2014

Identifying SUMO Protease Targets and Investigating E3 Ligase Interactions

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Identifying SUMO protease targets and investigating E3 ligase interactions

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A Thesis presented to the Graduate Faculty of the College of William and Mary in Candidacy for the Degree of Master of Science

Department of Biology

The College of William and Mary
January 2014
This Thesis is submitted in partial fulfillment of the requirements for the degree of

Master of Science

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Protocol number(s): IBC-2012-10-08-8156-opkers

Date(s) of approval: 2013-11-02
ABSTRACT

Posttranslational modification by the Small Ubiquitin-like MOdifier (SUMO) is a pervasive mechanism for controlling protein function. SUMO is conserved from yeast to man and is fundamental to eukaryotic life. Sumoylation is a dynamic process and regulation of SUMO conjugate levels is accomplished in two ways. First, SUMO can be removed from conjugate proteins by SUMO specific proteases. In budding yeast *Saccharomyces cerevisiae*, the SUMO protease Ulp1 is responsible for removing SUMO from target proteins and processing SUMO precursor peptides. Ulp1 is essential for cell cycle progression; however, few specific Ulp1 substrates have been identified. The first part of this thesis utilized a substrate-trapping mutant of Ulp1, known as the U-Tag, to identify candidate Ulp1 substrates. Our analysis has identified 32 candidate Ulp1 substrates including Bmh1, a regulatory molecule that interacts with the Anaphase Promoting Complex (APC). Bmh1 is a 14-3-3 protein that associates with an APC component and was previously shown to be sumoylated. These findings suggest a mechanism by which a SUMO protease can contribute to cell cycle regulation. The second method cells use to regulate SUMO conjugate levels is by destruction of sumoylated proteins in the ubiquitin proteasome system. Recent work has identified a novel class of SUMO-Targeted Ubiquitin Ligases (STUbLs) which selectively ubiquitylate polysumoylated proteins. In yeast, the STUbL heterodimer Slx5-Slx8 has an important role in SUMO-targeted degradation. Slx5 is the targeting domain of the STUbL complex and it usually resides in the nucleus where it plays a key role in genome stability and DNA damage repair. Continuing work in the lab has identified a physical interaction between Slx5 and the E3 SUMO ligase known as Siz1. The data herein describes the functional consequences of this interaction and demonstrates that slx5Δ cells accumulate sumoylated and phosphorylated Siz1. These data suggest that three different modifications are involved in Siz1 regulation (SUMO, ubiquitin, and phosphorylation). Finally, our data indicate that Siz1 is degraded in an Slx5 dependent manner when nuclear export is blocked during G2/M. In summary, part two of this thesis identifies targeting and localization domains in Slx5 and additionally provides evidence that STUbLs may regulate levels of an E3 SUMO ligase.
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ACKNOWLEDGEMENTS

This writer wishes to express his appreciation to Professor Oliver Kerscher, under whose guidance this investigation was conducted, for his patience, guidance and criticism throughout the investigation. The author is also indebted to Professor Lizabeth Allison, Professor Diane Shakes, and Professor Shantá Hinton for their careful reading and criticism of the thesis.
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Chapter 1. Introduction

Posttranslational modification

Eukaryotic cells enhance the diversity of their proteome through the covalent addition of chemical groups or peptides. For example, methyl groups, acetyl groups, nitrosyl compounds, carbohydrates, lipids, or small proteins can become covalently linked to the side chain of specific amino acids in a process known as posttranslational modification. It is estimated that over 5% of the proteome is dedicated to facilitating more than 200 types of posttranslational modification (Walsh, 2006).

Posttranslational modification can modulate protein function in response to internal cellular cues or external stimuli. The most common posttranslational modification is phosphorylation. Mediated by a group of enzymes called kinases, phosphorylation links an inorganic phosphate group donated by ATP to the side chain of specific amino acids; most commonly serine, threonine, and tyrosine. A phosphate group increases the local negative charge of residues to which they are linked. Changes in protein charge profile can induce changes in conformation or interactions which, in turn, modify protein function. One well studied example is receptor signaling carried out by members of the mitogen activated protein kinase (MAPK) family. MAPKs are well known for transmitting information across the plasma membrane and into the nucleus using a trio of kinases acting sequentially to begin a signal cascade. Phosphorylation is a reversible process
and removal of phosphate molecules is facilitated by a group of enzymes called phosphatases. In humans, there are more than 500 known kinases and over 150 phosphatases (Lothrop, Torres, & Fuchs, 2013; Walsh, 2006).

This thesis will focus on modification by two types of small peptide modifiers; SUMO and ubiquitin. Sumoylation and ubiquitylation are discussed in detail below.

**Ubiquitin**

One form of posttranslational modification that is widely used by eukaryotic cells is the attachment of small proteins to the side chains of specific residues such as lysine. The first protein modifier discovered, described in 1975, is a 76 residue peptide known as ubiquitin which was named for its ubiquitous distribution throughout the eukaryotic domain (Goldstein et al. 1975). Initially discovered as a means to target proteins for proteasomal degradation, ubiquitylation is now also known to regulate protein interactions, localization and activity (Jackson & Durocher 2013). In yeast, ubiquitin is expressed as a polyubiquitin fusion that must be processed by deubiquitylating enzymes (DUBs) into conjugation competent monomers (Finley et al. 2012; Ozkaynak et al. 1984).

