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Lost and Found: Localization of the Ubiquitin Ligase Slx5 to the Yeast Bud Neck

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

by

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**ABSTRACT**

Slx5 and Slx8 are two subunits of a SUMO-Targeted Ubiquitin Ligase (STUbL) in the budding yeast *Saccharomyces cerevisiae*. STUbLs are E3 ubiquitin ligases that target sumoylated substrates for ubiquitylation. Slx5 contains several SUMO-interacting motifs (SIMs) and is considered to be the targeting subunit of the Slx5·Slx8 STUbL. In yeast, Slx5 forms SIM-dependent nuclear foci, some of which co-localize with double-stranded DNA breaks. To study how these foci form in the nucleus, we performed a high-throughput screen for temperature-sensitive mutants. In this screen, we identified an *slx5* mutant that both formed nuclear foci and mislocalized to the bud neck.

Slx5 localization at the bud neck resembled the localization pattern of septin proteins. Septins are a class of proteins involved in cytokinesis and cellular division and are transiently sumoylated at the G2/M phase of the cell cycle, making them potential Slx5·Slx8 ubiquitylation targets when nuclear import of Slx5 is impaired. Here we present our data and analysis of this Slx5 septin mislocalization phenotype. The findings of this study have great implications for understanding the STUbL activity in humans.

**INTRODUCTION**

*Post-translational Modifications: Ubiquitylation and Sumoylation*

Post-translational modification through the covalent attachment of small molecules is an important feature that greatly increases the function of an organism’s proteome by altering a protein’s activity, localization, or interactions with other proteins. These covalent modifications include phosphorylation, acetylation, and methylation, among many others (Mann and Jensen, 2003). The two post-translational modifications
that are of interest for this study are ubiquitin and the small ubiquitin-like modifier (SUMO), two small, highly conserved proteins. Ubiquitin is best known for its function in targeting proteins for degradation by the proteasome (Kerscher et al., 2006). This occurs when ubiquitin becomes attached to the target substrate in the form of a polyubiquitin chain, which is then recognized by the receptors within the proteasome or by other proteins that later bind the proteasome. One example for ubiquitin’s function is the degradation of the proteins securin and cyclin B, which must be degraded for cell cycle progression (Cox et al., 2002).

Ubiquitin is synthesized as an inactive precursor that must be processed to expose a C-terminal diglycine motif that serves as the site of substrate attachment (Kerscher et al., 2006). Upon maturation of the ubiquitin, covalent attachment occurs through the action of three types of enzymes: the E1 activating enzyme, the E2 conjugating enzyme, and the E3 ubiquitin ligase. In the yeast *S. cerevisiae*, the activating enzyme is Uba1. The E1 first attaches a molecule of ATP to the C-terminus of the ubiquitin. This forms a mixed-anhydride bond that is attacked by the E1 active-site cysteine residue, resulting in the release of AMP and the formation of a high-energy thioester bond between the E1 and the ubiquitin. The ubiquitin is transferred to a thiol group on the E2, such as Ubc1-8, -10, -11, and Ubc13-Mms2 in yeast. The E3 then facilitates transfer of the ubiquitin to a lysine residue of a substrate through the formation of an isopeptide bond.

The E3 is key in conferring substrate specificity; three major classes of proteins have been identified as E3 ubiquitin ligases in yeast (Kerscher et al., 2006). The first class includes the RING E3s, which contain a zinc-coordinating RING motif. The U-box E3s, a structurally related class of proteins, are similar but lack the zinc ions. These first
two types of E3s act by binding both the E2 and the substrate, transferring the ubiquitin to the target protein. The final class of ubiquitin ligases, HECT E3s, function in a fundamentally different manner: The E2 transfers the ubiquitin to the HECT E3, which subsequently attaches the ubiquitin to the lysine side chain of the substrate.

Like ubiquitin, SUMO must be processed to a mature state before covalent attachment to its substrate. In *S. cerevisiae*, the precursor encoded by the gene *SMT3* must have the C-terminal amino acid residues removed in order to expose the diglycine motif necessary for conjugation. The protein Ulp1 is responsible for this cleavage process (Li and Hochstrasser, 1999). Once maturation is complete and the diglycine motif exposed, the SUMO can be covalently attached to a substrate’s lysine residues.

Sumoylation occurs in a fashion analogous to ubiquitylation, requiring E1, E2, and E3 enzymes to attach SUMO to a lysine residue, generally contained within the consensus sequence ψKxD/E, where ψ represents a hydrophobic amino acid (Kerscher et al., 2006). The E1 Uba2-Aos1 activates the SUMO before it is transferred to the E2 Ubc9 (Johnson and Blobel, 1997). At least three E3 SUMO ligases are known to exist in yeast: Siz1, Siz2, and Mms21 (Takahashi et al., 2003; Takahashi et al., 2001). Again, each E3 targets a specific set of proteins for sumoylation. Interestingly, Ubc9 has also been shown to possess E3-like activity; unlike E3 ubiquitin ligases, E3 SUMO ligases are not necessary for covalent attachment of SUMO to a substrate (Hochstrasser, 2001).

*Functions of Sumoylation*

Unlike ubiquitylation, sumoylation does not directly target substrates to the proteasome. Instead, this modification enhances the functionality of a protein by altering its activity, localization, or even its interactions with other proteins. Sumoylation is a
reversible process, with the presence of several specialized proteases allowing SUMO-conjugated proteins to exist in a dynamic state and potentially regulate various cellular processes. For instance, sumoylation seems to be involved in regulating cohesion at the centromeres in budding yeast (Bachant et al., 2002). SUMO proteases in S. cerevisiae are Ulp2 as well as Ulp1, the latter of which is the same protein responsible for cleaving the precursor to render SUMO conjugation competent (Kerscher et al., 2006).

A yeast cell without functional Ulp1 becomes inviable. In order to understand the mechanism behind ulp1A lethality, scientists utilized a temperature sensitive mutant of ULP1 called ulp1ts, in which Ulp1 functions at the permissive temperature but not above the nonpermissive temperature (Xie et al., 2007). Furthermore, when grown at the nonpermissive temperature, this mutant arrests in the G2/M phase of the cell cycle. A yeast genomic library was screened to find high-copy suppressors of ulp1ts. SLX5 was isolated from this screen as a gene that rescues the growth defect of the ulp1ts strain at high dosages (Xie et al., 2007). Because Ulp1 is normally responsible for SUMO processing, the ability of SLX5 overexpression to rescue the ulp1ts at the nonpermissive temperature unsurprisingly depended on expression of mature SUMO. Importantly, however, it was only overexpression of SLX5 and not its binding partner SLX8 that could rescue ulp1ts cells. Furthermore, the RING domain of Slx5 was also required to suppress the ulp1ts growth defect.

