Crosstalk between SUMO and Ubiquitin Systems: The STUbL Subunit Slx5 Interacts with the SUMO E3 Ligase Siz1

Brooke Campbell Matson
College of William and Mary

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Crosstalk between SUMO and Ubiquitin Systems:
The STUbL Subunit Slx5 Interacts with the SUMO E3 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

Brooke Campbell Matson

Accepted for ________________________________

__________________________
Oliver Kerscher, Director

__________________________
Lizabeth A. Allison

__________________________
Elizabeth J. Harbron

__________________________
Helen A. Murphy

Williamsburg, VA
May 2, 2011
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**Abstract**

SUMO-targeted ubiquitin ligases (STUbLs) are a newly defined class of enzymes that attach ubiquitin to sumoylated substrates. STUbLs are conserved from yeast to humans and have been implicated in DNA repair, transcriptional regulation, and genome stability. Importantly, studies on STUbLs provide insight into the crosstalk between the SUMO and ubiquitin conjugation pathways. The budding yeast STUbL Slx5-Slx8 has few confirmed ubiquitylation substrates, but many more are believed to exist. For example, Slx5-Slx8 ubiquitylates the DNA damage repair protein Rad52 in vitro; in vivo, the transcriptional regulator Mot1 and the transcription factor MATa2 appear to be promising substrates.

Recently, we observed a novel colocalization between Slx5 and a septin protein, Cdc3, as well as a two-hybrid interaction between Slx5 and the SUMO E3 ligase Siz1. The goals of this study, therefore, were to determine whether Slx5-Slx8 can ubiquitylate these substrates *in vitro* and to investigate the role that sumoylation plays in STUbL targeting. Here, we report that Slx5 interacts with the SUMO E3 ligase Siz1 in *in vitro* ubiquitylation and pulldown assays and that SUMO2 chains are required for the ubiquitylation of a substrate by the human STUbL RNF4.

**Introduction**

*Ubiquitin: A Post-translational Modification*

Post-translational modifications perform significant regulatory roles in the cell and can take the form of chemical groups or small protein modifiers. For example, phosphorylation, or the covalent attachment of a phosphate group, is critical for many
signal transduction pathways affecting transcription (reviewed by Karin and Hunter, 1995). Ubiquitin is another example of a common post-translational modification and is the founding member of the ubiquitin-like protein (Ubl) family (Kerscher et al., 2006). Ubls participate in regulating many cellular processes, including transcription, DNA repair, and cell cycle control, among others (Hochstrasser, 2009). At 76 amino acids in length, ubiquitin is one of only two chain-forming Ubls. Specifically, ubiquitin chains are known to earmark targets for degradation by the proteasome, a complex involved in protein quality control. First synthesized as inactive precursors, Ubls become active after their attachment site is exposed. The carboxyl group of the C-terminal diglycine motif comprises this attachment site, which predominantly targets lysine side chains on substrate proteins. Ubiquitin’s seven internal lysines – Lys6, 11, 27, 29, 33, 48, and 63 – enable it to form chains on targets (Peng et al., 2003). Conventionally, Lys48 chains uniquely flag ubiquitylation targets for degradation. However, there is evidence that Lys63 chains may also achieve this effect (Saeki et al., 2009).

Ubiquitin conjugation to target proteins is executed by a specific enzyme cascade – an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ligase (Kerscher et al., 2006). Ubiquitin is attached to the E1 in an energy-dependent exchange and then passed to the E2, which subsequently uses the E3 as a bridge to access the target. To ensure that this process occurs in an orderly, step-wise fashion, the E2 can only bind the E1 or the E3 at any one time (Eletr et al., 2005). Much of this pathway has been worked out in the budding yeast *Saccharomyces cerevisiae*. In this system, Uba1 is the only E1, whereas there are a handful of E2s – Ubc1-8, -10, -11, and Ubc13-Mms2. The RING (really interesting new gene) and HECT classes are the only two defined classes of E3 ligases.
Named for their RING domain, RING E3s and the similar U-box E3s simultaneously bind the E2 and the substrate. In contrast, HECT E3s conjugate ubiquitin to the target only after it has been transferred from the E2 to the E3.

**SUMO**

The other chain-forming Ubl is SUMO (small ubiquitin-like modifier), which is 18% identical to ubiquitin. At 11 kDa, SUMO adds approximately 20 kDa to modified proteins on a SDS-PAGE gel (Johnson, 2004). More than 1,000 proteins have been identified as potential SUMO-conjugation (sumoylation) targets (Makhnevych et al., 2009). However, despite the prevalence of targets, only about 1% of these target protein pools are actually sumoylated at any one time (Johnson, 2004). In contrast to ubiquitin, the conjugation of poly-SUMO chains to substrates does not flag them for degradation. Instead, sumoylation often changes a target’s conformation, activity, localization, or interactions with other proteins.

SUMO modifies proteins both covalently and noncovalently (Kerscher, 2007). To attach to a substrate covalently, SUMO recognizes a consensus motif on the target: "ψKXE, where ψ is a hydrophobic amino acid, and X is any amino acid. The noncovalent interaction between SUMO and its targets is mediated by SUMO-interacting motifs, or SIMs (Kerscher, 2007). While there are 16 ubiquitin-binding domains, only one SIM has been identified. Noncovalent modification by SUMO is especially important for proper PML (promyelocytic leukemia) body formation, for example; PML bodies are domains within the nucleus that recruit proteins involved in transcriptional regulation and DNA repair. Noncovalent sumoylation may explain why it appears that so few proteins are
covalently conjugated to SUMO. Importantly, the sumoylation pathway intersects with other post-translational modification pathways. For example, phosphorylation is typically considered to be antagonistic to SUMO. Likewise, SUMO and ubiquitin can compete for substrate lysines (Johnson, 2004).

Three types of SUMO exist in vertebrates: SUMO1, 2, and 3. SUMO2 and SUMO3, which are 98% identical, harbor the ψKXE consensus sequence and are capable of using this lysine to form chains (Ulrich, 2005). Therefore, the majority of free SUMO in the cell is in the form of SUMO2 or 3. On the other hand, only one type of SUMO exists in yeast – Smt3. Interestingly, smt3Δ can only be complemented by SUMO-1 (Johnson and Hochstrasser, 1997).

The SUMO conjugation pathway parallels that of ubiquitin but requires a different set of E1, E2, and E3 enzymes (Kerscher et al., 2006). First, Ulps make precursor SUMO conjugation-competent by freeing the C-terminal diglycine motif. These enzymes also cleave SUMO from substrates. Ulp1, for example, is a nuclear pore complex (NPC)- and bud neck-localized SUMO protease; deletion of this gene is lethal in yeast. While Ulp2/Smt4 doesn’t catalyze SUMO maturation, it is a nuclear protein with a more restricted set of desumoylation substrates; ulp2Δ strains exhibit stress-sensitivity and genome instability. In yeast, the E1 activating enzyme is the Aos1-Uba2 heterodimer. While the E2 enzyme, Ubc9, is sufficient to sumoylate substrates in vitro, the consensus is that sumoylation does occur through E3s even though they are nonessential in vitro (Schwarz et al., 1998, Kerscher et al., 2006).

