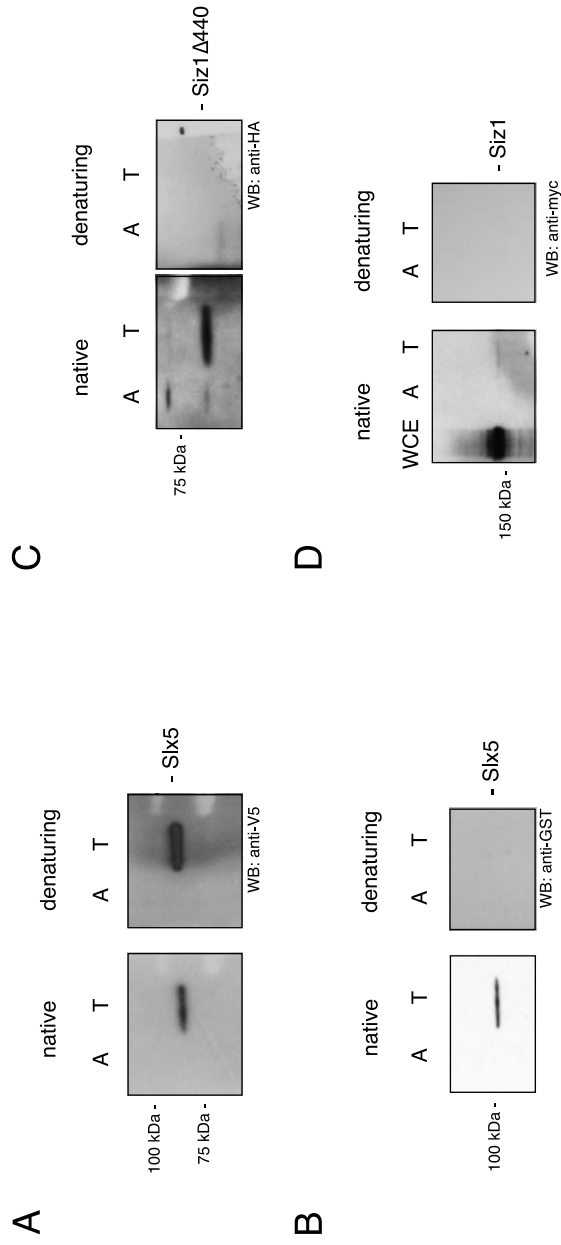


Figure 10: Purification of over-expressed RING-domain containing proteins with TALON metal affinity resin

Proteins were galactose overexpressed, extracted by bead beating and purified. Purification was performed under native and denaturing conditions, for which guanidinium hydrochloride was added to the sample to 6M before nutation with resin. Lysate was nutated with both amylose control resin (A) and TALON Metal Affinity resin (T). Samples were separated by SDS-PAGE and Western Blotted (WB) with an antibody to the appropriate epitope tag. A) Galactose-induced Six5-V5/6xHis (YOK 2096) was purified with TALON resin under both native and denaturing conditions B) Galactose-induced Six5-GST (YOK 2071) was purified with TALON resin in native, but not denaturing conditions. C) Galactose-induced Siz1Δ440-HA (YOK 2353) was also purified with TALON resin in native, but not denaturing conditions. D) Endogenously expressed Siz1-13xmyc (YOK 2397) was faintly purified in native, but not denaturing conditions. Less protein may have been purified due to endogenous, rather than overexpressed levels, or because the RING domain is less exposed. None of these proteins contain His tags, but each does contain a metal coordinating RING domain, which presumably interacts with TALON resin when properly folded.

Slx5 physically interacts with Siz1Δ440 in vivo

With the confirmed ability to extract and purify full-length Slx5 and Siz1Δ440, we were now equipped to perform a co-purification experiment. However, our previous observation that the RING domains of Slx5 and Siz1Δ440 intrinsically purify from TALON resin regardless of their affinity tag necessitated the use of an additional affinity resin. Thus, we decided to purify with glutathione resin instead, which binds the GST epitope tag. We galactose-overexpressed Slx5-GST and Siz1Δ440-HA both individually and together in a *ubc4Δ ubc6Δ* background strain to help stabilize any potential interaction between the two proteins (YOKs 2507, 2508 and 2509) (constructs F and B in Table 2). Proteins were extracted in Buffer H with the use of a bead ruptor as described in Materials and Methods. In addition to a protease inhibitor cocktail, N-ethylmaleimide was used to preserve sumoylation, which improves Slx5 targeting, and sodium orthovanadate was used to preserve phosphorylation, since work in the lab and recent literature indicates a role for phosphorylation in subsequent sumoylation of proteins and in Siz1's ability to interact with sumoylated targets (Ulrich, 2012; Stehmeier and Muller, 2009). Lysates were incubated with glutathione resin, which would bind the Slx5-GST but not the Siz1Δ440-HA. After washing the resin with Buffer I and eluting glutathione-bound proteins with Buffer J, samples were separated by SDS-PAGE. As expected, when Western Blotting with anti-GST, Slx5 is seen in the elution samples, so the glutathione purification was successful. Additionally, in the strain expressing both proteins, Siz1Δ440 co-purifies with Slx5 (FIG 11). When expressed alone, Siz1Δ440 does not purify with the glutathione resin, indicating that the interaction observed is legitimate and is not due to non-specific interactions with the resin.

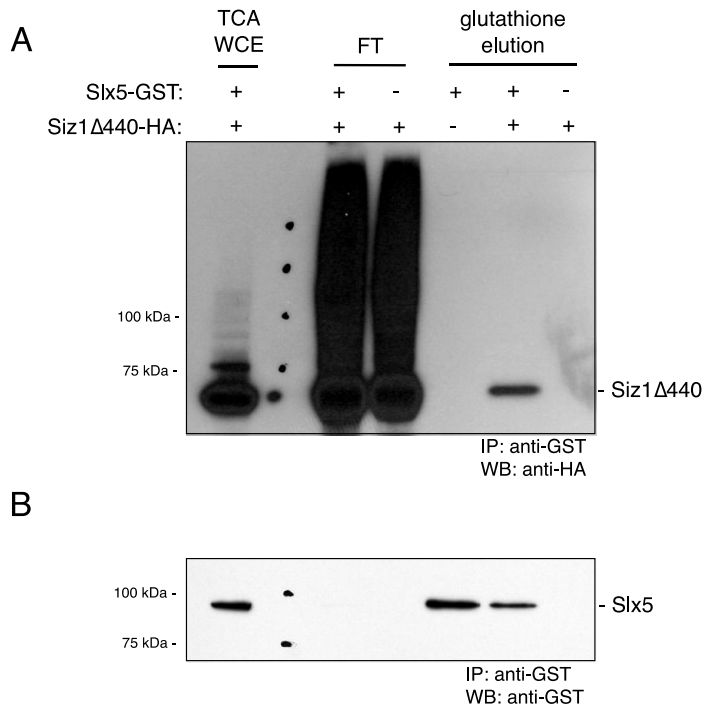


Figure 11: Slx5 interacts with Siz1Δ440 *in vivo*

20 ODs of cells from strains overexpressing GST-Slx5 and Siz1Δ440-HA in the indicated combinations (from YOK 2507, 2508, 2509) were harvested, and protein extracts were prepared by bead-beating in Buffer H. Whole cell extract (WCE) was prepared by TCA precipitation. Clarified lysate was incubated with glutathione agarose and bound proteins were eluted with Buffer J. Also shown is flow-through (FT) of unbound proteins. All samples were separated by SDS-PAGE and individual proteins were detected after western blotting (WB) using antibodies to the GST or HA tags as indicated. Note that Siz1Δ440 is only eluted when Slx5-GST is bound to the affinity resin, indicating an *in vivo* interaction between the two proteins. IP: immunoprecipitation.

Siz1Δ440 is auto-sumoylated in vivo

Given the evidence that Slx5 and Siz1Δ440 interact *in vivo*, we sought to confirm the sumoylation status of Siz1Δ440. Slx5 often interacts with its targets via SIMs, so if Siz1Δ440 were sumoylated, it could help explain their interaction. Additionally, while Siz1Δ440 has been confirmed to have many of the same properties as full-length Siz1 *in vitro*, the auto-sumoylation of the truncation construct has not been confirmed *in vivo*.

To determine if Siz1Δ440 is sumoylated *in vivo*, we performed a SUMO-FLAG shift assay. Siz1Δ440-V5/His₆ (construct A in Table 2) was galactose expressed in a *ulp1ts*

background containing either Smt3gg or FLAG-Smt3gg. The addition of the FLAG tag makes the Smt3gg slightly heavier, leading to a visible shift on a western blot of higher molecular weight bands on top of Siz1 Δ 440 if the protein's modification is SUMO. A noticeable shift was indeed detected, confirming that the modification on Siz1 Δ 440 is SUMO (FIG 12). Thus, Siz1 Δ 440 is appropriately sumoylated *in vivo*, making it an acceptable replacement for full-length Siz1 in protein interaction studies.

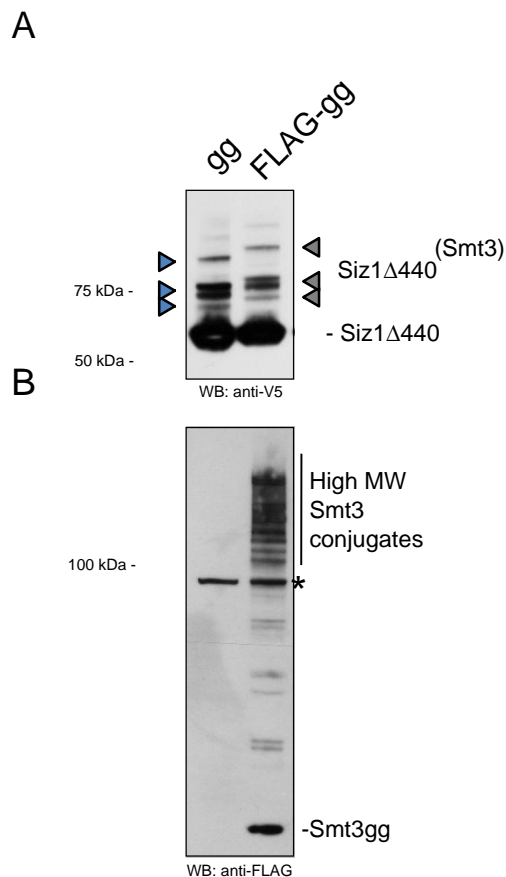


Figure 12: Siz1 Δ 440 is sumoylated in a FLAG-Smt3gg mobility shift assay.