Cells lacking Slx5 are slow-growing and display a nibbled colony morphology. Furthermore, slx5A cells are highly sensitive to genotoxic stress (Xie et al., 2007). This pointed to the idea that Slx5 may be involved in a DNA repair pathway. The sensitivity
to genotoxic agents can be rescued by inserting a plasmid containing \textit{SLX5} into a \textit{slx5A} strain; the plasmid fully complements the function of the deleted gene (Xie et al., 2007).

\textit{Slx5-Slx8 is a SUMO-Targeted Ubiquitin Ligase}

\textit{Slx5} interacts with its binding partner \textit{Slx8} to form a heterodimer that functions as an E3 ubiquitin ligase within the nucleus (Xie et al., 2007), known as a SUMO-targeted ubiquitin ligase (STUbL) (Perry et al., 2008). Both proteins bear a RING domain, a characteristic shared by many E3 ligases. \textit{Slx5} also contains several SUMO interacting motifs (SIMs) that presumably allow it to localize to SUMO-conjugated targets. SIMs are characterized by a core of hydrophobic amino acids often flanked by acidic amino acids. It is thought that the hydrophobic core of the SIM interacts with hydrophobic amino acid residues on SUMO, such as valine, phenylalanine, and leucine (Kerscher, 2007). Within the \textit{Slx5-Slx8} heterodimer, it is most likely \textit{Slx5} that primarily performs the targeting function using SIMs (called SIM A and B), while \textit{Slx8} functions as the core ligase to attach ubiquitin to sumoylated substrates (Xie et al., 2007). Poly-SUMO chains attached to a substrate activate the \textit{Slx5-Slx8} heterodimer, which appears to attach a single ubiquitin to the N-terminal SUMO on the chain (Mullen and Brill, 2008). In at least one other case, the ubiquitylation of \textit{Rad52}, ubiquitin appears to be attached to the substrate in chains (Xie et al., 2007).

One \textit{in vitro} target of this STUbL is the protein \textit{Rad52}, a protein involved in the DNA repair and homologous recombination pathway. \textit{In vivo}, SUMO becomes conjugated to \textit{Rad52} in response to double-stranded DNA breaks. This sumoylation promotes the recombination activity of \textit{Rad52} (Sacher et al., 2006). It is possible that
ubiquitylation by Slx5·Slx8 is important to terminate and regulate the DNA repair function of Rad52 and other DNA repair proteins.

Our lab recently showed that Rad52 and Slx5 both co-localize to double-stranded break sites within DNA, suggesting that Slx5 also has the ability to interact with sumoylated targets in vivo (Cook et al., 2009). While nuclear foci composed of Slx5 are also observed in cells with undamaged DNA, these observations suggest that at least some of the observed foci are involved in DNA repair. However, there remained several unanswered questions regarding this foci forming ability of Slx5. 1) Which proteins does Slx5 interact with in foci? 2) How does Slx5 get to the foci? 3) Does Slx5 have a nuclear localization sequence, and if so, which importins or other proteins are involved in transporting it through the nuclear pore complex? To address these questions, we attempted to generate a temperature-sensitive mutant of SLX5 for use in a high-copy suppressor screen (see Materials and Methods).

**Novel Localization of Slx5 to the Bud Neck**

Though we were unable to successfully isolate a temperature-sensitive slx5 mutant, we did isolate a strain with unusual Slx5 localization, slx5-ts49. In addition to forming nuclear foci, strain slx5-ts49 showed a distinct bud neck localization phenotype that is not observed in wildtype cells (Figure 1). The bud neck is the junction between the mother cell and the bud that eventually becomes the daughter cell.
Figure 1: Localization of wildtype Slx5 and slx5-ts49. Wildtype Slx5 forms distinct nuclear foci (left panel). In the strain containing slx5-ts49 (YOK1008), it forms nuclear foci and localizes to the bud neck, as indicated by arrow (right panel). YOK1008 was originally isolated in a high-throughput robotic screen for conditionally temperature-sensitive mutants of slx5.

Based on the fact that Slx5 localized to the bud neck, we hypothesized that the mutant Slx5 was localizing to the septins. Named for their role in cytokinesis and septum formation in *S. cerevisiae*, septins are highly conserved from yeast to humans, though they are absent in plants and certain protozoans (Douglas et al., 2005). *S. cerevisiae* expresses five septins (Cdc3, Cdc10, Cdc11, Cdc12, and Shs1/Sep7) during vegetative growth, and two (Spr3 and Spr28) only during sporulation. The five septins present during vegetative growth form a complex consisting primarily of elongated octamers containing Cdc3, Cdc10, Cdc11, Cdc12 (Cao et al., 2009). Shs1 is not required for the assembly of this octamer, but serves to stabilize it (Douglas et al., 2005). This complex assembles into a 10 nm filament, which subsequently forms a ring at the bud neck. Septins contain a characteristic GTPase domain, which is flanked by a basic region thought to facilitate binding to membranes containing phosphoinositide. GTP binding is thought to be important for the association of the pentameric complex into filaments (Douglas, 2005). The fact that yeast septin complexes have not been shown to hydrolyze
GTP reinforces the idea that GTP plays a primarily structural role in stabilizing the septin complex, rather than regulating septin function (Hall and Russell, 2004).

The septin ring is not regulated by a process of synthesis and degradation; rather, it is controlled by assembly and disassembly (Douglas et al., 2005). Approximately 15 minutes before a bud appears on the mother cell, the septins form a ring, which later extends into the developing daughter cell to form a collar structure. The ring then goes from its “fluid” state, where the septin subunits are interchangeable, to a “frozen” state in S, G2, and M, where the subunits are stable. The septins are disassembled early in G1 just to form the new collar structure in the next cell division. The fact that the septins assemble into very specific structures at specific points in cellular division suggests that septin dynamics are tightly linked to and important for yeast cell cycle progression.

Specifically, it has been shown that septins function as a scaffold to recruit various proteins involved in bud site selection, such as Bud2, Bud5, and Bud9. It also functions as a scaffold for the myosin ring at the neck, which eventually recruits actin to form the actomyosin contractile ring (Kusch et al., 2002). Importantly, the septin ring splits and traps proteins involved in cytokinesis and septum formation at the bud neck as the cell completes division (Douglas et al., 2005). The septins can also interact with actin and tubulin. Indeed, Kusch et al. (2002) found that they are used to capture microtubules and correctly position the spindle apparatus before the onset of cell division. Not surprisingly, cells lacking septins displayed nuclear migration defects. The septins are also used to sense the progression of bud morphogenesis as part of cell cycle checkpoints (Douglas et al., 2005).
Three of the five vegetative growth septins (Cdc3, Cdc11, and Shs1) are sumoylated very briefly from the onset of anaphase to cytokinesis, almost at the same time as the septin ring disassembly (Johnson and Blobel, 1999; Takahashi et al., 1999). Septins are the most abundant sumoylated species at G2/M, and cell cycle arrest with nocodazole greatly increases the observed number of cells with SUMO attached to the septins on the mother cell side of the bud neck (Johnson and Blobel, 1999). SUMO conjugation occurs with the aid of the E3 SUMO ligase Siz1 (Johnson and Gupta, 2001; Takahashi et al., 2001), which resides in the nucleus for the majority of the cell cycle but is briefly exported to the cytoplasm by Kap142/Msn5 at M phase, when it participates in septin sumoylation (Makhnevych et al., 2007). Immediately after mitosis, Siz1 re-enters the nucleus (Johnson and Gupta, 2001). SUMO is removed from the septins by the previously mentioned SUMO protease Ulp1, which is sequestered at the nuclear pore complex (NPC) through the karyopherins Kap121 and Kap96-Kap60 (Panse, 2003). Kap121 also helps target Ulp1 to the septin ring during mitosis, where Ulp1 can perform its SUMO protease activity upon the sumoylated septins (Makhnevych et al., 2007).