Sumoylation in budding yeast is executed by three E3 ligases – Siz1, Siz2, and Mms21. Siz1 and Siz2 belong to the Siz/PIAS (protein inhibitor of activated STAT)
family of SUMO E3 ligases and together carry out the vast majority of sumoylation in yeast cells (Reindel et al., 2006). PIAS family E3s contain SAP and SP-RING domains, the latter sharing similarity with the ubiquitin ligase RING domain (Hochstrasser, 2001). In vertebrates, a domain in the NPC protein RanBP2/Nup358 and a polycomb group protein Pc2, which belongs to a family of proteins involved in chromatin dynamics and gene silencing, both demonstrate SUMO E3 ligase activity (Pichler et al., 2002, Kagey et al., 2003).

**Crosstalk Between SUMO and Ubiquitin Systems**

Historically, there has been evidence for antagonism between the sumoylation and ubiquitylation pathways (Ulrich, 2005). For example, IκBβ activates the NFκB pathway upon its degradation, which is signaled by its phosphorylation and subsequent ubiquitylation (Karin and Ben-Neriah, 2000). Sumoylated IκBβ, on the other hand, isn’t degraded, preventing the activation of NFκB (Desterro et al., 1998). Another example of antagonism between these two pathways occurs in the context of the Huntingtin protein, which is involved in Huntington’s disease. While sumoylation of Huntingtin stabilizes the protein and exacerbates neurodegeneration, ubiquitylation destabilizes the protein and decreases its toxicity (Steffan et al., 2004).

However, cooperation between the SUMO and ubiquitin pathways is also evident. For example, following DNA damage, the IκB kinase regulatory subunit NEMO is first sumoylated and kept in the nucleus, where it is then phosphorylated, ubiquitylated, and finally degraded (Huang et al., 2003); this triggers the activation of the NFκB pathway. Especially interesting concerning the crosstalk between the SUMO and ubiquitin
pathways was the accumulation of simultaneously sumoylated and ubiquitylated proteins in the presence of proteasome inhibitors (Uzunova et al., 2008).

**Slx5-Slx8: A SUMO-targeted Ubiquitin Ligase**

The cooperative crosstalk between these two pathways has taken new directions with the discovery of a subclass of E3 ligases termed SUMO-targeted ubiquitin ligases (STUbLs), which attach ubiquitin to sumoylated targets. The yeast STUbL Slx5-Slx8 is comprised of two subunits, both of which contain RING domains (Xie et al., 2007). Slx5 serves as the targeting subunit of the heterodimer, while Slx8 executes the ubiquitin ligase function. Slx5-Slx8 was originally isolated from a synthetic lethal screen with the DNA helicase Sgs1 (Mullen et al., 2001). Synthetic lethality was also observed between Slx5 and Slx8 and components of the sumoylation machinery, establishing the first evidence of Slx5-Slx8’s involvement with the SUMO pathway (Wang et al., 2006). Additionally, Slx5 was identified as a high-copy suppressor of a *ulp1ts* mutant (Xie et al., 2007); at the nonpermissive temperature, the SUMO protease Ulp1 is nonfunctional and cannot cleave SUMO from substrates. The fact that Slx5 can rescue this mutant provided further evidence for a link between Slx5-Slx8 and the sumoylation pathway.

Mutations in Slx5 and Slx8 confer elevated rates of gross chromosomal rearrangements (GCRs) and DNA damage, implicating roles for Slx5-Slx8 in resolving stalled replication forks and DNA damage repair (Zhang et al., 2006). In support of these roles, the DNA damage repair protein Rad52 was identified as the first *in vitro* substrate of Slx5-Slx8 (Xie et al., 2007). *In vivo* substrates include the transcriptional regulator Mot1 and the transcription factor MATα2, which also serves as an *in vitro* target (Wang
and Prelich, 2009, Xie et al., 2010). Significantly, STUbLs are conserved from yeast to humans. Specifically, Slx5-Slx8’s human ortholog RNF4 has been shown to target the oncogenic fusion protein PML-RARα, which is involved in acute promyelocytic leukemia (APL) (Tatham et al., 2008). Arsenic trioxide serves as an effective therapeutic for this cancer by inducing the sumoylation of PML and its STUbL-mediated ubiquitylation. Insights into Slx5-Slx8, therefore, may shed light on RNF4 and its substrates.

A Structure-Function Analysis of Slx5

Recently, we and others demonstrated that Slx5 forms nuclear foci and localizes to sites of double-stranded DNA breaks (Nagai et al., 2008, Cook et al., 2009). In an attempt to understand how Slx5 is targeted to these nuclear foci, we performed a structure-function analysis of Slx5 (Westerbeck et al., manuscript in preparation). Six C-terminal truncations of Slx5 were generated and their localization patterns analyzed by fluorescence microscopy. Interestingly, we observed that the Slx5(C4) construct localized to the bud neck of dividing yeast cells (Figure 1).

Figure 1: Slx5(C4) localizes to the bud neck of dividing yeast cells. A GFP-tagged, C-terminal truncation of wild-type Slx5(C4) (1-621) was generated. Localization of wild-type (WT) Slx5 and Slx5(C4) were compared by fluorescence microscopy. While WT Slx5 formed nuclear foci, C4 staining was present in the cytosol and at the bud neck. SIMs 1-4 indicate SUMO interacting motifs 1-4. Figure by Jason Westerbeck.
Therefore, we hypothesized that Slx5 could be localizing to the septins, a class of GTP-binding proteins and a structural feature of the bud neck. While septins are absent in plants, they are highly conserved and are important for proper cytoskeleton organization (Spiliotis, 2010). In yeast, five septins – Cdc3, Cdc10, Cdc11, Cdc12, and Shs1/Sep7 – form hetero-oligomeric complexes that assemble into filaments and then into complex, higher order structures (Weirich et al., 2008). Of these, only Shs1 is nonessential. Investigation into this novel bud neck localization of Slx5 led to the observation that Slx5(C4) colocalized with the septin Cdc3 (Figure 2).

![Cdc3-YFP, Slx5-621-CFP, Merged](Figure 2: Slx5(C4) colocalizes with the septin Cdc3 at the bud neck. Slx5(C4) lacks the potential nuclear localization signal and exhibits diffuse cytoplasmic staining with enrichment at the bud neck, where it colocalizes with Cdc3. Figure by Cecilia Esteban.)