Siz1 Δ 440-V5/His₆ (BOK 794) was overexpressed in *ulp1ts* strains YOK 430 and YOK 428 also expressing high levels of conjugation competent Smt3 (gg) or FLAG-tagged Smt3 (FLAG-gg) as indicated. Proteins extracted from each strain were separated by SDS-PAGE and after western blotting (WB) were probed with an antibody to the V5 epitope (A) or the FLAG tag (B) as indicated. Arrows indicate the FLAG-dependent mobility shift of sumoylated Siz1 Δ 440. FLAG-Smt3gg expression leads to the formation of high molecular weight (MW) Smt3 conjugates. A non-specific protein cross-reacting with the anti-FLAG antibody serves as loading control (*). Molecular weights are indicated in kDa.

Siz1Δ440 is ubiquitylated in an Slx5-dependent manner

With the confirmation that Siz1Δ440 is sumoylated, and that Slx5 and Siz1Δ440 interact *in vivo*, we next decided to investigate if Siz1Δ440 is a substrate of Slx5-dependent ubiquitylation *in vivo* as well. Previous work in the lab showed that Siz1Δ440 was a substrate for Slx5 in an *in vitro* ubiquitylation assay, but the results had not been confirmed *in vivo*. Visualizing ubiquitylation on a single protein can be difficult, since ubiquitylation often leads to proteasome-dependent degradation. We overcame this challenge by expressing a modified version of ubiquitin in which the final glycine (amino acid 76) was mutated to an alanine. This ubiquitin G76A is still able to form those isopeptide bonds, at about 20% the usual rate, but unlike bonds with wild type ubiquitin, formation of these bonds is irreversible, preventing deubiquitylation by the proteasome (Hodgins et al., 1992). Thus, the irreversible addition of ubiquitin G76A makes it useful for studying potential ubiquitylation targets, such as Siz1Δ440.

To investigate the status of Siz1Δ440 ubiquitylation by this method, we overexpressed Siz1Δ440-V5/His₆ (construct A in Table 2) both with and without ubiquitin G76A-myc in either a wild type or *slx5Δ* background (YOKs 2379 and 2380). After probing whole cell extracts for Siz1Δ440, we saw a slight increase in modification of Siz1Δ440 in the presence of ubiquitin G76A compared to normal ubiquitin. Looking at strains expressing the ubiquitin G76A, we also saw a significant increase in modification of Siz1Δ440 in the wild type as compared to the *slx5Δ* background (FIG 13). These observations together may indicate that Siz1Δ440 is ubiquitylated in an Slx5-dependent manner.

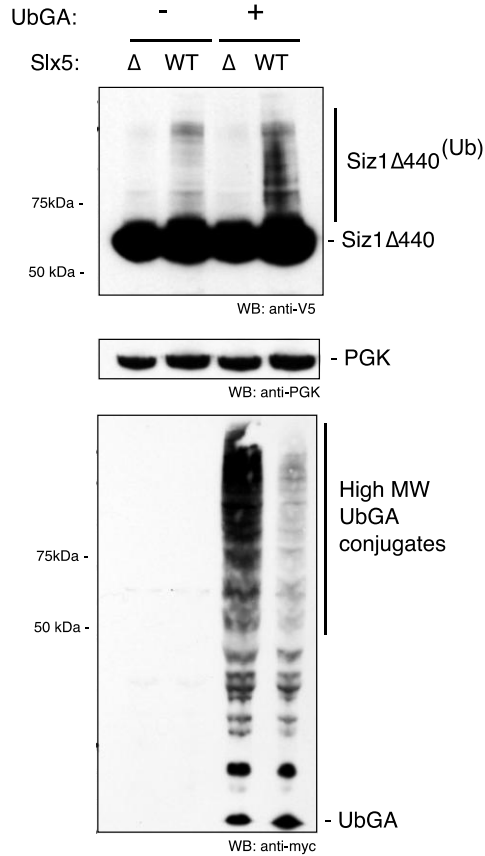


Figure 13: Siz1Δ440 ubiquitylation is Slx5 dependent.

Siz1Δ440-V5/His₆ (BOK 794) was overexpressed in Slx5Δ (Δ) and wildtype (WT) strains along with an empty vector (- UbGA) or a myc-tagged Ubiquitin-G76A (BOK309) (+ UbGA), which is still conjugated to its substrates, but not deubiquitylated by the proteasome. Proteins extracted from each strain were separated by SDS-PAGE and visualized by western blotting (WB) with an antibody to the V5 epitope. Siz1Δ440 is modified by ubiquitin in WT but not Slx5Δ strains, indicating that ubiquitylation of Siz1Δ440 is STUbL-dependent. Additionally, the presence of UbGA is confirmed in both +UbGA lanes by probing with an antibody to the myc tag. Equal loading of proteins is confirmed by PGK.

DISCUSSION

In this work, we study the interaction of STUbLs and SUMO E3 ligases, as well as describe an efficient method to extract and purify proteins from yeast cells under native conditions. In doing so, we serendipitously discovered the intrinsic ability of RING domain proteins to bind TALON metal affinity resin in the absence of a 6xHIS tag or other moieties, which could prove useful in further studies of RING-containing proteins. Development of this method allowed us to purify Slx5, a STUbL subunit, and

Siz1 Δ 440, a SUMO E3 ligase and reveal their interaction *in vivo*. Further investigation revealed that Siz1 Δ 440 is likely an *in vivo* substrate of the Slx5/Slx8 ubiquitin ligase.

Development of successful extraction and purification protocols

The methods of protein extraction and purification developed within this thesis were successful for several reasons. First of all, during extraction, use of the bead beater at 4°C to lyse cells minimized opportunities for cell extracts to heat up, which would cause proteins to denature and degrade. By contrast, the pulverizing and sonication method left ample opportunities for temperature increase as well as human error in manipulation of the cell pellet. Additionally, preparation of the whole cell extracts with TCA proved much more effective than with LDS Sample Buffer. With the TCA preparation, samples were washed successively with TCA to precipitate proteins while eliminating other macromolecules. By contrast, LDS Sample Buffer preparation did not contain wash steps, so the final sample contained a mix of proteins and other macromolecules, which affected its separation by SDS-PAGE. Finally, the basic buffer composition used in the final method consists of 50mM Hepes buffer to maintain the sample at a physiological pH, 200mM NaCl to keep the proteins soluble, mimic physiological conditions, and prevent nonspecific binding, and 1% Triton X-100 to prevent protein aggregation.

Is the sumoylation of Siz1 required for its interaction with Slx5?

Our demonstration that Slx5 and Siz1 Δ 440 interact *in vivo* raises several important questions. Primarily, how do they interact? Since our co-purification was performed with a C-terminal truncation of Siz1, that region is likely not involved in its

interaction with Slx5. However, we entertain several possibilities shown in FIG 14. First, sumoylated Siz1 could noncovalently interact with SIMs on Slx5 (FIG 14a). The SUMO-FLAG shift assay in this study revealed that Siz1 Δ 440 is sumoylated *in vivo*. Given the ability of Slx5 to interact with sumoylated substrates via SIMs, this possibility seems likely. However, there are also reports that sumoylation is not required for targets to interact with Slx5/Slx8 (Xie et al., 2010). In our case, there is evidence that sumoylation does enhance, but is not required for, the interaction between Siz1 and Slx5. A yeast two-hybrid assay between Siz1(SUMO no more (SNM)), a version of Siz1 with all lysines mutated to arginines to prevent sumoylation, and Slx5 showed a weak interaction between the two proteins (unpublished observation). Additionally, a fusion of Smt3 to Siz1 Δ 440 did not enhance Slx5/Slx8's *in vitro* ubiquitylation activity (Matson, 2011 thesis). In a second model (FIG 14b), SIMs present in Siz1 could recognize sumoylated Slx5 in a similar manner (Kerscher, unpublished observation), which would help explain why sumoylation of Siz1 doesn't seem necessary for the interaction.

Further experiments can be done to confirm these two models. Our *in vivo* co-purification was performed with N-ethylmaleimide to preserve sumoylation, supporting that sumoylation plays an important role for *in vivo* targeting. However, we could test this more specifically by attempting to co-purify Siz1(SNM) and Slx5. Additionally, we have been conducting experiments on the assumption that only Slx5 interacts with Siz1 since it is generally thought of as the targeting subunit of the Slx5/Slx8 heterodimer due to its SIMs. However, Siz1 also interacted with just Slx8 in *in vitro* pulldown assays (Matson, 2011 thesis). Thus, in a third model (FIG 14c), it is possible that Slx8 is directly involved in the heterodimer's interaction with Siz1. Slx5, Slx8 and Siz1 all

contain RING domains, which could help mediate an interaction between the three proteins. Hetero-RING complexes involving the ubiquitin ligase Mdm2 actually enhance its activity (Kawai et al., 2007). It would be interesting to attempt an *in vivo* pulldown assay with Slx8-GST and Siz1 Δ 440-HA to see if those two proteins interact as well. This result would also help explain why Siz1 sumoylation enhances its interaction with Slx5, but is not entirely necessary.

Is phosphorylation of Siz1 required for its interaction with Slx5?