However, while the mechanisms pertaining to septin sumoylation are well-studied, the function of septin sumoylation is not completely understood. It has been suggested that SUMO is a placeholder to block ubiquitylation of certain septins (Johnson and Blobel, 1999). Indeed, septins lacking SUMO consensus sites on Cdc3, Cdc11, and Shs1 show ring disassembly defects that manifest through the presence of extra septin rings co-localized with bud scars. However, mutation of the SUMO ligase Siz1 does not result in any major phenotypic defects, even though the septins remain unsumoylated (Johnson and Gupta, 2001). Additionally, only a small number of septin proteins are
sumoylated, a fact that seems to contradict the idea that SUMO is necessary for proper septin ring disassembly (Johnson and Blobel, 1999). It is possible that the ring disassembly defect may indirectly arise from the presence of so many mutated sites on the septin proteins (Takahashi et al., 2008). However, more research is needed to fully determine the effect a lack of sumoylation will have on the septins.

The original slx5 mutant exhibited diffuse cytoplasmic localization, nuclear foci, and potential localization to the septins. We hypothesized that the bud neck staining was a result of Slx5 interaction with septin-conjugated SUMO. In this study we show that release of GFP-tagged Slx5 from the nucleus results in the accumulation of this STUbL protein at the bud neck, potentially at the septin proteins. Release of Slx5 from the nucleus was accomplished in two ways. First, we generated a series of C-terminal truncation mutants, finding one that displayed primarily cytoplasmic and bud neck localization (J. Westerbeck, unpublished observations). In our second method, we took advantage of a nup170Δ strain, which displays normal nuclear localization of proteins at 30°C but leaks proteins across the nuclear envelope at temperatures near 0°C. Use of this nucleoporin mutant allowed us to assess whether wildtype, full-length Slx5 could exhibit a similar localization to cytoplasmic targets. We hypothesized that exclusion of Slx5 from the nucleus would result in localization to the septins. Our research had three main objectives: 1) Verify that Slx5 is indeed localizing to the septins; 2) Determine the nature of the interaction between septins and Slx5; and 3) Establish if there is a possible role for Slx5 at the septin ring.
MATERIALS AND METHODS

Strains and plasmids – Strains used in this study are listed in Table 1. YOK1322 (wildtype), YOK747 (slx5Δ::G418), and YOK1270 (nup170Δ::G418) were obtained from Open Biosystems and are in the BY4741 background. YOK747 was transformed with a library of SLX5 plasmids randomly mutated using XL1-Red mutagenic bacteria (Stratagene) and used in the high-throughput robotic screen for temperature sensitive slx5 mutants; YOK1008 (candidate slx5-ts49) was isolated from this screen. Upon further testing, we determined that this mutant was not actually temperature sensitive; however, it displayed the unusual bud neck localization.

We next extracted the mutant plasmid (BOK422) and transformed it back into the same slx5Δ::G418 strain (YOK747). However, the bud neck localization phenotype did not recapitulate in this newly transformed strain (YOK1200). It is possible that other defects in the background of the original mutant strain (YOK1008) may have acted in combination with the mutation on the slx5 plasmid to help exclude the Slx5 from the nucleus. Because the bud neck localization phenotype did not repeat, we looked for a new way to release Slx5 into the cytosol to see if it would indeed localize to the bud neck.

In order to more fully understand the SLX5 mutation underlying the initial mislocalization mutant, we looked closely at the sequence of both the wildtype and mutant Slx5 proteins. Slx5 must be imported into the nucleus in order to form its characteristic nuclear foci. Analysis of the wildtype Slx5 sequence revealed three areas rich in either lysine or arginine, which could potentially function as classical nuclear localization signals (cNLSs). These are well-characterized domains known to contain
stretches of basic amino acid residues (Ossovaskaya et al., 2008). One of the most common pathways for nuclear import with a cNLS involves the use of karyopherins, mediator proteins that help translocate target proteins across the nuclear pore.

Sequencing of the original mutant plasmid revealed a single amino acid switch from asparagine to aspartic acid in slx5-ts49, N570D. Interestingly, this amino acid switch occurred adjacent to one of the potential cNLSs, a lysine-rich stretch of amino acid residues. Introduction of the negatively charged residue to an otherwise positive region could alter the protein dynamics of that region. This strengthened our hypothesis that Slx5 might contain a cNLS at this site. Impaired function of this putative cNLS, compounded with other background mutations in the original mutant, could likely have resulted in the observed bud neck localization phenotype. Using the previously described SLX5-GFP/LEU2 plasmid (BOK370) (Cook et al., 2009) and primers for QuikChange Site-Directed Mutagenesis (Stratagene; procedure described below), we replaced three lysines within this potential cNLS with alanines (579-KKK-581 to 579-AAA-581). This mutant plasmid (BOK421) was transformed into an slx5Δ strain (YOK747). However, this putative cNLS mutant (YOK1203) failed to recreate the bud neck localization phenotype; the localization pattern of the Slx5 and the growth rate of the strain was essentially the same as the wildtype. We therefore searched for a new way to forcibly exclude Slx5 from the nucleus to see whether it would indeed localize to the bud neck.

The Slx5 C-terminal truncation mutant plasmid SLX5(1-621)-GFP/LEU2 (BOK505), which encoded the first 207 amino acids of Slx5, was acquired from Jason Westerbeck. To generate this mutant, fragments of the SLX5 gene were amplified through PCR. The PCR products were then individually inserted into the universal GFP
acceptor plasmid (Cook et al., 2009). A CFP-tagged version of this truncation (BOK507) was created by excising the GFP out of plasmid BOK505 and replacing it with the CFP fragment from an SLX5-CFP/LEU2 plasmid (BOK502) through ligation. Full-length SIM A/B-deficient Slx5 (BOK463) had been generated through QuikChange Site-Directed Mutagenesis (Stratagene) of a known SIM B mutant with SIM A* primers (Caitlin Cook, unpublished reagents). A SIM A/B-deficient truncation, SLX5(1-621)-SIM A/B-GFP/LEU2 (BOK527), was made using the same PCR primers that had been used to make BOK505. This PCR product was inserted into the universal GFP acceptor. These plasmids were used as follows.