Interestingly, septins are highly sumoylated during mitosis. The SUMO E3 ligase Siz2 was originally identified as a regulator of septin sumoylation (Johnson and Gupta, 2001, Takahashi et al., 2001a). However, observations of wild-type septin sumoylation in siz2Δ cells led to the conclusion that Siz1 is primarily responsible for the sumoylation of the septins Cdc3, Cdc11, and Shs1/Sep7 (Johnson and Gupta, 2001, Takahashi et al.,...
Therefore, given the colocalization we observed and the highly sumoylated state of the septins during mitosis, we considered the septins to be promising candidate STUbL substrates and worthy of further investigation.

We hypothesized that this potential interaction between Slx5 and Cdc3 may be involved in septin ring disassembly. Specifically, Slx5 may be executing the STUbL-mediated ubiquitylation of Cdc3, triggering its degradation. Therefore, to assess the effect of Slx5 on the septins, whole-cell protein extracts were isolated from slx5Δ cells. If sumoylated Cdc3 were serving as a STUbL substrate, we would expect to see an enhancement of Cdc3 sumoylation in slx5Δ cells. Strikingly, however, decreased septin sumoylation was observed in slx5Δ cells compared to wild-type cells (Figure 3).

![Figure 3: slx5Δ cells exhibit decreased levels of sumoylated septin species.](image)

We therefore concluded that Slx5 may be indirectly affecting the sumoylation state of the septins. Because Siz1 is known to sumoylate septins, we hypothesized that
there may be an interaction between Slx5 and Siz1. Indeed, we observed a strong two-hybrid interaction between this STUbL subunit and SUMO E3 ligase (Figure 4).

![Figure 4: Slx5 and Siz1 interact in a yeast two-hybrid screen. The Slx5 binding domain (BD) and the Siz1 activation domain (AD) interact, enabling growth on both -Trp -Leu (-T-L) and -Ade (-A) plates. Additionally, a mutant form of Slx5’s human ortholog RNF4 interacts with Siz1’s human ortholog, PIAS1. YOK1221 served as a positive control. White dashes indicate untested combinations. Figure by Jason Westerbeck.](image)

Significantly, we also observed a two-hybrid interaction between a mutant form of Slx5-Slx8’s human ortholog, RNF4, and Siz1’s human ortholog, PIAS1. This suggests that the Slx5-Siz1 interaction is conserved and that further characterization of this interaction may have important implications for humans.

**Specific Aims**

The specific aims of this thesis are as follows:
1. To test candidate STUbL substrates in an *in vitro* ubiquitylation assay. We will test the hypothesis that the septins and Siz1 serve as substrates of the yeast STUbL Slx5-Slx8.

2. To determine if these prospective targets *physically interact* with Slx5-Slx8. Specifically, we will test that hypothesis that if candidate substrates are ubiquitylated *in vitro* by Slx5-Slx8, they will also interact with Slx5 in an *in vitro* pulldown assay.

3. To investigate the role of SUMO chains in STUbL targeting. SUMO chains will be linked to a putative STUbL target to test whether this modification can enhance ubiquitylation.
**MATERIALS AND METHODS**

_Bacterial hosts and plasmid constructs_ – A complete list of bacterial hosts and constructs used in this study is available in Table 1. Protein overexpression for affinity purification was executed in BL21(DE3) cells (Stratagene) or *R cells (Sean Prigge, Johns Hopkins School of Public Health), which contain the chloramphenicol-resistant pRIL plasmid (BOK 473, BOK 579).

Our _in vivo_ sumoylation system consisted of two duet plasmids courtesy of James Wohlschlegel – pRSF-Duet-UBC9/SMT3 (BOK 470) and pACYC-Duet-UBA2/AOS1 (BOK 471) (Wohlschlegel et al., 2006). pGEX-2TZ-CDC3 (BOK 476) and pGEX-2TZ-CDC11 (BOK 477) served as sumoylation substrates in this _in vivo_ sumoylation strain and were also obtained from James Wohlschlegel. His6- and T7-tagged Rad52-SUMO, which we purified as a positive control for our _in vitro_ ubiquitylation assay, was expressed by pET21a-RAD52(K220R)-SUMO-His6 and was a gift from Mark Hochstrasser’s lab (Xie et al., 2007). Finally, we obtained an enzymatically active truncation of Siz1 – pT-77-SIZ1\(\Delta 440\)-His – from Yoshimitsu Takahashi through AddGene (BOK 591) (Takahashi et al., 2003); this construct was used to purify Siz1\(\Delta 440\) and clone _SIZ1\(\Delta 440\) into the pET-SUMO vector (Invitrogen) to create a Smt3-Siz1\(\Delta 440\) fusion.

_Cloning CDC3, CDC11, and SIZ1\(\Delta 440\) into the pET-SUMO vector_ – Invitrogen’s Champion pET-SUMO Protein Expression System was used to generate His6-tagged SUMO fusion proteins. _CDC3_ (BOK 476), _CDC11_ (BOK 477), and _SIZ1\(\Delta 440\) (BOK 591) were PCR-amplified with the following oligos: _CDC3_ – OOK 397 and OOK 398; _CDC11_ – OOK 395A and OOK 396A; _SIZ1\(\Delta 440\) – OOK 546 and OOK 547. After
ligating amplicons into the pre-gapped pET-SUMO vector, constructs were confirmed by restriction digest and sequencing with primers provided by Invitrogen. Plasmids were then transformed into BL21(DE3) cells or *R cells.

_Bacterial transformation with TSS_ (See Appendix) – His6-UBC4, MBP-SLX5, and MBP-SLX8 were transformed into BL21(DE3) cells using transformation and storage solution (TSS – 5% DMSO, 50 mM Mg$^{2+}$, 10% PEG at pH 6.5) as described previously in Chung et al., 1989. TSS was also used to develop an _in vivo_ sumoylation strain by individually transforming BOK 470, BOK 471, and either BOK 476 or BOK 477 into BL21(DE3) cells.

_Electroporation into *R cells_ – One µL of a 1:1,000 miniprep dilution was added to a 40 µL aliquot of *R cells in an electroporation cuvette (VWR), which was inserted into an electroporator (Eppendorf). Following electroporation at 1700 V, 200 µL of SOC (2% bacto-tryptone, 20 mM glucose, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, 10mM NaCl, 0.5% yeast extract) were added. These 241 µL were transferred to a microcentrifuge tube, rotated at 37°C for one hour, and plated on an LB plate containing the appropriate antibiotic. See Table 1 for each plasmid’s resistance gene. Carbenicillin was used at 60 µg/mL for ampicillin-resistant bacterial strains, kanamycin at 50 µg/mL, and chloramphenicol at 170 µg/mL.