Attachment of ubiquitin to target proteins is an energy dependent process carried out by a three enzyme cascade consisting of an E1 activating enzyme, and E2 conjugating enzyme and an E3 ligase (model 1). Ubiquitylation is reversible and processing of ubiquitin fusion protein is carried out by deubiquitylating enzymes (DUBs) (model 1). Ubiquitin is the founding member of
a conserved family of modifiers named ubiquitin-like proteins (Ubls). This family contains at least 10 proteins which are all modestly related in sequence but, in some cases, share a common three-dimensional structure (Kerscher et al. 2006).

SUMO

A second posttranslational modifier, known as SUMO, shares about 18% sequence identity with ubiquitin. There are three SUMO isoforms in humans but only one in yeast, Smt3. In this thesis, Smt3 will be referred to as yeast SUMO; the yeast version of this posttranslational modifier. SUMO is a 110-amino acid protein, and the second most widely characterized member of the Ubl family. It is highly conserved among eukaryotes and essential for life (Johnson, 2004). Sumoylation has been shown to play a key role in facilitating DNA repair, cell cycle progression, protein stability, transcription, and the stress response (Kerscher et al. 2006). However, it also holds important roles in the assembly of protein complexes.

SUMO processing and attachment

Initially, SUMO is expressed as a precursor that must be processed by a SUMO protease into its conjugation competent form. Processing in yeast involves the removal of three C-terminal residues by the SUMO-specific protease Ulp1. This cleavage makes SUMO conjugation competent by exposing a di-glycine repeat that is the site of substrate attachment. After processing, attachment is carried out by a three enzyme cascade reminiscent of the ubiquitin system described earlier (model 1). To initiate substrate modification, SUMO
must first be activated by an E1 activating enzyme which in yeast is a heterodimer consisting of Aos1 and Uba2. The E1 uses ATP to form a high energy thioester bond between the terminal glycine carboxylate and a catalytic cysteine residue of the enzyme. This high energy bond is then transferred to a catalytic cysteine residue on an E2 conjugating enzyme known as Ubc9. Finally, Ubc9 transfers the SUMO to a lysine side chain on its target protein, with the help of an E3 SUMO ligase that facilitates the conjugation onto specific proteins. Conjugation usually occurs within the consensus sequence ψ-K-x-D/E where ψ is hydrophobic, K is the lysine conjugated to SUMO, x is any amino acid, and D/E is an acidic residue (Kerscher et al. 2006). Single SUMO molecules can be attached to the same protein, or chains can form on residues within the tertiary structure of SUMO monomers.

Of particular interest are four SUMO ligases in yeast; Siz1, Siz2, Mms21, and the meiotic-specific Zip3. All four are members of the Siz/PIAS-RING (SP-RING) ligase family that shares sequence similarity to ubiquitin’s Really Interesting New Gene (RING) ligase family and function in a similar manner (Johnson & Gupta 2001; Takahashi et al. 2001; Zhao & Blobel 2005; Cheng et al. 2006). SUMO ligases recruit SUMO charged E2 enzymes into a complex with the substrate to facilitate conjugation. Although the SUMO E2 enzyme can catalyze sumoylation alone, SUMO E3 ligases enhance conjugation to specific substrates and deletions of Siz1 and Siz2 are non-lethal but have been linked to growth defects and sensitivity to environmental stress. Siz1 and Siz2 are responsible for over 90% of global sumoylation and they show considerable substrate overlap.
(Chen et al. 2011; Silver et al. 2011). There are, however, substrates specific to either Siz1 or Siz2. For example, Siz1 is required for sumoylation of the septin proteins Cdc3, Cdc11 and Shs1 (Johnson & Blobel, 1999).

**SUMO removal**

The covalent attachment of SUMO to specific substrates or other SUMO proteins is a reversible process. SUMO can be removed from its substrates by the same SUMO protease that renders it conjugation competent, Ulp1. This protease cleaves the isopeptide bond between the SUMO carboxy-terminal glycine and the lysine side chain to which it is linked. Cleavage releases SUMO monomer and the target protein in its initial conformation. A second SUMO protease in yeast is called Ulp2. Ulp1 and Ulp2, have overlapping but non-identical substrates specificities but only Ulp1 can process the SUMO precursor (Hickey et al. 2012). Both are cysteine proteases that contain a papain-like fold and operate using a conserved ~200 residue core domain (Ulp1 domain, or UD) that houses a catalytic cysteine residue. Ulp1 is necessary for viability owing to its ability to process precursor SUMO (Li & Hochstrasser 1999; Li & Hochstrasser 2000; Strunnikov et al. 2001). Ulp2 is non-essential but contributesto chromosome stability owing to its involvement in recombination repair, centromere cohesion and spindle formation (Lee et al. 2011; Schwartz et al. 2007; Baldwin et al. 2009). A third potential SUMO protease has recently been uncovered. The metalloprotease known as Wss1 displays weak SUMO-dependent isopeptidase activity *in vitro*. Interestingly, Wss1 also has deubiquitylating activity and shows a preference for cleaving ubiquitin from
SUMO chains. This activity, along with a newly discovered physical interaction with the proteasome, have suggested that Wss1 acts on sumoylated substrates entering the proteasome (Mullen, Chen, and Brill 2010).