The nup170Δ::HIS3 strain (YOK1198/YMB2060) was used in experiments investigating the mislocalization of full-length Slx5. YOK1198 was transformed with SLX5-GFP/LEU2 (BOK370) to make YOK1199 and YOK1310. Additionally, it was transformed with SLX5-SIM A/B-GFP/LEU2 (BOK463) to create YOK1311. Wildtype YOK1322 was transformed with SLX5(1-621)-CFP/LEU2 (BOK507) to form YOK1325. This new strain was subsequently transformed with SLX5(1-621)-CFP/LEU2 (BOK507) to form YOK1364. Additionally, Cdc3 was tagged with YFP in the Nup170-deficient YOK1270 to generate YOK1327. This strain was subsequently transformed with the plasmid BOK507 to make YOK1359.
Strain YOK1298, which contained smt3-R11,15,19-TRP, was acquired from Caitlin Cook. Primers OOK372 (SMT3 FWD) and OOK373 (SMT3 REV) were used to generate a smt3-R11,15,19-TRP mutant in wildtype strain MHY501.

Four new strains were generated from a cross between YOK821 (slx5Δ::G418 MATa) and YOK1327 (nup170Δ::G418 CDC3-YFP/HIS5). The strains YOK1397, YOK1398, YOK1402, and YOK1403 were isolated from this cross and subsequently used for protein extraction.

Standard gene names according to the Saccharomyces Genome Database were used. Yeast Rich (yeast extract/peptone/dextrose) (YPD) and minimal (SD) media preparation and manipulation of yeast cells were previously described in Guthrie and Fink (1991).

**Transformation of yeast cells with plasmids** – To make cells competent, yeast cells were inoculated in 5 mL of the appropriate media and grown overnight (30°C, shaken) to log phase (OD$_{600} =$ 0.5-1.5). Cells were spun down and the supernatant was discarded. 2 mL LiAce/TE was then added and the cells were incubated for at least 30 minutes. 5 ODs were transferred to a microcentrifuge tube and spun down, then the supernatant discarded. Cells were resuspended in the residual liquid, then mixed with 5 µL of denatured HS-DNA (5µg/mL), approximately 1-2 µL of plasmid, and 200 µL of LiAce/TE/PEG containing 20 µL of DTT. Transformations were incubated at 30°C for 30 minutes before heat shock at 42°C for 14 minutes. Cells were then plated on selective media and grown at 30°C for 3 days.

**Generating the YFP tag for the Cdc3-YFP fusion** – Forward and reverse primers were generated according to Wach et al, (1997) for PCR amplification of the YFP
fragment from pDH5 (Yeast Resource Center). This fragment was then inserted in frame with the gene of interest to generate a protein tagged at the C-terminus by using the transformation method described below.

*Transformation of yeast cells, inserting fragments into chromosome* – Cells were grown overnight in the appropriate media and made competent with LiAce/TE as described in the plasmid transformation section. After a 30-minute incubation with LiAce/TE, 2 ODs of competent cells were transferred to a microcentrifuge tube and spun down, then the supernatant discarded. Cells were resuspended in the residual liquid, then mixed with 5 µL of denatured HS-DNA (5µg/mL), 500 ng DNA fragment, and 500 µL of LiAce/TE/PEG. Transformations were incubated at 30°C for 30 minutes followed by addition of 20 µL DMSO and heat shock at 42°C for 15 minutes, with gentle swirling every 5 minutes. Cells were spun down and the supernatant discarded. 1 mL YPD was used to resuspend the cells, which were then incubated for 90 minutes (30°C, shaken). Transformed cells were next spun down and resuspended in 1 mL 0.9% NaCl. 100 µL of this mixture was plated onto the appropriate selective media and incubated until colonies appeared. Microscopy was used to confirm successful transformants if necessary.

*Western Blot* – Proteins were extracted as follows. Yeast cultures were spun down in a 15 mL conical tube. The supernatant was removed and the cells were resuspended in 800 µL of 20% TCA. This suspension was transferred to a 1.5 mL microcentrifuge tube, spun down for 30 seconds, washed in a further 400 µL of 20% TCA, and resuspended in a third addition of 400 µL 20% TCA with 200 µL glass beads. The mixture was vortexed at high speed for 4 minutes and the white supernatant transferred to a new tube before spinning down. The clear supernatant was carefully
removed and the pellet washed in 800 µL 2% TCA. After a final centrifugation, the supernatant was removed, the pellet resuspended in 200 µL TCA sample buffer containing BME, and the suspension was boiled for 2 minutes at 100°C.

Samples were run in a pre-cast Invitrogen NuPAGE Novex 4-12% Bis-Tris mini-gel in MOPS buffer at 200V for 50 minutes. A membrane was pre-soaked in methanol for one minute and semi-dry transfer buffer for another minute. Samples were then transferred to the membrane using semi-dry transfer apparatus for 20 minutes at 19 V. After transfer, the membrane was blocked in a solution of TBST containing 4% milk for 1 hour, then shaken with 4% milk in TBST containing primary antibody overnight. The membrane was given three TBST washes, each 5 minutes long, before addition of 4% milk in TBST containing secondary antibody. After addition of the secondary antibody, the membrane was placed in TBST for three 15-minute washes. Protein bands were visualized on X-ray film after treating the membrane with Millipore Immobilon Western Chemiluminescent HRP Substrate. The primary antibody used to visualize YFP-tagged Cdc3 was the mouse α-GFP JL8 (Clontech), which also crossreacts with YFP. The secondary antibody used was an anti-mouse IgG antibody conjugated to horseradish peroxidase (Roche Applied Science).

Ligation – To make the CFP-tagged SLX5(1-621) truncation (BOK507), backbone- (SLX5-CFP/LEU2, BOK502) and fragment-containing (SLX5(1-621)-GFP/LEU2, BOK505) plasmids were separately digested with NotI and SacII. Calf intestinal phosphatase was added to the backbone for 30 minutes at 37°C after the digestion. Both digestions were then run out on a 1% agarose gel for 40 minutes at 120 volts to purify. The backbone and fragment were then extracted from the gel using a
QIAGen Gel Extraction Kit and ligated using T4 DNA ligase for 3 hours at 25°C. The
ligation was transformed into One Shot Mach 1-T1R chemically competent *E. coli*
(Invitrogen), which were plated and allowed to grow overnight. The resulting colonies
were inoculated overnight, miniprepped, and digested with NotI and SacII to ensure that
the inserted fragment was present.

*Nocodazole arrest* – Transformed cells were grown overnight in 5 ml –LEU
media to log phase (30°C, shaken) in order to maintain selection for the SLX5-GFP
plasmid. The cells were then transferred to 5 ml fresh YPD media, and nocodazole
(Sigma-Aldrich) was added to a concentration of 15 µg/ml. The cultures were placed at
30°C for 120 minutes to arrest the cells at G2/M.