In addition to being sumoylated, Siz1 is also phosphorylated and exported with the help of the karyopherin Msn5 in G2/M to sumoylate the septins (Takahashi et al., 2001). Phosphorylation of Siz1 may also be involved in DNA damage signaling, since PIAS1 is phosphorylated by I κ B kinase alpha and may be involved in the ataxia-telangiectasia mutated (ATM)-mediated response (Kuo et al., 2012). Eight phosphorylation sites have been identified on Siz1 based on mass spectrometry sequencing. Several kinases, such as protein kinase A, casein kinase I and glycogen synthase kinase 3, may be involved in these events. Phosphorylation at S132, S139 and S811 are all M-phase regulated, but their functions are unknown (PhosphoGrid: YDR409W; FIG S2). Our lab has observed cell cycle specific phosphorylation of Siz1. Furthermore, in the absence of Slx5, Siz1 phosphorylation is also enhanced.

Therefore, in a fourth model (FIG 14d), phosphorylation may be involved in the interaction between Siz1 and Slx5. As mentioned earlier, RNF4 has a positively charged arginine rich region (ARR) following its four SIMs, which could mediate an interaction with a negatively charged phosphate group (Kuo et al., 2012). This region is conserved throughout the RNF4 family in other eukaryotic organisms. While the sequence of

budding yeast Slx5 does not line up perfectly with the rest of the eukaryotic RNF4 family, Slx5 does contain two putative ARR regions from amino acids 189-208 and 241-260 (FIG S3). Since the four SIMs of Slx5 are in between amino acids 24 and 158, the placement of these arginines seems consistent with an ARR following the SIMs, and could interact with a negatively charged phosphate on Siz1. Additionally, the second ARR falls within a region required for nuclear import and dimerization of Slx5 (Westerbeck et al., manuscript in preparation).

Our *in vivo* co-purification of Siz1 and Slx5 was successful when the phosphatase inhibitor sodium orthovanadate was added to preserve phosphorylation. Further experiments could attempt the pulldown without phosphatase inhibitors, or with a Siz1 serine mutant that cannot be phosphorylated. Additionally, if we determine that Siz1's phosphorylation does enhance its interaction with Slx5, we could mutate arginines in Slx5's putative ARR to see if that abolishes the interaction as well. Data gathered from the described experiments could support or refute the mechanisms of interaction postulated in our model. It is also likely that multiple mechanisms are involved in the targeting of Slx5 to Siz1 (FIG 14e).

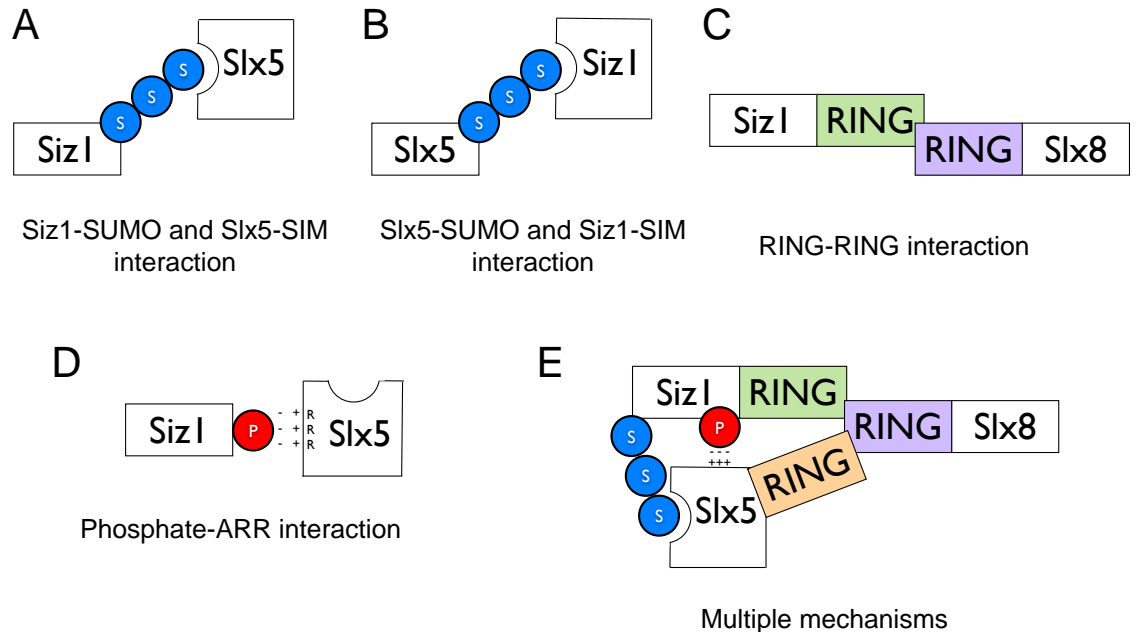


Figure 14: Potential mechanisms for mediating an interaction between Siz1 and Slx5

A) SUMO molecules on Siz1 interact noncovalently with SUMO-interacting motifs (SIMs) on Slx5. B) SUMO molecules on Slx5 interact noncovalently with SIMs on Siz1. C) The SP-RING domain on Siz1 interacts with the RING domain on Slx8. D) A negatively-charged phosphate group on Siz1 interacts with a positively-charged arginine rich region (ARR) on Slx5. E) A combinations of mechanisms may be possible.

Does Slx5 ubiquitylate Siz1 in vivo?

The evidence presented in this study suggests that Siz1 is ubiquitylated *in vivo* in an Slx5-dependent manner. In combination with the *in vivo* co-purification (FIG 12) and the *in vitro* ubiquitylation assay (Matson, 2011 thesis), this indicates that Siz1 may be ubiquitylated by Slx5. We sought to show ubiquitylation of Siz1 Δ 440 in whole cell extracts. For this purpose, we used ubiquitin-GA (UbGA), in which ubiquitin's final glycine is mutated to an alanine, allowing conjugation to substrates but preventing deubiquitylation by the proteasome (Hodgins et al., 1992). We saw increased modification on Siz1 Δ 440 in the presence of UbGA in the wild-type as compared to the *slx5* Δ , which suggests that the modification on Siz1 Δ 440 is indeed ubiquitin. However, probing for ubiquitin to confirm this was not useful, because it just revealed the total

level of ubiquitin in the cell. Therefore, we cannot exclude the possibility that the modification on Siz1 Δ 440 in our figure could be a moiety other than ubiquitin (e.g., SUMO).

To more definitively demonstrate that Siz1 is actually ubiquitylated, we attempted to purify Siz1 and then probe for ubiquitin. Proteasome inhibitors (e.g., MG132) are often used in studies of ubiquitin. Due to the impermeability of the yeast cell wall, mutant strains (e.g. *pdr5* Δ) that confer increased permeability or reduced drug efflux must be used. However, these strains can be difficult to create, especially in an existing mutant background. To this end, we employed a novel growth condition to inhibit the proteasome with MG132 to preserve any ubiquitylated proteins that would normally be targeted for degradation (Fig S4; Liu et al., 2007). However, our attempts to purify Siz1 were unsuccessful, and we are currently repeating the experiment with a different Siz1-V5/His construct. Purification of ubiquitylated Siz1 would offer solid evidence of a functional *in vivo* interaction between Slx5 and Siz1.

Slx5 may help coordinate cell-cycle specific roles of Siz1

As the main SUMO ligase in budding yeast, Siz1 has numerous targets in various locations, many of which have been discussed earlier. In sum, Siz1 remains nuclear for much of the cell cycle due to its DNA-binding SAP domain, where it interacts with transcription factors, PCNA, and DNA damage repair (DDR) proteins, among other targets. In G2/M, Siz1 is exported to the cytoplasm, where it sumoylates the septins. In order to carry out all of its roles, Siz1 must be dynamically regulated. Here we propose a model in which the interaction between Siz1 and Slx5 coordinates Siz1 functions (FIG 15).

During S-phase, Siz1 or PIAS1 sumoylates PCNA, which recruits the DNA helicase Srs2, preventing unwanted homologous recombination. If DNA damage occurs, resulting in a dsDNA break (DSB), a cascade of events occurs. ATM phosphorylates several proteins, including MDC1, H2AX, and possibly Siz1 (Kuo et al., 2012). However, it is important to note that Siz1 phosphorylation does not increase its *in vitro* ligase activity, and instead may regulate *in vivo* protein interactions (Johnson and Gupta, 2001). Siz1 then sumoylates DDR proteins (e.g. BRCA1), facilitating their assembly and functions (Kuo et al, 2012), while also possibly auto-sumoylating itself. Next, Slx5/8 or RNF4 is recruited to the DSB (Cook et al., 2009; Galanty et al., 2012) likely via its interaction with phosphorylated and sumoylated Siz1, as well as its SIM-mediated interaction with other sumoylated DDR proteins. Slx5 ubiquitylates those DDR proteins, creating a hybrid SUMO-ubiquitin chain that is recognized by both ubiquitin-interacting motifs (UIMs) and SIMs on Rap80 (Guzzo et al., 2012). Rap80 then recruits the BRCA1 complex, which mediates homologous recombination to fix the break. Once damage is repaired, ubiquitylation of DDR proteins and Siz1 could send them for degradation by the proteasome, thus eliminating excessive SUMO chains, which are toxic to the cell. Additionally, ubiquitylation of sumoylated Siz1 by Slx5 creates a hybrid chain on Siz1, which may be recognized by the Cdc48-Ufd1-Npl4 (Cdc48-UN) complex via a SIM on Ufd1 and the ability of the complex to interact with ubiquitin (Nie et al., 2012). Cdc48 unravels protein complexes, and therefore may release Siz1 from its complex with DDR proteins. Once released, Siz1 could be degraded by the proteasome, or could be free to carry out its other functions.

With the DNA damage repaired, highly sumoylated proteins removed and Siz1 available for export, the cell cycle continues. At G2/M, Siz1 becomes phosphorylated, likely at a different residue than the one involved in DNA damage. With the help of Msn5, Siz1 is exported from the nucleus into the cytoplasm where it sumoylates the septins. This modification regulates the dynamics of septin ring formation and disassembly, which is critical for microtubule capture and the completion of mitosis (Johnson and Blobel, 1999; Takahashi et al., 2001). Thus, in the proposed model, the interaction between Slx5 and Siz1 helps coordinate DNA damage repair, as well as Siz1 localization and function, enabling the yeast cell to progress through the cell cycle while maintaining its genomic integrity.