_Induction of protein overexpression with IPTG_ (See Appendix) – One colony of BL21(DE3) or *R cells expressing the recombinant protein to be purified was inoculated in 4 mL of LB broth containing the appropriate antibiotic and grown up overnight by rotating at 37°C. This 4 mL culture was transferred to a flask containing 200 mL SOC and grown up at 37°C to OD$_{600}$ 0.3-0.4. A 1 mL sample of this uninduced culture was
collected and centrifuged. After discarding the supernatant, the cell pellet was resuspended in 60 µL of 1x LDS Sample Buffer (Invitrogen) containing BME (40 µL/1 mL sample buffer). This sample was boiled in a 110°C heat block for three minutes and then frozen in liquid nitrogen. This was done a total of three times.

After adding 80 µL of 1 M IPTG to the remaining 203 mL of bacterial culture, these cells were transferred to a 30°C shaker, where they were induced for 3.5 – 5 hours. Another 1 mL sample was collected as described above. To analyze protein induction, whole-cell protein extracts from uninduced and induced cell cultures were compared by running 10 µL of each 1 mL collection on a pre-cast NuPAGE Novex 4-12% Bis-Tris mini-gel (Invitrogen) for 55 minutes at 200 V in MOPS Buffer (Invitrogen). These gels were rinsed in distilled water, stained in either GelCode Blue (Pierce) or SimplyBlue Safe Stain (Invitrogen) for one hour, and destained in distilled water for another hour. Gels were placed on wet Whatman paper, covered with plastic wrap, and placed in a gel dryer for approximately 45 minutes at 80°C. The remainder of the cell culture was centrifuged at 4°C for 15 minutes at 5,000 RPM. After discarding the supernatant, cell pellets were kept on ice and gently resuspended by pipetting in 4 mL of 1x Halt Protease Inhibitor Cocktail (PI) (Thermo), diluted in 1x PBS. These 4 mL cultures were then spun down into screw-cap tubes and frozen in liquid nitrogen.

Recombinant protein purification (See Appendix) – Frozen cell pellets of IPTG-induced BL21(DE3) or *R cells were resuspended in 800 µL 1x Halt Protease Inhibitor Cocktail by pipetting. 1x PBS was then added to 2 mL. Cells were sonicated three times using a Branson sonifier. Sonicated cells were then centrifuged at 4 °C for 8 minutes at approximately 15,000 RPM. Protein extracts were added to a 15 mL conical and diluted
in 4 mL 1x PBS. Affinity columns (Pierce) were assembled per the manufacturer’s instructions. His6-tagged proteins (Ubc4, Smt3-Cdc3, Smt3-Cdc11, Siz1A440, and Smt3-Siz1A440) were purified on Talon beads (Clontech), MBP-tagged proteins (Slx5, Slx8, and RNF4) on amylose resin (New England Biolabs), and GST-tagged proteins (Cdc3 and Cdc11) on glutathione resin (GE). His6- and GST-tagged proteins were eluted with elution buffer (His6: USB; GST: BD Biosciences), and MBP-tagged proteins were eluted with 100 mM maltose. Glycerol was added to 15%. Elutions were then analyzed by gel staining as described previously. Proteins were frozen in liquid nitrogen and stored at -80°C.

In vitro ubiquitylation assay – For sizing and quantitation of enzymes and substrates used in our in vitro ubiquitylation assays, see Figure S2 and Table 2; these analyses were performed with a Protein 230 kit on the Agilent 2100 Bioanalyzer according to the manufacturer’s instructions. 10x ubiquitylation buffer, E1 enzyme (Uba1), ATP, and 20x ubiquitin were provided in a commercial ubiquitylation kit (Enzo). Ubiquitylation buffer, IPP (100 U/ml), DTT (50 µM), E1 (Uba1), E2 (Ubc4), and E3 enzymes (Slx5-Slx8 or RNF4) were combined with purified substrate protein and ubiquitin. Reactions totaled 27 µL and were incubated in a 30°C heat block for three hours. Reactions were stopped by adding an equal volume of SUTEB sample buffer (0.01% bromophenol blue, 10 mM EDTA, 1% SDS, 50 mM Tris at pH 6.8, 8 M Urea) containing DTT (5 µL of 1 M DTT/1 mL SUTEB sample buffer). Protein products were boiled in a 65°C heat block for ten minutes, analyzed by Western blot as described below, frozen in liquid nitrogen, and stored at -80°C.
In vitro pulldown assay – MBP-Slx5, MBP-Slx8, and T7-Siz1Δ440 were overexpressed in BL21(DE3) or *R cells by IPTG induction as described above. Fifty ODs of cells overexpressing either MBP-Slx5 or MBP-Slx8 were each combined with 50 ODs of cells overexpressing T7-Siz1Δ440. Fifty ODs of cells overexpressing T7-Siz1Δ440 were also collected. Whole-cell protein extracts were then isolated and passed through a column containing amylose resin as described above (Recombinant protein purification). Proteins bound to the amylose resin were eluted with 1x LDS Sample Buffer (Invitrogen) and analyzed by Western blot as described below.

Western blot – Proteins were separated on a pre-cast NuPAGE Novex 4-12% Bis-Tris gel for 55 minutes at 200 V in 1x MOPS buffer. Proteins were then transferred to a polyvinylidene difluorine (PVDF) membrane (Millipore) by semi-dry transfer in semi-dry transfer buffer (192 mM glycine, 250 mM Tris Acetate at pH 8.8, 20% methanol) for 30 minutes at 19 V. The blot was blocked in TBS (150 mM NaCl, 50 mM Tris-HCl at pH 7.4) containing 4% milk for an hour and then in 4% milk containing primary antibody overnight. α-Smt3R11B6 (Hochstrasser Lab, Yale), α-GST (Abcam), α-avidin-HRP (Pierce), α-T7 (Novagen), α-SUMO2 (Michael J. Matunis, JHSOM), and α-MBP (New England Biolabs) primary antibodies were used, all at a concentration of 1:10,000. After three, five-minute washes in large volumes of TBS containing TWEEN20 (1 mL TWEEN20/1 L TBS), blots were incubated in α-rabbit HRP (Abcam) or α-mouse HRP (GE) for 90 minutes and then washed again in large volumes of TBST three times for five minutes each. Chemiluminescent substrate (Millipore) was added to the blot, which was then wrapped in saran wrap and exposed to film.
RESULTS

Development of an in vitro ubiquitylation assay system to test candidate STUbL substrates – The yeast STUbL Slx5-Slx8 has only two confirmed ubiquitylation targets, the DNA damage repair protein Rad52 and the transcription factor MATα2 (Xie et al., 2007, Xie et al., 2010). The transcriptional regulator Mot1 is also a promising candidate (Wang and Prelich, 2009). However, many more substrates are believed to exist. We are very interested in identifying these potential targets and testing their efficacy to further our understanding of Slx5-Slx8’s role in genome stability. Therefore, we sought to develop an in vitro ubiquitylation assay for the analysis of prospective STUbL substrates.

To determine whether Slx5-Slx8 could ubiquitylate a candidate target, we combined components of the ubiquitylation cascade: the E1 enzyme (Uba1), the E2 enzyme (Ubc4), the E3 enzyme (the Slx5-Slx8 STUbL), the substrate protein in question, and ubiquitin (Figure 5A).