*Nocodazole/benomyl arrest* – Cells were grown overnight to log phase and diluted
to an OD of 0.3 in YPD. Nocodazole and benomyl (Sigma-Aldrich) were added to a
concentration of 15 µg/ml and 10 µg/ml, respectively. Cells were rotated at 25°C for 150
minutes to induce arrest at G2/M.

*Temperature shift* – Cells underwent a temperature shift from 30°C to 0°C. This
was accomplished by placing the tubes containing arrested cells in an ice water bath for a
period of 30 minutes.

*Microscopy* – 400-ml samples of cells were removed, washed, and concentrated
by centrifugation. If cells were treated with nocodazole or benomyl, they were spun
down at 5000 rpm; otherwise, cells were centrifuged at top speed. 6-µl samples of
concentrated cells were placed on glass slides and observed at room temperature using a
Zeiss Axioskop with a 100x oil immersion objective. Images were captured using a
Retiga-SRV camera (Q-vision), iVision software (BioVision Technologies), and a
Uniblitz shutter assembly (Rochester, NY). Filter sets used for this study included CZ909 (GFP), XF114-2 (CFP), and XF104-2 (YFP) (Chroma Technology Group). Images were processed using Adobe Photoshop.

Yeast crosses – Parent strains (mating types a and α) were grown separately and then mixed in a patch on a YPD plate. Small samples from this patch were then streaked out onto a new plate to obtain single colonies. Those that contained diploids were then patched out onto a sporulation plate and allowed to sporulate for 5 days. Small amounts of cells were taken from the plates and suspended in 50 µL of 1M sorbitol before treatment with 8 µL of zymolase for twelve minutes without agitation. Tetrads were dissected and allowed to grow until colonies were visible. Strains generated from the cross were genotyped by replica plating onto selective media, and genotypes were confirmed by PCR.

QuikChange Site-Directed Mutagenesis – PCR primers were generated according to the Stratagene website. Instructions from the QuikChange kit, acquired from Stratagene, were subsequently followed to generate the mutants of interest.

Results

Discovery of the original bud neck localization mutant – We first discovered the septin mislocalization phenotype during a high-throughput robotic screen whose primary aim was to generate a temperature-sensitive mutant of Slx5. In this screen, we transformed a slx5Δ::G418 strain (YOK747) with a library of randomly mutated SLX5-GFP/LEU2 plasmids. Approximately 1500 transformants were then robotically arrayed on plates containing 0.1M hydroxyurea (HU) and grown at two temperatures, 30°C and
37°C. Exposure to HU causes S-phase arrest, mimicking DNA damage, and thus can be used in place of UV to test for functional Slx5 at the two temperatures. Potentially temperature-sensitive mutants that grew on HU plates at 30°C but not 37°C were subjected to further testing and microscopy, during which we identified the Slx5 mutant (YOK1008) that mislocalized the GFP-tagged protein to the bud neck of dividing cells. We estimated that this phenotype occurred in approximately 0.5% of the cells.

Based on the localization pattern, we hypothesized that Slx5 was possibly localizing to the bud neck-localized septins, which are highly sumoylated at G2/M. Arresting yeast cells at the stage of the cell cycle using nocodazole, which interferes in microtubule polymerization, greatly increases the prevalence of sumoylated septin proteins (Johnson and Blobel, 1999). We therefore arrested the mutant with nocodazole and observed that 11% of the population now displayed the bud neck localization phenotype.

Attempts to repeat mislocalization – We attempted to repeat the mislocalization phenotype by transforming the original mutant plasmid into a fresh slx5Δ strain and also by mutating a putative NLS of Slx5. These efforts (described in the Materials and Methods) were unsuccessful, so we developed other methods to localize Slx5 to the nucleus.

A C-terminal truncation mutant and full-length Slx5 localize to the bud neck – Graduate student Jason Westerbeck designed primers to synthesize GFP-tagged C-terminal truncations of the Slx5 protein at lengths of 50, 107, 207, 310, 414, and 517 amino acids each. The plasmids containing the GFP-tagged truncations were individually transformed into a wildtype strain of yeast (YOK1322), and localization of each mutant
was observed using fluorescence microscopy. Truncation mutants of the lengths 51, 107, and 207 amino acids localized primarily to the cytoplasm, and mutants of the lengths 310, 414, and 517 amino acids localized to the nucleus, indicating that an NLS potentially resides between amino acid positions 207 and 310 on the protein. The mutant Slx5(1-621)-GFP (YOK1375; named based on nucleotide length), which expresses the first 207 amino acids of Slx5, exhibited bud neck localization in some cells. When we arrested this same strain with nocodazole and benomyl, a reagent that similarly interferes in microtubule polymerization, the bud neck localization phenotype increased dramatically to near 80% (Figure 2). Knowing that this C-terminal truncation mutant displayed the desired phenotype, we next sought to determine whether full-length Slx5 could localize to the bud neck.

![Figure 2: Localization of truncation Slx5(1-621)-GFP.](image)

G2/M arrested cells exhibit distinct Slx5(1-621) staining at the bud neck of the dividing cell, indicated by arrows.
To accomplish this, we utilized strains of yeast lacking the nucleoporin Nup170 (YOK1198, YOK1270). Deletion of Nup170 results in defects of NPC assembly, in which certain structural nucleoporins do not become properly incorporated into the NPC (Terry and Wente, 2009). This results in a “leaky” nuclear phenotype, in which relatively large molecules can diffuse across the nuclear membrane more easily than in cells with normal NPCs. In a $nup170\Delta$ mutant, proteins with an NLS are actively transported into the nucleus and display normal localization at 30°C (Shulga et al., 2000). At 0°C, active import into the nucleus is inhibited, but passive diffusion continues. At these low temperatures, proteins equilibrate more rapidly across the nuclear envelope in $nup170\Delta$ cells than that of wildtype cells due to the enlarged nuclear pore. A strain of cells lacking Nup170 can therefore be used to study the mislocalization of proteins that typically reside in the nucleus.

At 98 kDa, Slx5 is too large to diffuse into the nucleus of a wildtype cell, so it must be actively transported into the nucleus regardless of whether the process involves the use of a cNLS and karyopherins. This need for active transport into the nucleus makes the STUbL subunit Slx5 a suitable candidate for this $nup170\Delta$-temperature shift assay.