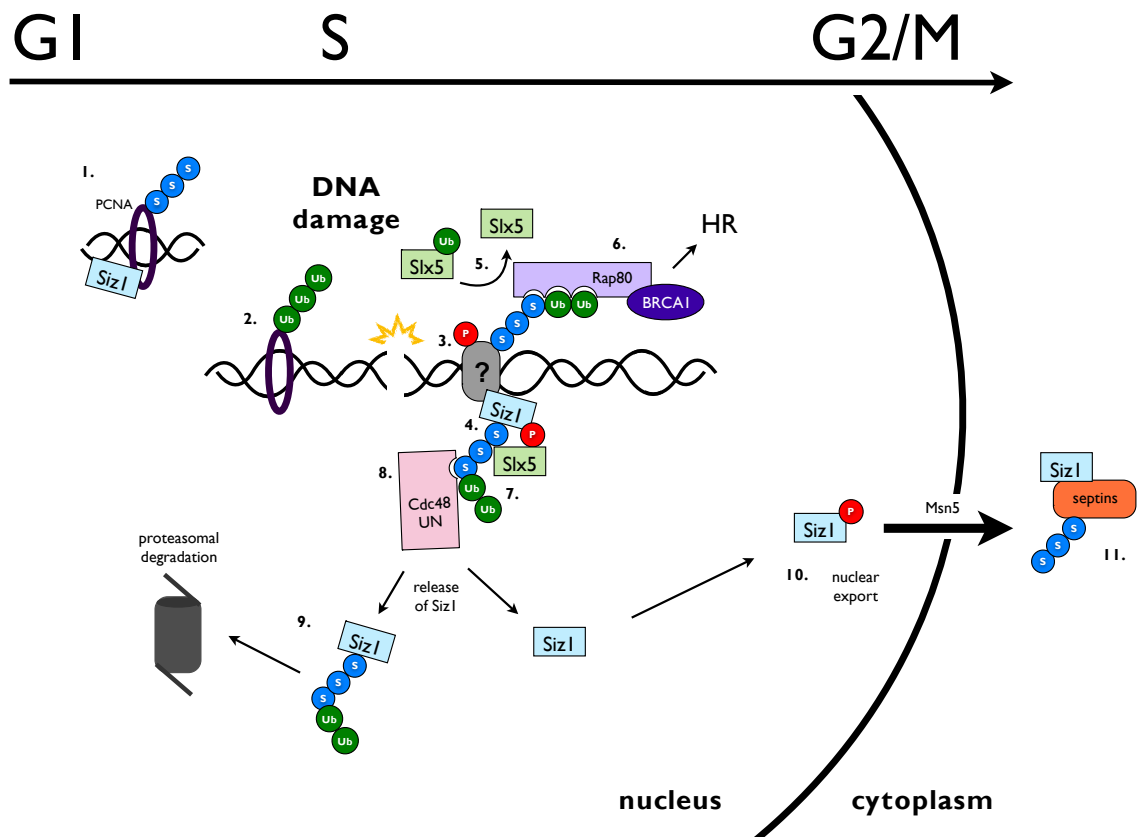


Figure 15: Coordination of cell-cycle specific roles of Siz1 by interaction with Slx5

Siz1 changes sub-cellular locations throughout the cell cycle, corresponding to its various targets and functions. During S-phase in the absence of DNA damage, Siz1 interacts with DNA via its SAP domain and with PCNA via its PINIT domain, and sumoylates PCNA (1). In the case of DNA damage, leading to dsDNA breaks, PCNA becomes ubiquitylated instead (2). Various proteins (e.g., H2AX, MDC1 and potentially Siz1) become phosphorylated by ATM (3). Siz1 sumoylates damage repair proteins, coordinating their assembly at the break, while also auto-sumoylating itself (4). Sumoylated proteins may be targets for Slx5, which ubiquitylates them (5). Hybrid SUMO-Ubiquitin chains bind Rap80, which recruits BRCA1, leading to homologous recombination (HR) (6). Meanwhile, perhaps via both sumoylation and phosphorylation, Slx5 also interacts with and ubiquitylates Siz1 (7). This also forms hybrid chains, which may be recognized by Cdc48-UN (8). Cdc48 unravels the complex, releasing Siz1, which may then be degraded by the proteasome (9). Free Siz1 is also becomes available for phosphorylation and nuclear export by Msn5 as the cell cycle continues into G2/M (10). Once in the cytoplasm, Siz1 sumoylates the septins (11).

Intrinsic RING domain purification

During our attempts to purify Slx5 and Siz1, we serendipitously discovered that natively folded RING-domain containing proteins bind to TALON metal affinity resin. We hypothesize that this is due to the natural ability of the RING domain to coordinate metal ions (FIG 1). This is supported by our data, which show that RING-containing proteins bind TALON resin solely under native conditions, and do not bind uncharged amylose resin. Siz1-myc purified at much lower levels than the overexpressed RING proteins tested, which is likely due to the fact that Siz1-myc was expressed from an endogenous promoter instead of by galactose overexpression, so there were lower levels of the tagged protein in the first place. Additionally, the RING domain falls in the middle of full-length Siz1, rather being exposed at the end as in Slx5 or Siz1 Δ 440, which could make it less accessible for binding to TALON resin.

While the results so far are promising, further experiments would be helpful to determine how widespread this ability is. We only tested Slx5, Siz1, and Siz1 Δ 440 for intrinsic binding, and it would be useful to purify other RING-containing proteins. Additionally, proteins that do not contain RING domains and are known not to interact with RING-containing proteins should be tested as a negative control. If those experiments continue to support our hypothesis, we could perform site-directed mutagenesis of cysteines and histidines in a RING domain, followed by an attempt at TALON purification as before. This would more definitively indicate that it is indeed the RING domain responsible for this binding.

A logical biochemical explanation exists for this binding ability. TALON metal affinity resin consists of a tetradentate chelator charged with a cobalt ion (Co^{2+}), which

has high affinity for histidine-tagged proteins. The imidazole group on the histidine contains a nitrogen with a lone pair of electrons that forms a bond with the metal cation. RING domains contain both histidine and cysteine residues, which normally coordinate zinc ions (Zn^{2+}). Similar to the imidazole group, the thiol group on cysteine contains two lone pairs of electrons that are available to form bonds with multiple metal cations. Both Zn and Co are transition metals that lose two electrons from the 4s orbital when they ionize. Given these similar properties, it is reasonable that a RING domain could bind Co^{2+} just as it binds Zn^{2+} . It would be interesting to also test the ability of RING domains to bind Ni^{2+} affinity resin.

This intrinsic binding ability is useful for several reasons. First of all, if attempting purification and co-purification studies, it is important to know that RING-domain proteins may bind to TALON and other metal affinity resins. For instance, one of the reasons it was such a challenge to show *in vivo* co-purification of Slx5 and Siz1 was because both proteins were independently purified by the TALON resin regardless of tag or interactions. Second, while in some situations the intrinsic binding ability may be frustrating, in others it could prove useful to purify a RING-domain protein of interest that is not tagged. Though other RING proteins may purify as well, the majority of whole cell proteins can be washed away. Third, RING-domain proteins often have ubiquitin or SUMO ligase activity. After purification in their native condition without a tag, they could then be used in subsequent *in vitro* conjugation assays. In sum, this finding is both biochemically interesting and useful for the study of RING-domain proteins.

SUPPLEMENTAL FIGURES AND TABLES

1 MINLEDYWEDETPGPDREPTNELRNEVEETITLMELLKVSELKDICRSVSFPVSGRKAVL
61 QDLIRNFLQNALVVGKSDPYRVQAVKFLIERIRKNEPLPVYKDLWNALRKGTPLSAITVR
121 SMEGPPTVQQQSPSVIRQSPTQRRKTSTTSSTRAPPPTNPDASSSSSSFAVPTIHFKES
181 PFYKIQRLLPELVMNVEVTGGRGMCSAKFKLSKADYNLLSNPNSKHRLYLFSGMINPLGS
241 RGNEPIQFPFPNELRCNNVQIKDNIRGFKSKPGTAKPADLTPHLKPYTQQNNVELIYAFT
301 TKEYLFGYIVEMITPEQLLEKVLQHPKIIKQATLLYLKKTLDREDEEMGLTTTSTIMSLQ
361 CPI SYTRMKYPSKS INCKHLQCFDALWFLHSQLQIPTWQCPVCQIDIALENLAISEFVDD
421 ILQNCQKNVEQVELTSDGKWTAILEDSDSDSDSNDGSRSPKGTKEFDPAFLYKVVMG
481 CRNSISSLGGMYPYDVPDYAGYPYDVPDYAGSYPYDVPDYAAQC*

Figure S1: Sequence of Siz1 Δ 440-HA

Sequencing of Siz1 Δ 440-HA (BOK 795) confirms that the construct contains three HA tags (highlighted in green) and no polyhistidine tag. The C-terminal end of this truncated protein contains an exposed SP-RING domain (highlighted in yellow), which harbors several cysteine and histidine residues (underlined) that could be involved in RING-domain binding to TALON metal affinity resin.