![Figure 5: An in vitro ubiquitylation assay. A. E1 (Uba1), E2 (Ubc4), and E3 (the Slx5-Slx8 STUbL) enzymes of the ubiquitylation cascade are combined with a substrate protein (X) and ubiquitin. B. Completed reactions are analyzed by immunoblotting with an antibody to a tag on the substrate or to the substrate itself. If the candidate substrate is ubiquitylated in vitro by Slx5-Slx8, a ladder of bands will be absent in the negative control lane lacking E3 enzyme (lane 1) but present in the experimental lane containing all components of the ubiquitylation cascade (lane 2). These bands represent mono-, di-, and polyubiquitylated substrate [X-(Ub)n].]
Several of these components were purified from bacteria, while others were provided in a commercial kit (See Materials & Methods). If Slx5-Slx8 ubiquitylated the candidate target, we would be able to observe ubiquitin-modified substrate proteins on a Western blot probed with an antibody to the substrate (Figure 5B).

First, we affinity-purified His-Ubc4, MBP-Slx5, and MBP-Slx8 (Figure 6). To confirm the activity of the E2 enzyme, Ubc4, we took advantage of our observation that SUMO2 chains are efficient substrates of ubiquitylation by Slx5-Slx8’s human ortholog RNF4 (Figure S1). In tandem with this newly purified Ubc4, RNF4, also cloned and purified in our lab, ubiquitylated SUMO2 chains in vitro, confirming the activity of Ubc4.

![Figure 6: Purification of His6-Ubc4, MBP-Slx5, and MBP-Slx8.](image)

Identification of the septins as candidate STUbL substrates – As part of previous STUbL analysis, we observed an interesting mislocalization of Slx5 to the bud neck of dividing yeast cells (Westerbeck et al., manuscript in preparation). We then demonstrated
the in vivo colocalization of Slx5 and Cdc3, one of several septin proteins. Septins are a class of proteins that form a structural feature of the bud neck and are among the most highly sumoylated proteins in the yeast cell during mitosis. Based on the colocalization we observed and on Slx5's binding of sumoylated proteins, we considered septins to be promising STUbL substrates.

In vivo sumoylation of septins as prospective STUbL targets – To test the efficacy of the septins Cdc3 and Cdc11 as STUbL substrates, we attempted to sumoylate septins in vivo and purify these septin-SUMO conjugates out of bacteria. First, we tried to sumoylate the septins in a bacterial host harboring all the components of the sumoylation machinery (Wohlschlegel et al., 2006) (Figure 7). A plasmid expressing a SUMO E3 ligase was not included in this bacterial strain because Ubc9 has been shown to conjugate Smt3 to substrates directly (Schwarz et al., 1998).

![Figure 7: In vivo reconstitution of sumoylated septins. Three plasmids were transformed into BL21(DE3) cells: one expressing the two subunits of the E1 enzyme (Uba2 and Aos1); one expressing the E2 enzyme (Ubc4) and yeast SUMO (Smt3); and one expressing either GST-Cdc3 or GST-Cdc11. Septin overexpression was induced with IPTG. The septins were then sumoylated in vivo and available for affinity-purification. Red stars indicate GST-tagged septins.](image-url)
Next, we expressed GST-tagged septin-SUMO conjugates in this bacterially reconstituted expression system as described previously. GST-Cdc3 and GST-Cdc11 were then affinity-purified. Our analysis of the eluates by gel staining (Figure 8A) and by Western blot with α-GST and α-Smt3 antibodies (Figure 8B) suggested that we were successful in purifying both unsumoylated (~80 kDa) and sumoylated Cdc3 (~100 kDa). First, we observed a band at approximately 100 kDa in the second and third elutions of Cdc3. Second, the α-GST antibody detected the expected 20 kDa sumoylation shift between these two forms of Cdc3 (Johnson, 2004). Finally, both the α-GST and α-Smt3 antibodies detected a band at approximately 100 kDa, the anticipated size of GST-Cdc3-Smt3.

On the other hand, we did not observe a band at the appropriate size for GST-Cdc11 or GST-Cdc11-Smt3 on the stained gel (Figure 8A). However, according to the α-GST blot, we were successful in purifying unmodified Cdc11 (Figure 8B, left). Given the absence of bands detected by the α-Smt3 antibody at the expected size for GST-Cdc11-
Smt3 conjugates, we concluded that Cdc11 did not exhibit the previously published sumoylation (Wohlschlegel et al., 2008). Because STUbL ubiquitylation activity is not always SUMO-dependent – for example, Slx5-Slx8 can ubiquitylate an unsumoylated form of the transcription factor MATa2 in vivo (Xie et al., 2010) – we decided to proceed with testing Cdc3 and Cdc11 to determine whether Slx5-Slx8 could ubiquitylate septins in vitro.

*Testing the septins in our in vitro STUbL assay* – Purified GST-Cdc3-Smt3 and GST-Cdc11 were used in in vitro ubiquitylation reactions by combining them with E1 (Uba1), E2 (His6-Ubc4) and E3 (MBP-Slx5 and MBP-Slx8) enzymes, ATP, and ubiquitin at 30°C. After three hours, we separated the proteins products on SDS-PAGE gels and performed a Western blot, using an α-GST antibody to probe for the GST-tagged septins and an α-avidin-HRP antibody to probe for ubiquitin. Unfortunately, after experimenting with several reaction conditions, we did not observe any ubiquitylation of the purified septins (Figure 9). We suspected that the bands on the α-avidin-HRP blot were simply due to the formation of free ubiquitin chains (Figure 9, right).

![Figure 9: Attempt to ubiquitylate sumoylated septins by Slx5-Slx8. E1 (Uba1), E2 (Ubc4), and E3 (the Slx5-Slx8 STUbL) enzymes were combined with sumoylated septins, ATP, and ubiquitin for three hours at 30°C (lanes 1, 3). Reactions lacking the E3 enzyme were assembled as negative controls (lanes 2, 4). Protein products were analyzed by immunoblotting with anti-GST antibodies and anti-avidin-HRP antibodies to probe for ubiquitin.](image-url)
Given the uncertainty surrounding the sumoylation state of the purified septins, we decided to purify His6-tagged SUMO-septin fusion proteins. Cdc3 and Cdc11 ORFs were each cloned into the pET-SUMO vector (BOK 548, BOK 550) and then transformed into BL21(DE3) cells. We overexpressed Smt3-His6-Cdc3 and Smt3-His6-Cdc11 and affinity-purified these proteins (Figure 10).