First, we transformed the $SLX5$-GFP/LEU2 plasmid into $nup170\Delta::HIS$ and wildtype strains. We then treated the strains (YOK1199, YOK1202) with nocodazole and benomyl to ensure that the majority of the cells were arrested in G2/M, when the septins are most highly sumoylated. The cells were then shifted to an ice water bath to halt active transport into the nucleus. Finally, we performed fluorescence microscopy on the cells to observe the localization of the Slx5-GFP.
Our observations showed that the \textit{nup170A} strain exhibited two distinct localization patterns at the two different temperatures. At 30°C, \textit{nup170A} exhibits only nuclear foci, but at 0°C, it shows bud neck localization, diffuse cytoplasmic localization, and nuclear foci co-existent in the same cell in 45.6% of cells. By contrast, the wildtype strain exhibited only nuclear foci at both temperatures (Figure 3). Use of the \textit{nup170A} mutant successfully recreated the bud neck localization phenotype with full-length Slx5, though this phenotype was far less prevalent than in the strain containing the C-terminal truncation mutant. Indeed, results of this experiment were slightly inconsistent; reasons for this will be discussed below.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Comparison of Slx5-GFP localization in wildtype and \textit{nup170A} cells. Wildtype and \textit{nup170A} cells were arrested in G\textsubscript{2}/M (nocodazole) and shifted to 0°C for 30 minutes. Wildtype cells displayed normal Slx5 localization at both temperatures. In cells lacking the nucleoporin Nup170, bud neck localization was exhibited at both temperatures, but was dramatically increased when the cells were shifted to 0°C due to increased equilibration of proteins across the nuclear membrane. Note that at 0°C, the bud neck localization phenotype is greatly increased.}
\end{figure}
The C-terminal truncation mutant and full length Slx5 co-localize with the septin subunit Cdc3 – In order to perform a colocalization assay, we first had to fuse YFP to the C-terminus of a septin subunit within a wildtype strain (YOK1322) and the strain nup170Δ::G418 (YOK1270). The nucleoporin mutant nup170Δ::HIS3 used in the nup170Δ-temperature shift assay could not be used, as the YFP fragment contained a HIS5 marker. In this case, Cdc3 was tagged with YFP. Successful transformants were confirmed by fluorescence microscopy.

The CDC3-YFP/HIS5 strain (YOK1325) was next transformed with the CFP-tagged truncation mutant plasmid SLX5(1-621)-CFP/LEU2 (YOK1364). A nocodazole/benomyl arrest was performed on successful transformants before microscopy. Images revealed that in many of the G2/M arrested cells, the C-terminal Slx5 truncation was co-localized with the septin subunit Cdc3 (Figure 4).

![Image](image.png)

**Figure 4:** The truncation Slx5(1-621) co-localizes with the septin ring subunit Cdc3. YFP, CFP, and brightfield images were collected and were subsequently merged in Adobe Photoshop. Bud neck localization by the C-terminal truncation mutant Slx5(1-621) is here shown to be septin ring staining. Slx5(1-621)-CFP and Cdc3-YFP were cotransformed into strain YOK1322. This strain was arrested at G2/M before image capture. Most cells displayed co-localization of slx5(1-621) with the septin ring.
We decided to investigate whether full-length Slx5 showed similar co-localization. For this experiment, the *nup170Δ::G418 CDC3-YFP/HIS5* (YOK1327) strain was transformed with the plasmid *SLX5-CFP/LEU2*, which contains full-length Slx5 tagged with CFP. These cells (*nup170Δ::G418 CDC3-YFP/HIS5 + SLX5-GFP/LEU2*) (YOK1359) were then arrested with nocodazole and benomyl and shifted to low temperatures before microscopy.

![Cdc3-YFP and Slx5-CFP images](image)

**Figure 5: Full-length Slx5 co-localizes with the septin subunit Cdc3.** Shown are *nup170Δ* cells containing YFP-tagged Cdc3 and CFP-tagged Slx5. Cells were arrested with nocodazole and benomyl and shifted to 0°C before microscopy. Only cells fully arrested at G2/M – when septins are most highly sumoylated – display co-localization of Slx5 with the septin ring (septin overlays indicated by arrow). At right are three additional sets of images displaying the same co-localization pattern (septin overlays indicated by arrows).
YFP, CFP, and brightfield images were taken and merged in Adobe Photoshop, as described previously. While bud neck localization was again less prevalent than with the truncation mutant, several images revealed that full length Slx5 colocalized with Cdc3 (Figure 5). Significantly, Slx5 only co-localized with Cdc3 in cells that were fully arrested at G2/M.

*SUMO-interacting motifs are important for septin localization of Slx5 –* Previous work by Xie et al. (2007) had shown that two of the SIMs in Slx5, SIM A and SIM B, are important for the SUMO-targeting ability of the Slx5 STUbL subunit. Results of the experiments described in this paper had shown that increasing septin sumoylation by nocodazole/benomyl arrest also greatly increased the incidence of the septin localization phenotype. Taken together, these two pieces of data suggest that SUMO is very important for the septin localization of Slx5.

To evaluate whether SIM A and SIM B were involved in the septin localization phenotype, we transformed cells of the background *nup170Δ::HIS3* cells with a GFP-tagged SIM A/B mutant of *slx5*, in which the SIMs were no longer functional (YOK1311). We also transformed the full-length wildtype *SLX5-GFP/LEU2* plasmid into the *nup170Δ::HIS3* strain as a control (YOK1310). We then performed a nocodazole/benomyl arrest and temperature shift on these cells and temperature shift to force the SIM A/B-deficient Slx5 into the cytoplasm. We then used fluorescence microscopy to determine the localization of the SIM-deficient Slx5. Wildtype Slx5 protein localized to the septins, as expected. However, when SIM A and SIM B were deleted from Slx5, the localization to septins was essentially eliminated (Figure 6A).
Figure 6: SIMs are important for septin localization of Slx5. A) Full-length Slx5 lacking SIM A and SIM B does not display septin localization. Data were collected using the *nup170Δ* temperature shift assay described previously. B) The truncation mutant Slx5(1-621), in which SIM A and SIM B are intact, displays septin localization, as described previously (upper panel; indicated by white arrows). By comparison, the SIM A/B-deficient truncation mutant (lower panel) displays much reduced septin localization. Yellow arrows indicate the bud neck, where possible septin localization is just barely discernible.

As mentioned previously, the C-terminal truncation mutant Slx5(1-621)-GFP displayed septin localization far more reliably than the full length Slx5. We therefore generated a plasmid containing a GFP-tagged truncation lacking functional SIM A/B.
This plasmid was transformed into a wildtype strain and its localization observed (YOK1522). Septin localization was found to be either eliminated or significantly reduced (Figure 6B).

*The presence of SUMO chains enhances septin localization of Slx5* – Up to this point, our data suggested that Slx5 localizes to the septins and that SIMs are crucial for this localization. We next sought to determine whether SIMs of Slx5 were interacting with single SUMO molecules or poly-SUMO chains covalently attached to the septin proteins. To accomplish this, we used a strain containing an *smt3* mutant whose chain-forming lysines had been switched for arginines and transformed the *SLX5(1-621)-GFP/LEU2* plasmid into it. Upon G2/M arrest and microscopy, we discerned that while septin localization of the Slx5 was not eliminated, it occurred less frequently and with far less intensity than in cells with both fully functional SUMO and the Slx5 truncation (Figure 7).