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1  MINLEDYWEDETPGPDREPTNELRNEVEETITLMELLKVSELKDICRSVSFPVSGRKAVL
61  QDLIRNFIQNALVVGKSDPYRVQAVKFLIERIRKNEPLPVYKDLWNALRKGTPLSAITVR
121  SMEGPPTVQQQSPSVIRQSPTQRRKTSSTTSSTSRAPPPTNPDASSSSSSFAVPTIHFKES
181  PFYKIQRLIPELVMNVEVTGGRGMCSAKFKLSKADYNLLSNPNSKHRLYLFSGMINPLGS
241  RGNEPIQFPFPNELRCNNVQIKDNIRGFKSKPGTAKPADLTPHLKPYTQONNVELIYAFT
301  TKEYKLFYIVEMITPEQLLEKVLQHPKIIKOATLLYLKKTREDEEMGLTTTSTIMSLQ
361  CPISYTRMKYPSKSINCKHLQCFDALWFLHSQLQIPTWQCPVCQIDIALENLAISEFVDD
421  ILQNCQKNVEQVELTSDGKWTAILDDDDSDSDSNDGSRSPKGTSVSDHHCSSSHPSEP
481  IIINLSDDDDEPNGNPNPHVTNNHDDSNRHSNDNNNSIKNNDSHNKNNNNNNNNNNND
541  NNSIENDSNSNKHHDHGSRSNTPSHNHTKNLMDNDDDDDDRLMAEITSNHLKSTNTD
601  ILTEKGSSAPSRTLDPKSYNIVASETTTPVTNRVIPEYLGNSSSYIGKQLPNILGKTPLN
661  VTAVDNSSHLISPDVSVSSPTPRNTASNASSSALSTPPLIRMSSLDPRGSTVDPKTIRPP
721  INSNSYTASISDSFVQPQESSVFPFPREQNMDMSFPSTVNSRFNDPRLNTRFPDSTLRGA
781  TILSNGLDQRNSLPTTEAITRNDVGRQNSTPVLPPTLPQNVPIRTNSNKSGLPLINNEN
841  SVENPPNTATIPLQKSRLIVNPFIPRRPYSNVLPQKRQLSNTSSTSSPIMGTWKTQDYGKK
901  YNSG*

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Figure S2: Sequence of Siz1

The sequence of Siz1 was obtained through the Saccharomyces Genome Database. The DNA binding SAP domain stretches from amino acids 34-68 (highlighted in green). Potential sumoylation sites are underlined in and around this domain (K 38, 43, 57, 76, 86, 94, 102, 110). The PINIT domain, important for Siz1's interaction with PCNA, is from amino acids 181-349 (highlighted in blue). The SP-RING domain, which is essential for Siz1's SUMO ligase activity is from amino acids 361-403 (highlighted in yellow). A bona fide SIM is mapped at amino acids 480-491 (highlighted in purple). Several phosphorylation sites have been identified by mass spectrometry: S132, 134, 139, 691, 794, 881 and 886, and T696 (highlighted in red). S132, 139 and 811 are M-phase regulated. Additionally, S139 is part of a protein kinase A motif, S691 is part of a glycogen synthase kinase 3 motif, and S886 is part of a casein kinase 1 motif. However, the function of each phosphorylation site is not reported (PhosphoGrid: YDR409W).

1 MHSDTNGR TKSNNSP SDNNPNET **VILL**IDS DKEEDAS IREANLPVRLYPDRRVGRRRDALN
 61 RFVRSDSRSRNSQRTHITASSERPDFQANND **ITII**REVGRFFGDDGPIDPSAHY **VDLDQ**
 121 EPGSETLETPRTIQVDNTNGYLNDNGNNE SDDG **LTIV**EERTTRPRVTLNLPGGERLEVT
 181 ATTTDIP I **RR**SFEFQEDLGAS **RR**QL **RR**SATRARNL FVDRSDENEDWTDDTHNLPEAIQ
 241 **RARRE** **SR****MR****MS****RR**IAER **QR**VQQRVSSDENISTSI R LQS IRERI QSYTPDIRSAFHRAE
 301 SLHEFRSILQNVAPITLQECEEELMALFTEFRNQLLQNW AIDRV RNTQEEALRLHREALE
 361 RQERTAGRVFHRGTLRESITNYLNFNGEDGFLSRLW SGPALSDADEERHTQNIIDMIQER
 421 EERERDVVMK NLMNKTRAQQEEFEARAASLPEGYSASFDTTPKMKLDITKNGKEETIIVT
 481 DDDLAKTLEDIPV **CCLCGAELGVGIPDDFTGISQKDRGVSFEGLVSKYK FHC PYQTLARP**
 541 **SMLDRDLSKRTFIASCGHAF CGRCFARIDNAKKKSKMPKKKLAQLKGS AHPDNYGPKLCP**
 601 **ADSCCKLIRSRGRLKEVYF***

Figure S3: Sequence of Slx5

The sequence of Slx5 was obtained through the Saccharomyces Genome Database. The C-terminal RING domain of Slx5 stretches from amino acids 494-619 (highlighted in yellow). Cysteine and histidine residues that are involved in RING domain binding to TALON metal affinity resin are underlined (Li et al., 2007). Additionally, Slx5 contains four N-terminal SIMs in between amino acids 24 and 158 (highlighted in purple). Two putative arginine rich regions (ARRs) from amino acids 189-208 and 241-260 could help facilitate interactions with phosphorylated proteins. Each potential ARR is underlined, and arginines are highlighted in blue.

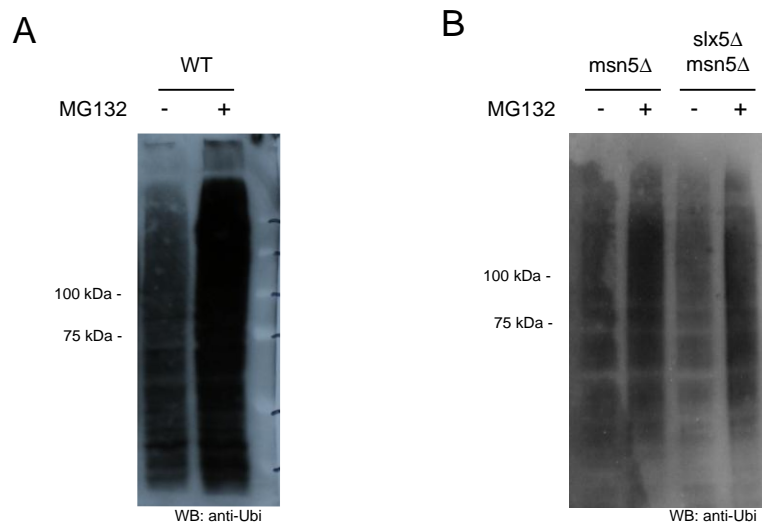


Figure S4: Use of MG132 to inhibit proteasomal degradation

Inhibition of the proteasome with MG132 can be difficult, requiring a *pdr5Δ* deletion strain that does not transport the drug back out of the cell. We employed a new method described by Liu et al, 2007 with a slight modification of our own to permeabilize wild-type yeast cells to MG132 (see Appendix A). Yeast cells were grown, harvested, and extracted by bead beating as described in Materials and Methods. After western blotting (WB), blots were probed with an antibody to ubiquitin (anti-Ubi, 1:4000). More whole cell ubiquitination is seen in MG132 treated samples (+) than in untreated ones (-). (A) Samples from YOK 2377. (B) Samples from YOKs 2514 and 2592, respectively.

Table 3: Yeast strains

Name	Relevant Genotype or Parent Strain	Plasmid(s) or Cassette insertion	Reference
YOK 724	Slx5Δ::kanMX	pYES2.1-GAL-lacZ-TOPO	
YOK 2062	JD52		Erica Johnson
YOK 2071	JD52	GAL1/10-GST-Slx5 (BOK 629, OpenBiosystems Yeast GST collection YSC4515-202484078)	
YOK 2096	JD52	pYES2.1-GAL-Slx5-V5/His ₆ -TOPO (BOK 390)	
YOK 2250	JD52	pYES2.1-GAL-HALO-Slx5(1-207)-V5/His ₆ -TOPO (BOK 830)	This study (Appendix B)
YOK 2251	JD52	pYES2.1-GAL-HALO-Slx5(1-517)-V5/His ₆ -TOPO (BOK 829)	This study (Appendix B)
YOK 2265	JD52	pYES2.1-GAL-Slx5(1-207)-Halo-V5/His ₆ -TOPO (BOK 830); FLAG-Smt3gg (BOK 700)	This study
YOK 2266	JD52	pYES2.1-GAL-Slx5(1-517)-Halo-V5/His ₆ -TOPO (BOK 829); FLAG-Smt3gg (BOK 700)	This study
YOK 2299	JD52	pRS426/URA (BOK 344)	This study (Appendix B)
YOK 2306	<i>slx5Δ::kanMX4</i> in MHY500 (YOK 2286)	pYES2.1-GAL-Slx5(1-207)-Halo-V5/His ₆ -TOPO (BOK 830)	This study (Appendix B)
YOK 2307	<i>slx5Δ::kanMX4</i> in MHY500 (YOK 2286)	pYES2.1-GAL-Slx5(1-517)-Halo-V5/His ₆ -TOPO (BOK 829)	This study (Appendix B)
YOK 2308	JD52	pYES2.1-GAL-Slx5-V5/His ₆ -TOPO (BOK 390); FLAG-Smt3gg (BOK 700)	This study (Appendix B)
YOK 2319	JD52	pYES2.1-GAL-Slx5(1-	This study