![Figure 10: Purification of SUMO-septin fusion proteins.](image)

Newly purified SUMO-septin fusion proteins were added to our in vitro STUbL assay. Unfortunately, we failed to observe any modification of these SUMO-septin fusion proteins (Figure 11). Specifically, when all of the ubiquitylation reaction components were present, an upshift of Smt3-Cdc3 and Smt3-Cdc11 due to ubiquitylation did not emerge. While the α-Smt3 antibody detected one band slightly higher than Smt3-Cdc3, this band was also present in the negative controls. Therefore, we cannot claim that this band is a ubiquitylated species of sumoylated septin.
At this time, we began to examine other septin-associated proteins and evaluate their potential to serve as STUbL substrates. Specifically, we observed that levels of SUMO-modified septins were significantly decreased in slx5Δ cells, drawing our attention to the SUMO ligase Siz1 (Westerbeck et al., manuscript in preparation). Siz1 sumoylates septins during mitosis and accounts for the vast majority of sumoylation in the yeast cell (Johnson and Gupta, 2001, Takahashi et al., 2001a). Before we utilized Siz1 as a substrate protein in our *in vitro* STUbL assay, we affinity-purified Rad52-SUMO – a confirmed, *in vitro* target of Slx5 (Xie et al., 2007) – to use as a positive control (Figure 12A). We also purified an enzymatically active truncation of Siz1 (Siz1Δ440) as well a SUMO-Siz1 fusion protein.

**Figure 11: Attempt to ubiquitylate SUMO-septin fusion proteins by Slx5-Slx8.**

E1 (Uba1), E2 (Ubc4), and E3 (the Slx5-Slx8 STUbL) enzymes were incubated with a SUMO-septin fusion protein and ubiquitin for three hours at 30°C (*lanes 6-7*). Control reactions were also performed (*lanes 1-5*). Reactions products were analyzed by immunoblotting with an anti-Smt3 antibody.
We then performed an *in vitro* ubiquitylation assay with Rad52-SUMO as a positive control and Siz1Δ440 and Smt3-Siz1Δ440 as experimental substrates. We observed a slower-migrating adduct and smear above Rad52-SUMO, signaling ubiquitylation of Rad52-SUMO and confirming its utility as a positive control (Figure 13A). After substituting Siz1Δ440 as the substrate, the α-T7 antibody detected several, strong bands above Siz1Δ440, suggesting mono-, di-, and polyubiquitylation (Figure 13B). Finally, we observed monoubiquitylation of Smt3-Siz1Δ440 (Figure 13C).

*Siz1 interacts with both Slx5 and Slx8 in an in vitro pulldown assay* – After demonstrating ubiquitylation of Siz1Δ440 and Smt3-Siz1Δ440 by Slx5-Slx8 in an *in vitro* ubiquitylation assay, we decided to investigate whether Slx5 and Siz1 would interact in an *in vitro* pulldown assay. In order to accomplish this, we individually overexpressed MBP-Slx5, MBP-Slx8, and T7-Siz1Δ440-His6 in BL21(DE3) or *R cells (BOK 500, BOK 501, BOK 758). We then combined these cell cultures as depicted in Figure 14.
Figure 13: Slx5-Slx8 ubiquitylates Siz1Δ440 and Smt3-Δ440 in vitro. E1 (Uba1), E2 (Ubc4), and E3 (the Slx5-Slx8 STUbL) enzymes; ATP; substrate (Rad52-SUMO, Siz1Δ440, or Smt3-Siz1Δ440); and ubiquitin were combined for three hours at 30°C. Reactions were analyzed by immunoblotting with an anti-T7 antibody. A. Polyubiquitylation of Rad52-SUMO, a positive control. B. Polyubiquitylation of Siz1Δ440 (lane 5). C. Monoubiquitylation of Smt3-Siz1Δ440 (lane 5).
After extracting all of the proteins from these new, combined bacterial cultures, we ran the protein extracts through a column containing amylose resin, which would bind the MBP-Slx5 and MBP-Slx8. After eluting the proteins bound to the beads and performing a Western blot by probing with an α-T7 antibody, we observed in vitro interaction of Siz1Δ440 with both MBP-Slx5 and MBP-Slx8 (Figure 15). As expected,
bands were absent in the Siz1Δ440 lane, demonstrating that Siz1Δ440 alone did not bind to the amylose resin and was not responsible for the interaction we observed.

**Figure 15: Slx5 and Siz1 interact in an in vitro pulldown assay.** Whole-cell extracts from cell cultures containing overexpressed MBP-Slx5 and Siz1Δ440; MBP-Slx8 and Siz1Δ440; and Siz1Δ440 were passed through a column containing amylose resin. Inputs and eluates were run on 4-12% Bis-Tris gels for either staining with SimplyBlue SafeStain or immunoblotting with an anti-T7 antibody.

**SUMO2 chains are necessary for in vitro ubiquitylation of substrates by the human STUbL RNF4** – After demonstrating Slx5-Siz1 binding in *in vitro* ubiquitylation and pulldown assays, we probed further into the targeting of STUbLs to their substrates. Specifically, we were curious to determine the role that SUMO chains play in the *in vitro* STUbL-mediated ubiquitylation of targets. Previously, we showed that SUMO2 chains are efficient substrates of ubiquitylation by RNF4 (Figure S1). For our studies on the role of SUMO2 chains in *in vitro* ubiquitylation, a graduate student in the lab, Zac Elmore, purified the substrate-interacting domain (SID) of the SUMO protease Ulp1 on amylose
beads (Figure 16A). Fused to MBP, this “U-tag” could bind, but not cleave, SUMO chains (Figure 16B). We performed our in vitro ubiquitylation assay as previously described with both Slx5-Slx8 and MBP-RNF4 as E3 enzymes and the U-tag with and without SUMO2 chains as substrates. We then used Western blotting and α-SUMO2 and α-MBP antibodies to analyze our reactions.

![Figure 16: MBP-U-tag binds, but does not cleave, SUMO chains and may stimulate STUbL targeting and subsequent ubiquitylation.](image)

The α-SUMO2 antibody revealed that RNF4, but not Slx5-Slx8, ubiquitylated the SUMO2 chains conjugated to the U-tag (Figure 17). Further, the α-MBP antibody suggested that only in the presence of SUMO2 chains is the MBP-U-tag ubiquitylated by RNF4. Perhaps the RNF4-specific ubiquitylation of the U-tag is due to species specificity between RNF4 and SUMO2 chains. Further experiments should clarify whether the MBP or the U-tag is being ubiquitylated and whether the stabilization of SUMO2 chains by the U-tag enables ubiquitylation of other, U-tag-associated substrates.
Figure 17: SUMO2 chains are necessary for substrate ubiquitylation by the STUbL RNF4. An in vitro ubiquitylation assay was performed by combining purified MBP-U-tag-SUMO2\textsuperscript{n} with E1 (Uba1), E2 (Ubc4), E3 (SIX5-SIX8 or RNF4), and ubiquitin for 3 hours at 30°C. Control reactions lacking SUMO2 chains (lane 1), E3 enzyme (lane 2), and U-tag (lane 3) were also performed. Protein products were visualized by both anti-SUMO2 and anti-MBP immunoblotting.
**DISCUSSION**

In this study, we set out to test candidate STUbL substrates in an *in vitro* ubiquitylation assay. Prospective targets included two septins and a SUMO E3 ligase, Siz1. Of these, the yeast STUbL Slx5-Slx8 ubiquitylated Siz1 *in vitro*. We were also able to demonstrate the interaction between Slx5 and Siz1 in an *in vitro* pulldown assay. In an attempt to advance our understanding of the role of SUMO chains in STUbL targeting, we also showed that non-covalent association of a protein is sufficient for STUbL targeting to this substrate.