![Slx5(1-621)+R11,15,19 vs. Slx6(1-621) + SUMO](image)

**Figure 7: SUMO chains enhance the septin localization of Slx5.** In a strain containing *smt3-R11,15,19*, which can no longer form SUMO chains (left panel), septin localization occurs with far less frequency and intensity than in a strain containing fully functional SUMO (right panel). Both strains shown contain the Slx5(1-621) truncation, which contains functional SIMs.
Slx5 may aid in turnover of sumoylated septin species – Our data have established that Slx5 can localize to the septins and that SIMs and SUMO are required for this interaction. We next sought to determine why Slx5 might be localizing to the septins. Knowing from previous studies that Slx5 functions as part of a STUbL, we hypothesized that it might be performing STUbL function at the septins.

We performed a Western blot to see if sumoylated septin species were affected by Slx5. Through a cross between slx5Δ::G418 MATa (YOK821) and nup170Δ::G418 CDC3-YFP/HIS5 MATa (YOK1327), we managed to generate the following strains: CDC3-YFP/HIS5 (YOK1398), slx5Δ::G418 CDC3-YFP/HIS5 (YOK1397), nup170Δ::G418 CDC3-YFP/HIS5 (YOK1403), and slx5Δ::G418 nup170Δ::G418 CDC3-YFP/HIS5 (YOK1402). These strains were grown in culture overnight to log phase. Each culture was then divided into two separate flasks; half was arrested with nocodazole and benomyl, and the other half was left to continue growing without treatment. Samples of cells from each culture were collected, then the proteins extracted. We performed a Western blot of the whole cell extracts and probed using an α-GFP antibody that bound the YFP fused to the Cdc3. We attempted to use an α-Cdc3 antibody at one point; however, the α-GFP antibody gave far cleaner results.

As expected, all G2/M-arrested cells displayed a number of dark bands indicative of sumoylated septins. The sumoylated species were greatly reduced without nocodazole/benomyl treatment. Significantly, however, the untreated slx5Δ and slx5Δnup170Δ strains exhibited a greater amount of higher-molecular weight species than untreated wildtype or nup170Δ cells (Figure 8). While it is likely that these higher-molecular weight species are indeed sumoylated septins, further investigation, for
instance by reprobing the original blots with α-SUMO antibody, is needed to confirm whether this is true.

**Figure 8:** Comparison of high-molecular weight septin species in cells arrested vs. unarrested at G2/M. A yeast cross was used to generate the following strains, which all contained Cdc3-YFP: Wildtype, slx5Δ::G418, nup170Δ::G418, and slx5Δ::G418 nup170Δ::G418. All strains were grown in the presence or absence of nocodazole and benomyl. Strains deficient in Slx5 display an increase in high-molecular weight septin species in unarrested cells. It is possible that this is an accumulation of sumoylated Cdc3; however, more investigation is needed to determine whether this is the case.

**DISCUSSION**

Our research set out to characterize a novel localization phenotype of the SUMO-targeted ubiquitin ligase Slx5. The findings of our study can be summarized as follows: First, a subpopulation of nuclear excluded Slx5 colocalizes with the septin subunit Cdc3 at the bud neck. Second, the SIMs of Slx5 and SUMO chains are important for the colocalization of Slx5 and Cdc3. Finally, absence of Slx5 in cells leads to a greater accumulation of potentially sumoylated septin species.

For our analysis we adopted the previous observation that nup170Δ mutants can be used to study the mislocalization of a nuclear protein – in this case, the 98 kDa Slx5. The diffusion channel in the yeast nuclear pore complex is typically about 10 nm in diameter; in a nup170Δ mutant, it can be as large as 40 nm across (Shulga et al., 2000).
This makes the *nup170Δ* mutant a particularly useful tool to study a potential role for Slx5 outside the nucleus. Indeed, the development of this assay was critical for our study, allowing us to define a potential novel extranuclear role for STUbLs.

Using the *nup170Δ* mutant allowed us to visualize Slx5 foci formation, cytoplasmic staining, and bud neck localization. As previously mentioned, results of this assay were sometimes inconsistent. It is possible that the way that cells were prepared for microscopy played a role. It has been suggested that SUMO chains rapidly break down if cells are immersed in water for as little as one minute (Erica Johnson, in press). Our research has shown that interaction with SUMO, particularly in the form of poly-SUMO chains, is key for septin localization of Slx5. It is possible that inconsistencies in our results are due to the breakdown of SUMO chains during the pre-microscopy wash. Since the localization of Slx5-GFP to septins is critically dependent on SUMO chains, these washes should be conducted using minimal (SD) glucose-containing media. Because the media contains sugar, it is more likely that SUMO chains will remain intact.

We were able to obtain far more consistent results with the truncations of Slx5. The reason for this is likely twofold: this mutant did not require any treatment to display cytoplasmic localization, and contains both intact SIM A (amino acids 24-27) and SIM B (amino acids 93-96). The *slx5* mutants longer than this particular truncation may have contained an NLS, as they all displayed nuclear localization; mutants shorter than Slx5(1-621)-GFP, however, may have all been truncated too close to SIMs for the SIMs to fold correctly (Figure 9). Essentially, the Slx5(1-621)-GFP mutant lacks the ability to enter the nucleus but also possesses intact SIM A and SIM B, allowing it to easily interact with sumoylated septins.
### Figure 9: Schematic of Slx5 truncations.

The C-terminal truncation Slx5(1-621) used in this study contains SIMs that are intact but lacks the putative NLS. Shorter truncations may have misfolded SIMs, and longer truncations may contain the putative NLS. Truncations were generated and initially analyzed by Jason Westerbeck.

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Overall, our results with full length Slx5 clearly indicate the Slx5 can co-localize with the septin ring in cells at G$_2$/M. Observations of the truncation Slx5(1-621) appeared far more consistent and support this same conclusion. It is therefore possible that Slx5 may occasionally localize to septins in wildtype cells. Indeed, we rarely observed an untreated wildtype cell containing GFP-tagged Slx5 that had localized to the septins (data not shown). It may be that the interaction is simply too brief to observe unless the SUMO chains at the septins are maintained by arresting the cells in G$_2$/M. One example of a similarly transient interaction involves Ulp1, the SUMO protease that is typically sequestered at the nucleus until it participates in the desumoylation of septins following cytokinesis (Makhnevych et al., 2007). Even in G$_2$/M arrested cells, we observed that Ulp1 was primarily localized to the nuclear periphery, and managed to find only a single cell in which it was localized to the septins (data not shown).