		207)-Halo-V5/His ₆ -TOPO (BOK 830); SUMO-GFP (BOK 642)	(Appendix B)
YOK 2320	JD52	pYES2.1-GAL-Slx5(1-517)-Halo-V5/His ₆ -TOPO (BOK 829); SUMO-GFP (BOK 642)	This study (Appendix B)
YOK 2321	JD52	pYES2.1-GAL-Slx5-V5/His ₆ -TOPO (BOK 390); SUMO-GFP (BOK 642)	This study (Appendix B)
YOK 2353	JD52	pAG425-GAL1-ccdB-Siz1Δ440-HA (BOK 795)	This study
YOK 2354	JD52	pAG425-GAL1-ccdB-Siz1Δ440-HA (BOK 795); pYES2.1-GAL-Slx5-V5/His ₆ -TOPO (BOK 390)	This study
YOK 2371	D7 <i>ulp1Δ + ulp1ts</i> , FLAG-Smt3gg (YOK 428)	pYES2.1-GAL-Siz1Δ440-V5/His ₆ -TOPO (BOK 794)	This study
YOK 2372	D8 <i>ulp1Δ + ulp1ts</i> , Smt3gg (YOK 430)	pYES2.1-GAL-Siz1Δ440-V5/His ₆ -TOPO (BOK 794)	This study
YOK 2373	JD52	<i>slx5Δ::kanMX4</i>	This study
YOK 2376	JD52	<i>slx5Δ::kanMX4</i>	This study
YOK 2377	JD52	pYES2.1-GAL-Siz1Δ440-V5/His ₆ -TOPO (BOK 794); pRS424 (BOK342)	This study
YOK 2379	JD52	pYES2.1-GAL-Siz1Δ440-V5/His ₆ -TOPO (BOK 794); CUP1-Ubg76A-myc (BOK 309)	This study
YOK 2380	<i>slx5Δ::kanMX4</i> in JD52 (YOK 2376)	pYES2.1-GAL-Siz1Δ440-V5/His ₆ -TOPO (BOK 794); pRS424 (BOK342)	This study
YOK 2381	<i>slx5Δ::kanMX4</i> in JD52 (YOK 2376)	pYES2.1-GAL-Siz1Δ440-V5/His ₆ -TOPO (BOK 794); CUP1-Ubg76A-myc (BOK 309)	This study

YOK 2396	<i>slx5Δ::kanMX4</i> in JD52 (YOK 2373)	Siz1-13xmyc/HIS5 (endogenously tagged)	Made by M. Guillotte, 2012
YOK 2397	JD52	Siz1-13xmyc/HIS5 (endogenously tagged)	Made by M. Guillotte, 2012
YOK 2501	MHY3765, alpha mating type, <i>ura3-52</i> , <i>lys2-801</i> , <i>trp1-Δ63</i> , <i>his3-Δ200</i> , <i>leu2-Δ1</i>	<i>ubc4Δ::HIS3</i> ; <i>ubc6Δ::TRP1</i> ; <i>mat-alpha2Δ::kanMX</i>	Xie et al., 2010
YOK 2505	JD52	<i>msn5Δ::hygromycin</i>	This study (not used in figures)
YOK 2507	<i>ubc4Δ::HIS3</i> ; <i>ubc6Δ::TRP1</i> ; <i>mat-alpha2Δ::kanMX</i> (YOK 2501)	GAL1/10-GST-Slx5 (BOK 629, OpenBiosystems Yeast GST collection YSC4515-202484078)	This study
YOK 2508	<i>ubc4Δ::HIS3</i> ; <i>ubc6Δ::TRP1</i> ; <i>mat-alpha2Δ::kanMX</i> (YOK 2501)	pAG425-GAL1- <i>ccdB</i> -Siz1Δ440-HA (BOK 795)	This study
YOK 2509	<i>ubc4Δ::HIS3</i> ; <i>ubc6Δ::TRP1</i> ; <i>mat-alpha2Δ::kanMX</i> (YOK 2501)	GAL1/10-GST-Slx5 (BOK 629, OpenBiosystems Yeast GST collection YSC4515-202484078); pAG425-GAL1- <i>ccdB</i> -Siz1Δ440-HA (BOK 795)	This study
YOK 2510	<i>ubc4Δ::HIS3</i> ; <i>ubc6Δ::TRP1</i> ; <i>mat-alpha2Δ::kanMX</i> (YOK 2501)	pYES2.1-GAL-Siz1-V5/His ₆ -TOPO (BOK 898); pRS425 (BOK 343)	This study
YOK 2514	Siz1-13xmyc/HIS5 (YOK 2397)	<i>msn5Δ::hygromycin</i>	This study (not used in figures)
YOK 2592	<i>msn5Δ::hygromycin</i> in JD52 (YOK 2514)	<i>slx5Δ::kanMX4</i>	This study (not used in figures)
YOK 2720	<i>slx5Δ::kanMX4</i> in JD52 (YOK 2373)	pYES2.1-GAL-Siz1-V5/His ₆ -TOPO (BOK 898)	This study
YOK 2721	JD52	pYES2.1-GAL-Siz1-V5/His ₆ -TOPO (BOK 898)	This study

APPENDIX A: Protocols

Yeast Cell Growth Media

2x YEP

- 6g Yeast Extract (Oxoid, LP0021)
- 12g Peptone (Fisher Scientific, BP1420-500)
- 300ml ddH₂O
- Stir until dissolved, filter sterilize

3x YEP:

- 30g Yeast Extract
- 60g Peptone
- 700ml ddH₂O
- Autoclave
- Add 6% galactose to individual aliquots of 3x YEP when ready to use

Dropout media

- 6.7 g Yeast Nitrogen Based without amino acids (Sigma, Y0626-250G)
- 1.7g amino acid drop-out mixture
- 500ml ddH₂O
- Stir until dissolved, filter sterilize

Creation of a Deletion Strain

Preparation of Cassette from Existing Deletion Strain

- Fast Yeast Genomic DNA prep lab protocol
 - Stop after chloroform extraction
- PCR up the deletion cassette
 - 9x reaction

Reagent	Amount
ddH ₂ O	Up to 360µl total
dNTPs (1.25 mM)	56.25µl
Elongase Buffer B (5x)	90µl
Ook 275 (100µM)	1.8µl
Ook 276 (100µM)	1.8µl
gDNA	< 250ng

Elongase Enzyme Mix	0.75µl per tube
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- PCR Program (optimized for *sIx5Δ*)

Temperature	Time	Purpose
94°C	45 seconds	Initial denature
94°C	45 seconds	Denature
50°C	30 seconds	Anneal
66°C	7 minutes	Extend
68°C	5 minutes	Final extension
4°C	∞	hold

} 32 cycles

- Combine 8 tubes of PCR reaction
- Confirm PCR product by gel electrophoresis
 - o 120V, 20-30 minutes (adjusted as necessary)
- Clean PCR product (IBI Scientific, IB47020)
 - o Clean all product in one DF column with one wash
 - o Elute DNA into 30µl of elution buffer

Transformation into receiving strain

- Inoculate receiving strain, grow until late log phase (~1.5 OD/ml)
- Spin down cells, resuspend in 800µl of LiAce/TE, pack on ice in 4°C for ~24 hours (makes cells competent for transformation)
- Spin down cells, wash in 500µl of LiAce/TE
- Add 5µl of ssDNA (boiled for 5 minutes, then on ice for at least 2 minutes beforehand)
 - o Gently resuspend by pipetting up and down
- Add 15µl of clean deletion cassette PCR product
- Add 200µl of LiAce/TE/PEG/DTT
- Incubate in 30°C heat block for 30 minutes
- Incubate in 42°C heat block for 20 minutes
- Spin down, resuspend in 1 ml YPD
- Spin on rotator at room temperature for ~24 hours
- Plate cells on selective media plate - 300µl on one plate, 700µl on another
- Incubate at 30°C
- Pick colonies, extract genomic DNA, and check presence of deletion cassette by PCR as above. Be selective when growing up strains – add 200µM G418 or 200 µM hygromycin as appropriate to liquid culture.

Extraction of Proteins by TCA

- Grow cells as desired
- Centrifuge 4ODs of cells
- Resuspend cell pellet in 800µl of 20% TCA and transfer to a microcentrifuge tube
 - o Cells can be stored at -80°C at this point until the protocol is continued
- Centrifuge at 15,000rpm for 30 seconds in 4°C, decant supernatant
- Add 200µl of small acid washed glass beads and 400µl of 20% TCA
- Vortex on a foam shaker for 4 minutes at 4°C
- Let contents of the tube settle, then transfer white supernatant to a new tube
- Centrifuge at 15,000rpm for 2.5 minutes in 4°C, decant supernatant
- Resuspend pellet in 800µl of 2% TCA
- Centrifuge at 15,000rpm for 2.5 minutes in 4°C, decant supernatant
- Add 200µl of TCA Sample Buffer + BME, vortex to resuspend
- Incubate in 100°C heat block for 2-5 minutes
- Centrifuge at 15,000rpm for 30 seconds to pellet insoluble materials
- Store at -80°C until further use

TCA Sample Buffer

- 15% glycerol
- 80mM Tris Base (non-pH'd)
- 3.5% SDS
- Bromophenol blue "to taste"
- Before use: add 40µl of β-mercaptoethanol (BME) to 1ml TCA Sample Buffer

MG132 Proteasome Inhibition

slightly modified from Liu et al., 2007

Media – Proline as Nitrogen Source

- 6.7g yeast extract without ammonium sulfate (1.34%) [Sigma, Y1251-100G]
- 0.5g of proline (to 0.1%)
- 1.7g amino acid dropout mix (0.34%)
- Up to 500ml with ddH₂O
- Filter sterilize

Growth

Note: SDS makes the cell membranes permeable to MG132, which itself inhibits the proteasome

- Inoculate cells in media/2% sugar source (sucrose, dextrose) and grow 30°C o/n
- Knock down to 0.5 OD in fresh proline media with 0.003% SDS (electrophoresis grade)
- Grow ~3 hr at 30°C to log phase
 - At this point, cells may be cell cycle arrested (e.g. nocodazole) or induced (e.g. galactose)
- Add 75µM MG132 or DMSO (control)
- Grow 30 minutes at 30°C
- Harvest cells

Purification of 6xHIS-tagged proteins expressed in budding yeast cells under native conditions (Szymanski and Kerscher, accepted manuscript)

1. Growth of Yeast Cells and Induction of Protein Expression:

(Modified from Gelperin et al., 2005)

1.1) Transform cells of a Gal⁺ yeast strain with a plasmid encoding a galactose-inducible 6xHIS-tagged protein of choice. For example, see reagents list.

1.2) Inoculate transformants in 5ml of appropriate selective media (e.g. SD-uracil) containing 2% sucrose. Incubate at 30°C overnight, rotating.