*Are septins targets of the yeast STUbL Slx5-Slx8? –* In our *in vitro* STUbL assay, we observed the formation of free ubiquitin chains, confirming the enzymatic activity of Slx5 and Slx8. However, it is likely that the purified septins from our *in vivo* sumoylation reconstitution system were, in fact, not sumoylated at all. Specifically, the transiency of sumoylation may have rendered the purification of septin-SUMO conjugates difficult; with the exception of RanGAP1, only 1% of SUMO substrates in the cell are sumoylated at any one time (Johnson, 2004). Therefore, because STUbL-mediated ubiquitylation can be SUMO-dependent (Lallemand-Breitenbach et al., 2008), the unsumoylated state of the purified septins could have prevented them from serving as efficient substrates of ubiquitylation. In an attempt to eliminate uncertainty surrounding the sumoylation state of the septins, we purified SUMO-septin fusion proteins, but we did not observe ubiquitylation of these conjugates either. Altogether, our observations suggested that the septins may not be *bona fide* STUbL substrates.

*How does Siz1 interact with Slx5? –* The finding that levels of sumoylated septins are decreased in slx5Δ cells (Westerbeck et al., manuscript in preparation) prompted us to
consider the enzyme that sumoylates the septins – Siz1 – to be a prospective STUbL substrate. After observing a two-hybrid interaction between Slx5 and Siz1 and their human orthologs RNF4 and PIAS1, respectively, we decided to test Siz1 in our *in vitro* STUbL assay. Specifically, we tested an enzymatically active, C-terminal truncation of Siz1 – Siz1Δ440 [Siz1(1-464)] (Takahashi et al., 2003). Indeed, we observed polyubiquitylation of Siz1Δ440 by Slx5-Slx8 as well as interaction between Siz1 and *both* Slx5 and Slx8 in an *in vitro* pulldown assay.

Given the two-hybrid interaction between Slx5 and Siz1 and the ubiquitylation of Siz1 by Slx5 *in vitro*, interaction between these two proteins in our *in vitro* pulldown assay was expected. However, very unexpected was the interaction between Slx8 and Siz1, which may suggest a previously undescribed role for Slx8 in substrate selection and specificity. Conventionally, SUMO-interacting motifs (SIMs) facilitate the noncovalent interaction between STUbLs and their sumoylated substrates (Kerscher, 2007). Slx5, with its two SIMs, is therefore considered the targeting subunit of the heterodimer, while Slx8 harbors ubiquitin ligase activity. However, considering that STUbL-mediated ubiquitylation can be SUMO-independent *in vitro* (Xie et al., 2007) and *in vivo* (Xie et al., 2010), it is entirely possible that Slx5 and Slx8 may both shoulder substrate selection responsibilities.

Interestingly, fusion of Smt3 to Siz1Δ440 did not enhance Slx5-Slx8’s ubiquitylation activity, supporting the hypothesis that there may be domains apart from Slx5’s SIMs that are important for Slx5-Slx8’s targeting of Siz1. In fact, Siz1 contains an SP-RING domain, which exhibits similarity to the RING domain characteristic of ubiquitin E3 ligases (Hochstrasser, 2001). Given that Slx5 and Slx8’s RING domains are
essential for their dimerization, perhaps Slx8’s RING domain and Siz1’s SP-RING domain enable the novel interaction we observed (Mullet et al., 2001).

The Slx5-Siz1 interaction may be physiologically relevant. – While wild-type Slx5 does not localize to the bud neck under physiological conditions, this previously undescribed interaction between Slx5 and a septin-associated protein may be physiologically relevant. Given Siz1’s sumoylation of the septins during mitosis, we implicate Slx5 in the control of septin sumoylation through Siz1. Slx5 and Siz1 are both nuclear proteins, so their interaction is not entirely surprising. However, following Siz1’s phosphorylation during G2/M, Siz1 is exported from the nucleus to sumoylate the septins during mitosis (Johnson and Gupta, 2001, Takahashi et al., 2001a). Therefore, it has been proposed that septin sumoylation is controlled by Siz1 localization, perhaps specifically by phosphorylation (Johnson, 2004). While the karyopherin Msn5 appears to be an important factor in Siz1’s export, the initial export signal is unclear (Takahashi and Kikuchi, 2005, Makhnevyych et al., 2007).

Additionally, there has been speculation about a possible role for autosumoylation and subsequent ubiquitylation in Siz1’s export (Takahashi et al., 2008). Our data are in support of this paradigm, in which the Slx5-Siz1 interaction is important for controlling the proper sumoylation or ubiquitylation state of Siz1 to enable its export into the cytoplasm. In the absence of Slx5, hypersumoylation of Siz1 may prevent this export event. Alternatively, a lack of Slx5 may deplete the cellular pool of free SUMO, preventing proper septin sumoylation (Figure 18).
Ubiquitylation is known to be capable of altering a protein’s localization. Specifically, monoubiquitylation affects protein transport across the cell membrane and in the endocytic pathway as well as in the nuclear-cytoplasmic shuttling of tumor suppressors like p53 (Hicke and Dunn, 2003, Salmena and Pnadolfi, 2007). However, this paradigm doesn’t automatically exclude a role for phosphorylation in Siz1 localization and septin sumoylation (Johnson and Gupta, 2001); it is possible that these two post-translational modifications may work in tandem to ensure Siz1’s proper localization.

*Why are there differences between the substrate ubiquitylation by Slx5-Slx8 and RNF4 in the presence of SUMO2 chains?* – We confirmed previous observations that RNF4, but not Slx5-Slx8, ubiquitylates human SUMO2 chains *in vitro*. Perhaps this
could be explained by species-specific interactions between SUMO2 chains and RNF4. We could test this possibility by using Smt3 chains in our in vitro ubiquitylation assay.

Interestingly, SUMO2 chains drastically enhanced the ubiquitylation of MBP-U-tag in vitro. Repeating the in vitro ubiquitylation assay with a SUMO mutant that cannot form chains could shed light on differences between mono- and polysumoylation in STUbL targeting. While it is clear that ubiquitin was conjugated to both the SUMO2 chains and the MBP-U-tag, additional experiments will clarify where the MBP or the U-tag is being ubiquitylated. If the MPB is ubiquitylated, the U-tag could, in theory, sumoylate any substrate and serve as a powerful tool to further our understanding of sumoylation and its effect on substrates.