Knowing that Slx5 could co-localize with septins, we next sought to determine the nature of this interaction. Arresting the cells in G$_2$/M increases the sumoylation of septins (Johnson and Blobel, 1999) and resulted in an increase of the Slx5 septin...
localization, indicating that SUMO might be important for this interaction. Previous research had showed that the Slx5 SIMs A and B are required for the targeting activity of Slx5. We therefore used the nup170Δ mutant to examine the localization of full-length slx5 mutant containing defective SIM A and SIM B. We did not find the septin localization pattern in this mutant; however, when we used a similarly SIM-defective truncation of Slx5, we were able to see a very faint septin localization. The faint septin localization in the SIM A/B-mutated truncation could be due to the existence of other putative SIMs in the truncation sequence, or another Slx5 interacting protein that also localizes to the septins. For example, Jason Westerbeck recently showed that Slx5 can interact with another septin-localized SUMO ligase.

We then sought to determine whether the Slx5 septin localization was dependent on the cell’s ability to form poly-SUMO chains. When we transformed the Slx5 truncation into a strain containing mutant smt3-R11,15,19, which could no longer form SUMO chains, we observed a reduction in septin localization of Slx5. This suggests that while single SUMO molecules can interact with Slx5 at the septins, Slx5 preferentially localizes to poly-SUMO chains. These findings corroborate those of Mullen and Brill (2008), who found that the Slx5·Slx8 heterodimer is activated by poly-SUMO chains.

Because Slx5 colocalizes with the septins when forced into the cytosol, we were curious to see whether Slx5 may possess a role in the cytosol, particularly at the septins, in certain instances. It is possible that Slx5 may still function as part of the Slx5·Slx8 STUbL, resulting in ubiquitylation and subsequent degradation of cytosolic sumoylated targets; however, we did not investigate the localization of Slx8 in this study. We reasoned that sumoylated Cdc3 may be an Slx5 target. Therefore, we compared the
levels of sumoylated septin species in Slx5-containing cells and Slx5-deficient cells. We found a noticeable increase of sumoylated septin species in untreated Slx5-deficient cells. This suggests that Slx5 may have a role in the turnover of sumoylated septin species. In G2/M arrested cells, levels of sumoylated septins may be lower due to the slower growth rate of slx5A cells.

We hypothesize that efficient nuclear import of Slx5 may prevent interactions with the sumoylated septins during regular cellular division. However, the fact that high-copy Slx5 is able to rescue ulp1ts mutants (Xie et al, 2007) may indicate that SUMO, and therefore Slx5, may have a role at the septins. Ulp1 is an NPC-localized SUMO protease that is responsible for the desumoylation of septin proteins (Panse, 2003). Slx5, which functions as part of a STUbL with Slx8, may linger in the cytosol when overexpressed in a ulp1ts mutant. The heterodimer might then localize to the septins and act in place of Ulp1 by ubiquitinating the sumoylated septin proteins, facilitating septin ring disassembly. A small amount of modified septins may be degraded or relocalized upon disassembly of the septin ring, which may be congruent with the small amount of septin proteins that are sumoylated (Johnson and Blobel, 1999). It is therefore possible that Slx5 – as part of the STUbL – rescues ulp1ts by bypassing the desumoylation step typically performed by Ulp1 at the septins. Instead, it interacts with the SUMO directly and the Slx8 part of the complex attaches ubiquitin to the septins (Figure 10). More research, however, is needed to verify the exact mechanism of Slx5 and septin interaction. These studies have shown a potential new role for Slx5 at the septins. Since Slx5 is highly conserved from yeast to humans, these findings may have important consequences for our understanding of STUbL function in humans.
Figure 10: Model of STUbL function at the septins. In this model, the Slx5·Slx8 STUbL interacts with poly-SUMO chains at the septin ring, resulting in ubiquitylation of sumoylated septin subunits and subsequent septin ring disassembly.
## Table 1: Yeast Strains

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<td>smt3-R11,15,19</td>
<td>SLX5(1-621)-SIM A/B-GFP/LEU2</td>
<td>This Study</td>
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<td>smt3-R11,15,19</td>
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<tr>
<td>BOK Number</td>
<td>Pertinent Genes</td>
<td>Description</td>
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<td>BOK370</td>
<td>SLX5-GFP/LEU2</td>
<td>WT GFP-tagged Slx5</td>
<td>pRS315</td>
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<td>BOK502</td>
<td>SLX5-CFP/LEU2</td>
<td>WT CFP-tagged Slx5</td>
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<tr>
<td>BOK421</td>
<td>SLX5**-GFP/LEU2</td>
<td>PKKKLAQ to PAAALAQ NLS mutant</td>
<td>BOK370</td>
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<tr>
<td>BOK422</td>
<td>SLX5*-GFP/LEU2</td>
<td>Plasmid extracted from YOK1008; XL1-Red mutated</td>
<td>BOK370</td>
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<tr>
<td>BOK463</td>
<td>SIM A/B mutant</td>
<td>Generated from a SIM B<em>12 mutant (BOK 287) using QuikChange with SIM A</em> primers</td>
<td>BOK370</td>
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<td>BOK507</td>
<td>SLX5-c207-CFP/LEU2</td>
<td>WT CFP-tagged Slx5 truncation</td>
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<tr>
<td>BOK505</td>
<td>SLX5-c207-GFP/LEU2</td>
<td>WT GFP-tagged Slx5 truncation</td>
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<td>BOK527</td>
<td>SLX50c207-SIM A/B-GFP/LEU2</td>
<td>SIM mutant truncation</td>
<td>BOK463</td>
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<tr>
<td>OOK Numbers</td>
<td>Schematic</td>
<td>Use</td>
<td></td>
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<tr>
<td>OOK267 (FWD) OOK268 (REV)</td>
<td>![OOK267 schematic]</td>
<td>To generate SIM A/B mutant by QuikChange PCR of SIMA on SIMB mutant (BOK287)</td>
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<tr>
<td>OOK340 (FWD) OOK341 (REV)</td>
<td>![OOK340 schematic]</td>
<td>For generating putative NLS mutant of Slx5 by QuikChange PCR of SLX5-GFP (BOK370)</td>
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<td>OOK375 (FWD) OOK376 (REV)</td>
<td>Generated as described in Wach et al. (1997); FWD is CD3-STOP-40 with YFP overhang; REV is CDC3-STOP+40 with YFP overhang</td>
<td>For generating YFP tag for CDC3 by PCR of YFP/HIS5 (pDH5)</td>
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<td>OOK387 (FWD) OOK391 (REV)</td>
<td>![OOK387 schematic]</td>
<td>For generating Slx5(1-621) truncation and Slx5(1-621)-SIM A/B mutant</td>
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<td>OOK276 (FWD) OOK275 (REV)</td>
<td>![OOK276 schematic]</td>
<td>For PCR confirmation of slx5A mutants from yeast cross</td>
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<td>OOK379 (FWD) OOK380 (REV)</td>
<td>![OOK379 schematic]</td>
<td>For PCR confirmation of nup170A mutants from yeast cross</td>
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REFERENCES