1.3) Dilute overnight culture to OD₆₀₀ = 0.3 in 33ml of selective media with 2% sucrose. Grow at 30°C, shaking (~150 rpm).

1.4) When the culture has reached OD₆₀₀ = 0.8-1.5, induce by adding 17ml of 3x YEP with 6% galactose (Recipe in Table 1), for a final concentration of 1x YEP with 2% galactose. Total culture volume is now 50ml. Incubate, shaking, at 30°C for an additional 5-6 hours.

Note: the culture volume can be varied. In step 3, dilute into two-thirds of your desired final volume. In step 4, add one-third the final volume of 3x YEP/6% galactose.

1.5) Measure the OD₆₀₀ of induced culture and centrifuge ~150-200 ODs of cells for 5 minutes at 5,000 rpm at 4°C.

1.6) Resuspend cell pellet with 1ml ice-cold 1x PBS with 1x protease inhibitor cocktail and transfer to a 2ml screw cap tube.

1.7) Centrifuge cells for 1 minute at 15,000 rpm at 4°C. Decant supernatant.

1.8) Snap freeze cell pellet in liquid nitrogen and store at -80°C until further use.

OPTIONAL: Use logarithmically growing yeast cultures expressing protein of interest instead of the galactose-induced cultures above.

2. Homogenization of Yeast Cells and Extraction of Proteins

2.1) To the frozen cell pellet from the previous step, add 200µl of acid-washed glass beads and 500µl of ice-cold Lysis Buffer (Recipe in Table 1 or use cell lysis buffer of choice).

2.2) Briefly pipet up and down. It is not required to fully resuspend the cell pellet. Keep tubes on ice at all times.

2.3) In the cold room, place the tube(s) with cells into the bead mill, balance, lock, and run the machine as per manufacturer's instructions.

2.4) Bead beat the tube(s) for 20 seconds at 5.5 m/s, then place on slushy ice for 1 minute. Repeat six times in total.

2.5) Clarify the extracted proteins by centrifugation for 15 minutes at 15,000 rpm at 4°C. OPTIONAL: remove small particulates by centrifugation through a SpinX filter.

2.6) Prepare a sample of the whole cell extract (WCE) to check presence of your protein by Western Blot:

2.6.1) Add WCE (corresponding to 2 ODs of cells) to 800µl 20% trichloroacetic acid (TCA). Vortex to resuspend.

2.6.2) Centrifuge for 2.5 minutes at 15,000 rpm at 4°C. Decant the supernatant, but be careful to retain the pellet.

2.6.3) Add 800µl of 2% TCA, vortex, then centrifuge for 2.5 minutes at 15,000 rpm at 4°C. Decant the supernatant, but be careful to retain the pellet.

2.6.4) Add 100µl of TCA Sample Buffer (Recipe in Table 1), vortex to dissolve pellet.

2.6.5) Incubate in a 100°C heat block for 2-5 minutes.

2.6.6) Vortex again to fully dissolve if remnants of pellet are still present. Pellets prepared by this method are notoriously difficult to fully dissolve. It may take ~10 minutes of vortexing to completely dissolve pellets.

2.6.7) Store sample at -80°C until further use.

2.7) Snap freeze aliquots of clarified WCE in liquid nitrogen and store at -80°C until further use.

3. Batch Purification of Proteins from Yeast Cell Homogenates.

Note: This purification method was optimized for purification of 6xHIS-tagged proteins on Co²⁺ metal affinity resin.

3.1) Resin Equilibration

3.1.1) For a sample with approximately 30 ODs worth of clarified WCE, add 50-100µl of affinity resin to a microcentrifuge tube. Uncharged agarose beads may be used as a control for non-specific binding.

3.1.2) Wash resin five times with 1ml of Wash Buffer: invert top-over-bottom until resin is resuspended, and then spin for 1 minute at 5,000 rpm at 4°C. Aspirate the supernatant.

Note: if performing extraction and purification on the same day, resin equilibration can be performed prior to extraction.

3.2) Protein Binding for Affinity Purification

3.2.1) Add 100-200µl of clarified lysate to 50-100µl washed beads, and bring the total volume up to 1ml with Lysis Buffer.

3.2.2) Nutate at 4°C for 2-5 hours.

3.2.3) Spin for 1 minute at 5,000 rpm at 4°C.

3.2.4) If desired, save a sample of the remaining supernatant. TCA precipitate as detailed above for the WCE (Step 2.6).

3.2.5) Wash resin with bound proteins five times with 1ml of Wash Buffer, followed by a spin for 1 minute at 5,000 rpm at 4°C. Keep samples cold during washes.

3.3) Elution of Bound Proteins

3.3.1) Add 150µl Elution Buffer to resin, nutate in cold for 5 min, spin for 1 minute at 5,000 rpm at 4°C and save the supernatant in a new tube. OPTIONAL: Repeat twice and pool elutions.

3.3.2) Prepare elution sample for Western Blot: To 25µl of eluted proteins, add 25µl 2x LDS Sample Buffer with 2µl β-mercaptoethanol (BME) and incubate in a 100°C heat block for 2 minutes.

3.3.3) Snap freeze excess eluted protein in liquid nitrogen.

OPTIONAL: strip remaining proteins from resin with an equal volume of 2x LDS Sample Buffer at 65°C for 5 minutes, *then* add 2µl BME.

3.3.4) Store samples at -80°C until further use.

3.4) Western Blot and probe with appropriate antibodies to visualize proteins.

3.4.1) Load 10-20µl of each sample and 3-10µl of a protein ladder in an SDS-PAGE gel of choice. We routinely use 4-12% Bis-Tris and 8% Tris-Glycine.

3.4.2) Run gel at 200V for 50 minutes.

3.4.3) Transfer proteins from gel to a PVDF membrane by semi-dry transfer at 19V for 20-30min (Recipe in Table 1)

3.4.4) Block membrane in 4% milk/1x Tris Buffered Saline-TWEEN (TBST) for 1 hour at room temperature (Recipe in Table 1).

- 3.4.5) Incubate membrane with primary antibody to your epitope-tagged protein of interest in 4% milk/1x TBST for 1-3 hours at room temperature or overnight at 4°C.
- 3.4.6) Wash membrane three times for five minutes each with 1x TBST.
- 3.4.7) Incubate membrane with appropriate secondary horseradish peroxidase (HRP) – conjugated antibody for 1-3 hours at room temperature.
- 3.4.8) Wash membrane three times for 15 minutes each in a large volume of 1x TBST.
- 3.4.9) Cover membrane with ECL substrate and wrap in saran wrap.
- 3.4.10) Expose membrane to film and develop to visualize proteins.

APPENDIX B: Additional data

Slx5 Truncation studies

After structure function studies of Slx5 (FIG 3; Westerbeck et al., manuscript in preparation) revealed that different truncations of the proteins have different sub-cellular localizations and different interaction properties, we thought that these constructs might be useful for further STUbL studies. For instance, the Slx5(1-517) construct still maintains many interactions with other proteins, but doesn't contain the catalytic RING domain, so those protein interactions might be stabilized. The Slx5(1-207) construct could be used to bind sumoylated proteins, since it contains four SIMs and exits the nucleus to bind the highly sumoylated septins. Additionally, many of these constructs have the ability to bind other Slx5 proteins, forming homodimers that could disrupt the formation of the functional Slx5/Slx8 heterodimer. This disruption could potentially lead to a dominant negative effect, in which we could induce an Slx5 Δ phenotype by overexpressing Slx5 constructs.

Over-expression of Slx5 constructs leads to induction of high molecular weight SUMO chains

Given these potential uses for the truncations, we decided to further investigate the effect of overexpressing them. We found that overexpression of Slx5(1-517) and full-length Slx5 (dubbed Slx5(FL)), but not Slx5(1-207) induced high molecular weight SUMO conjugates when co-expressed with Smt3gg (FIG B1). These adducts could possibly indicate that the Slx5/Slx8 heterodimer was dysfunctional, allowing sumoylated proteins to accumulate.

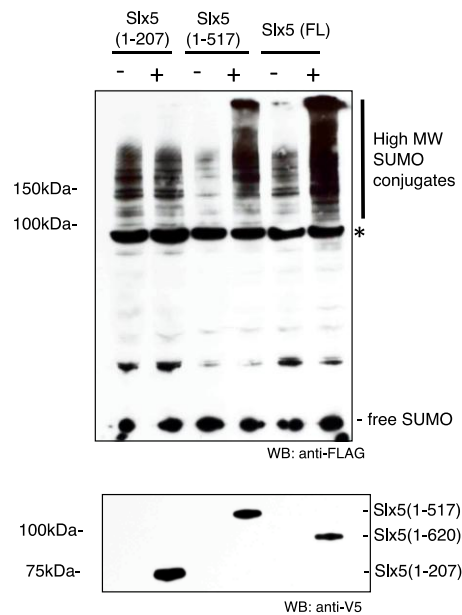


Figure B1: Differential levels of SUMO conjugates with overexpression of various truncations of Slx5

Smt3gg-FLAG was cotransformed with plasmids containing various truncations of Slx5 plus a V5 epitope tag under the control of a galactose promoter (YOK 2265, 2266, and 2308). Slx5(1-207) contains four SIMs and is unable to dimerize, localize to the nucleus, or interact with Slx8. Slx5(1-517) is identical to the full-length protein except the C-terminus RING domain. Slx5(FL) is the full length protein. When induced, overexpression of Slx5(1-207) does not cause high molecular weight (MW) SUMO conjugates, whereas overexpression of Slx5(1-517) and Slx5(FL) does. Expression of the Slx5 constructs was confirmed with an anti-V5 antibody. Slx5(1-207) and (1-517) additionally contain the HaloTag, accounting for their increased MW.