In summary, our data support interaction between the yeast STUbL subunit Slx5 and the SUMO E3 ligase Siz1. We hypothesize that the STUbL activity of Slx5-Slx8 may enable the ubiquitylation of autosumoylated Siz1 and its subsequent export into the cytoplasm to sumoylate the septins, ensuring proper cell division. This feedback loop may provide a previously realized pathway for the regulation of protein sumoylation in the cell. Furthermore, insights into Slx5’s function and interactors may have important implications for its human ortholog RNF4 and its role in genome stability.
Figure S1: SUMO2 chains are efficient substrates of ubiquitylation by the STUbL RNF4. An in vitro ubiquitylation assay was assembled as previously described with S1x5-S1x8 or RNF4 as the E3 enzyme and SUMO2 chains as the substrate protein. Control reactions lacking E3 enzyme were also assembled (lanes 2, 4). Protein products were analyzed by immunoblotting with an anti-SUMO2 antibody.
Figure S2: Analysis of purified recombinant proteins used in *in vitro* ubiquitylation assays: Purified recombinant proteins were sized and quantitated on the Agilent 2100 Bioanalyzer with a Protein 230 kit according to the manufacturer's instructions. See Appendix A for approximate concentrations and volumes used in *in vitro* ubiquitylation reactions.
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* Size and concentration estimated by comparison to a protein ladder and BSA standards, respectively, on a SYPRO Ruby-stained gel using the GE Storm 845 phosphoimager.

° Concentration and volume of a 2 µM dilution (20 µL RNF4 stock/280 µL 1x PBS).
Appendix: Protocols

Bacterial Transformation with TSS
1. Grow cells in LB containing the appropriate antibiotic to OD$_{600}$ 0.3-0.4.
2. Add an equal volume of cold 2x TSS.
   - 2x TSS (at pH 6.5): 180 mL 2x LB
   - 20 g PEG
   - 10 mL DMSO
   - 10 mL 1 M MgCl$_2$
3. Add 100 µL of plasmid DNA.
4. Incubate for 30 minutes at 4°C.
5. Add 900 µL LB or TSS containing 20 mM glucose.
6. Rotate at 37°C for one hour.
7. Plate.

IPTG Induction of Protein Overexpression in BL21(DE3) or *R Cells
1. Rotate one bacterial colony overnight at 37°C in 4 mL LB containing the appropriate antibiotic.
2. Add these 4 mL of bacterial culture to 200 mL SOC.
3. Grow in a 37°C water bath or shaker to OD$_{600}$ 0.3-0.4.
4. Remove 1 mL of bacterial culture.
   a. Spin down.
   b. Discard supernatant.
   c. Add 60 µL of sample buffer + BME (40 µL/1 mL sample buffer).
   d. Boil in a 110°C heat block for 3 minutes and freeze in liquid nitrogen.
   e. Repeat (d) twice.
5. Add 80 µL 1 M IPTG.
6. Shift the 204 mL bacterial culture to a 30°C water bath or shaker and grow for 3.5 – 5 hours.
7. Remove 1 mL of bacterial culture and perform steps 4a-e.
8. Spin down the remaining 202 mL at 4 °C for 15 minutes at 5,000 RPM.
9. Keeping cells on ice, resuspend in 4 mL PBS + protease inhibitors (PI).
10. Spin these 4 mL into screw-cap freezer tubes, and try to resuspend.
11. Freeze cell pellets in liquid nitrogen.

Recombinant Protein Purification
1. Resuspend the frozen cell pellet in 800 µL PBS + PI by pipetting.
2. Add PBS to 2 mL
3. Sonicate: 3 times on 20% duty cycle for a total of 20 seconds each time, alternating 2 seconds “ON,” 2 seconds “OFF.”
4. Spin at top speed for 8 minutes at 4°C.
5. Move supernatant to a 15 mL conical tube.
6. Add 4 mL PBS. Mix gently by inverting several times.
7. Assemble column underwater by using the plunger provided with the affinity columns to push the disc to the bottom of the column.
   a. *Cut the tip of the column if necessary to draw liquid through the column.
8. Wash disc 3 times with 5 mL PBS.
9. Add beads to column.
10. Wash beads 3 times with 5 mL PBS.
11. Add diluted protein (in 4 mL PBS in 15 mL conical) to column.
12. Wash proteins 3 times with 5 mL PBS.
13. Elute 3 or 4 times with the appropriate elution buffer.
14. Add glycerol to 15% and aliquot elutions.
15. Freeze in liquid nitrogen.

**In vitro Ubiquitylation Reaction**

See Figure S2 and Table 2 for size and quantitation of purified recombinant proteins used in this assay. An example *in vitro* ubiquitylation reaction is assembled as follows:

1. Assemble a master mix:
   
   \[
   \begin{align*}
   &3x \\
   &3.75 \text{ uL} \quad 10x \text{ buffer} \\
   &15 \text{ uL} \quad \text{IPP (100 U/mL)} \\
   &1.5 \text{ uL} \quad 50 \text{ mM DTT} \\
   &3.75 \text{ uL} \quad \text{E1 enzyme (Uba1)}
   \end{align*}
   \]

2. Add 8 uL of this 3x master mix to each reaction.
3. To each reaction, add:
   
   \[
   \begin{align*}
   &1.25 \text{ uL} \quad \text{ATP} \\
   &2.5 \text{ uL} \quad \text{E2 enzyme (Ubc4)} \\
   &4.5 \text{ uL} \quad \text{Slx5} \\
   &1.25 \text{ uL} \quad \text{Slx8} \\
   &5 \text{ uL} \quad \text{Substrate} \\
   &2 \text{ uL} \quad \text{Ub}
   \end{align*}
   \]

Note: For each control reaction, replace the reaction component with an equivalent volume of 1x PBS.

4. Incubate reactions at 30°C for three hours.
5. Stop reaction with an equal volume of SUTEB buffer + DTT (5 uL 1 M DTT in 1 mL SUTEB buffer).
6. Boil at 65°C for 10 minutes.
7. Load approximately 12 uL of each reaction onto a pre-cast NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen).
8. Run for 55 minutes at 200 V in MOPS buffer.
10. Rinse two blotting pads, PVDF membrane, and gel in semi-dry transfer buffer.
11. Assemble semi-dry transfer and run for 30 minutes at 19 V.
12. Block in TBS containing 4% milk for one hour.
13. Incubate in primary antibody overnight.
14. Wash blot three times in TBST for five minutes each.
15. Incubate blot in secondary antibody for 90 minutes.
16. Wash blot three times in TBST for five minutes each.
17. Add chemiluminescent substrate and expose to film.
REFERENCES