Over-expression of Slx5 causes differential growth dynamics

We wondered if these accumulated proteins had negative effects on the health of the cells. While growing the cells in liquid media, we had noticed that different strains had different growth dynamics. To further investigate this, we performed a spotting assay on untreated plates and plates that were treated with hydroxyurea (HU), to which *slx5Δ* strains are very sensitive. Plates were also made with either dextrose, as a negative control, or galactose, to induce the constructs. These assays revealed that upon the induction of Slx5(FL) and Slx5(1-207) on both untreated and HU plates, cells were extremely unhealthy as compared to wild-type, but upon the induction of Slx5(1-517), cells were just as healthy as the wild-type strain (FIG B2). This seemed to be inconsistent with our hypothesis that excess sumoylated proteins are unhealthy for the

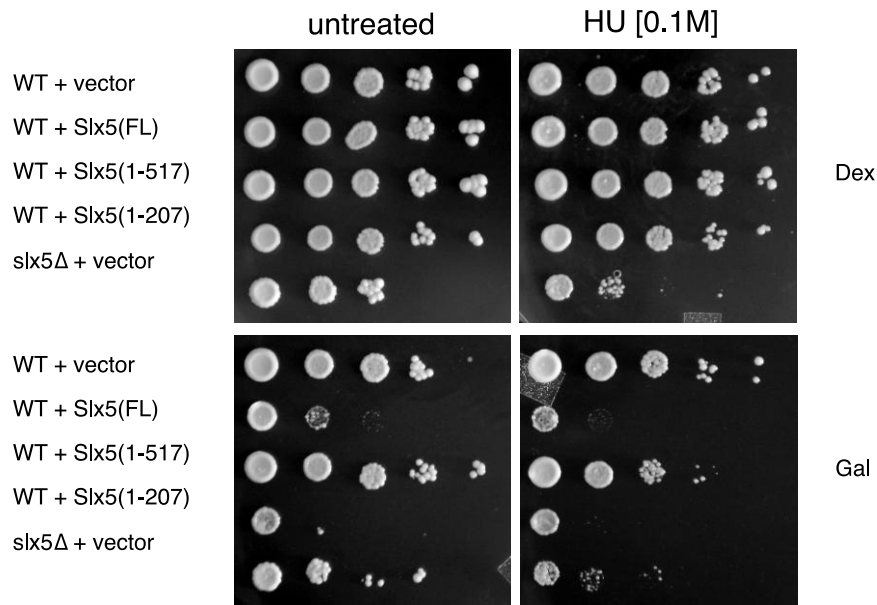


Figure B2: Growth phenotypes of strains overexpressing Slx5 truncation constructs

Wild-type (WT) yeast strains containing galactose-inducible plasmids for Slx5(FL), Slx5(1-517), Slx5(1-207) and an empty vector, as well as an *slx5Δ* strain containing an empty vector were grown (YOK 2299, 2096, 2251, 2250, and 724). Cells were diluted ten-fold and plated on various combinations of plates as shown above. Dex: dextrose; Gal: galactose, to induce expression of the protein; HU: hydroxyurea. When induced, in both HU and untreated conditions, Slx5(1-517) grew as well as the WT, while Slx5(FL) and Slx5(1-207) grew worse than the *slx5Δ*.

cell. It is possible that Slx5(1-207) and Slx5(FL) are each lethal for different reasons, or that the explanation is independent of sumoylated proteins.

Over-expression of Slx5 causes differential localization of SUMO within the cell

Due to our previous findings, we wondered whether the localization of sumoylated proteins would change upon induction of the various constructs. We co-transformed SUMO-GFP with Slx5(1-207), Slx5(1-517), or Slx5(FL), induced expression of the proteins, and then performed confocal microscopy to visualize SUMO. The pool of SUMO normally resides indistinctly in the nucleus, with the septin ring becoming highly sumoylated and visible during G2/M phase. In cultures expressing Slx5(1-207), SUMO was seen exclusively indistinctly in the nucleus and at the septin ring in the cells that were alive. In cultures expressing Slx5(FL), the majority of cells showed foci of SUMO in the nucleus, perhaps indicating an aggregation of sumoylated proteins, which is consistent with the high molecular weight SUMO conjugates we observed in the western blot, and might also explain the lethality of this construct. In cultures expressing Slx5(1-517), SUMO was seen in multiple locations. In some cells, SUMO was only enriched at the septins; in some, it formed bright foci in the nucleus; and in others, it formed both foci and enriched at the septins. This is also consistent with our previous results that Slx5(1-517) causes some accumulation of sumoylated proteins (in foci) but also properly sumoylates its septins to allow the cell cycle to continue (FIG B3). A summary of our findings regarding overexpression of Slx5 truncations can be found in Table B1.

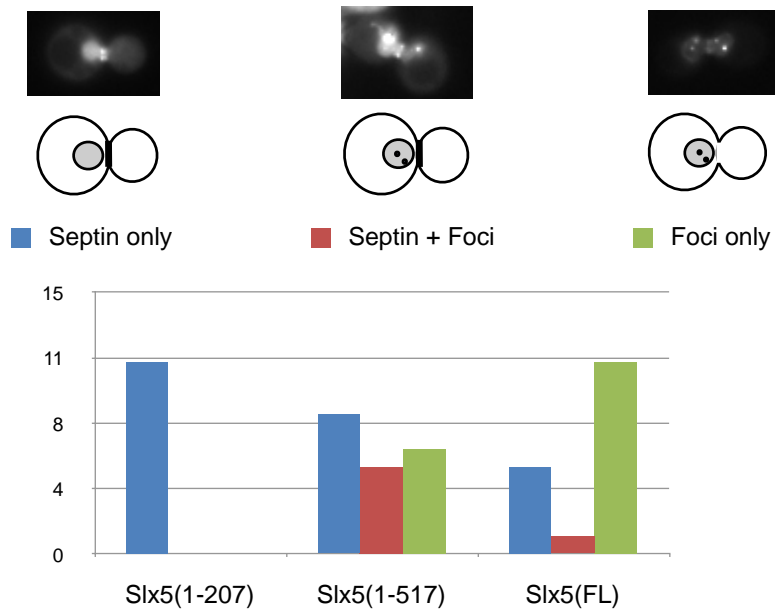


Figure B3: Differential localization of SUMO with various truncations of Slx5

SUMO-GFP was expressed along with Slx5(FL), Slx5(1-517) and Slx5(1-207) (YOKs 2319, 2320 and 2321). During logarithmic growth, cells were observed under the microscope, and the localization of SUMO was noted. When expressed with Slx5(1-207), SUMO was only seen enriched at the septin ring. When expressed with Slx5(1-517), SUMO was seen at the septin ring, in foci in the nucleus, or in both locations. When expressed with Slx5(FL), SUMO was mostly seen in foci in the nucleus, but also at the septin ring and in both locations. Exact percentages can be found in Table S1.

	Slx5(1-207)	Slx5(1-517)	Slx5(FL)
Western Blotting	No high MW SUMO chains	High MW SUMO chains	High MW SUMO chains
Growth Phenotypes	Dies (on both +HU and -HU)	Alive (on both +HU and -HU)	Dies (on both +HU and -HU)
Microscopy: SUMO-GFP	100% at septins	42% - septins 26% - septins and foci 32% - foci only	29% - septins 6% - septins and foci 65% - foci only

Table B1: Summary of results from Slx5 truncation experiments

A summary of the results obtained for Slx5(1-207), Slx5(1-517) and Slx5(FL) galactose overexpression. Data was obtained from western blotting of strains expressing Smt3gg along with an Slx5 truncation, from a spotting assay of each Slx5 truncation on dextrose or galactose plates that were untreated or treated with hydroxyurea (HU), and from microscopy of cells expressing SUMO-GFP along with an Slx5 truncation. MW: molecular weight.

The effect of over-expression of Slx5 constructs on Siz1 sumoylation and phosphorylation

To determine if over-expression of the Slx5 truncations affected the sumoylation of a specific target of Slx5, Siz1, we transformed Slx5(1-207) and Slx5(1-517) into a background strain endogenously expressing Siz1-13xmyc. Upon induction of the constructs, extraction of proteins by TCA preparation and subsequent western blotting with anti-myc, we observed a decrease in Siz1 sumoylation in cells expressing the Slx5 truncations (FIG B4). This is consistent with our hypothesis that overexpression of Slx5 constructs could act as an inducible dominant negative, mimicking the *slx5Δ* phenotype. We also decided to look at the effect of the Slx5 constructs on the phosphorylation of Siz1. We transformed Slx5(1-207), Slx5(1-517), or Slx5(FL) into a JD52 background strain endogenously expressing Siz1-13xmyc, induced the constructs, extracted the proteins by TCA preparation, and western blotted with anti-myc. These results were much less conclusive (unpublished observations). While their full value is yet to be determined, these Slx5 constructs could still prove useful in further STUbL studies.

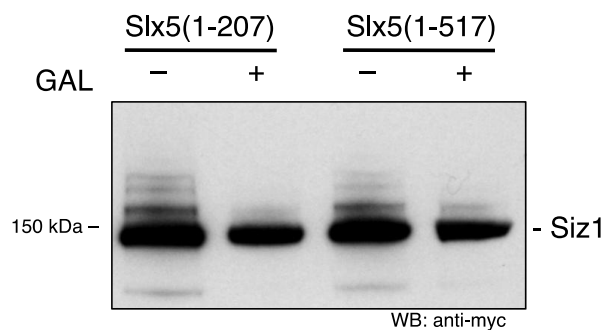


Figure B4: Differential sumoylation of Siz1 with the expression of Slx5 truncations

To investigate the potential dominant negative effect of Slx5 truncation overexpression on a putative target of Slx5 (Siz1), we overexpressed Slx5(1-207) or Slx5(1-517) in a Siz1-myc background strain (YOK 2306 and 2307). Cells were uninduced or induced with galactose (GAL -/+), and after western blotting (WB) were probed with an antibody to the myc epitope. In the uninduced strains, Siz1 shows several sumoylated adducts, while in the Slx5 overexpression strains, Siz1 modification is reduced.

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