Loss of Ulp1 mediated deconjugation results in cells arresting with large buds, presumably late in the cell division cycle (Li & Hochstrasser 1999; Hickey et al. 2012). Conversely, loss of Ulp2 is tolerated, but cells display sensitivity towards DNA damage and heat stress along with defects in meiosis (Hickey et al. 2012). Additionally, cells expressing a temperature-sensitive mutant of Ulp1 build up Clb2, a B-type cyclin that promotes the transition from G2 to M phase and is targeted for destruction after mitosis (Kerscher, O.; unpublished observation). To date, no substrates of Ulp1 involved in cell cycle progression have been identified.

Roles for SUMO

Sumoylation plays diverse roles in a wide range of cellular functions. In broad terms, attachment of SUMO may do one of three things. Firstly, sumoylation can obscure interaction domains of its conjugate and act as an antagonist to other substrates binding. For example, in yeast, the proliferating cell nuclear antigen (PCNA) undergoes sumoylation during S-phase. This sumoylation event occurs predominantly on lysine 164; a residue which can undergo both mono- and poly-ubiquitylation as well. Sumoylation on K164 obscures the residue from the ubiquitylation machinery and, in doing so, prevents PCNA-dependent DNA repair (Pfander et al. 2005; Hoege et al. 2002). Secondly,
sumoylation can lead to conformational changes in conjugate proteins, exposing or hiding key interaction domains within the target. For example, in humans, thymine DNA glycosylase (TDG) initiates base excision repair by removing thymine from sites of nucleotide mismatch, generating an abasic site. SUMO conjugation to TDG induces a conformational change which promotes TDG dissociation from the abasic site (Steinacher & Schär 2005; Baba et al. 2005).

Thirdly, sumoylation can recruit interacting partners to conjugate protein. Recruitment of novel interacting partners can be done by creation of a new interaction domain at the SUMO interface or by direct non-covalent interactions with attached SUMO. As an example, the yeast SUMO-Targeted Ubiquitin Ligase (STUbL) complex Rad18 interacts specifically with sumoylated PCNA using SUMO-interacting motifs (SIMs) near the amino-terminus of the ligase. This SUMO facilitated interaction stimulates mono-ubiquitylation of PCNA in response to DNA damage (Parker & Ulrich 2012). Sumoylation is required for viability and changes made to a protein by sumoylation have broad biological importance. To date, the yeast SUMO proteome consists of more than 500 proteins effecting 15 major biological pathways (Tan et al. 2013). However, the function of only a handful of sumoylated proteins is known.

Chains of SUMO and Ubiquitin

One hallmark of modification by SUMO and ubiquitin is the ability of these Ubls to form chains. Both SUMO and ubiquitin present internal lysine residues that can be used to assemble polymeric chains. Poly-modifier chains can be linear when linked to the same residue uniformly, or branched chains can form
when multiple lysines are used. Assembly and disassembly of chains are carried out using the same enzymatic machinery as single-moiety modification (model 1) (reviewed by Ulrich 2008; Kerscher et al. 2006).

For ubiquitin, seven internal lysines as well as the N-terminus are targets for polyubiquitylation (Finley et al. 2012). The most prominent function of polyubiquitylation is the initiation of protein degradation; polyubiquitylation by K48 linked ubiquitin chains directs conjugated proteins to the proteasome (Chau et al. 1989). Research into the roles of ubiquitin chain formation is ongoing, but distinct outcomes for polyubiquitylation have been identified. For example, polyubiquitylation by K63 linked ubiquitin chains activates kinases in the NF-κB pathway leading to NF-κB activation (Chen 2005). Ubiquitin is an information rich molecule, and construction of distinct chains results in a “ubiquitin code”; a structure of ubiquitin molecules readable by a large family of ubiquitin receptors (Finley et al. 2012; Randles & Walters 2012).

In contrast to ubiquitin chains, relatively little is known about the function of poly-sumoylation. Yeast SUMO contains consensus SUMO attachment sites at K11, K15, and K19 which act as acceptors for SUMO polymerization (Ulrich 2008). Current reasoning suggests that poly-sumoylation creates additional binding surfaces on the modified target allowing for more robust non-covalent interactions of an effector protein containing SUMO-interacting motifs (SIMs) (Ulrich 2008). For example, the promyelocytic leukemia protein (PML) is a poly-sumoylated protein which also contains a SIM in its C-terminus. Poly-sumoylation
of PML promotes nucleation of PML and assembly into nuclear bodies (NBs) through SUMO-SIM interactions (Kerscher 2007; Gao et al. 2008).

**SUMO-mediated interactions**

SIMs are noncovalent SUMO-binding elements within SUMO-interacting proteins. SIMs allow SIM-containing proteins to interact with their sumoylated binding partners. One SIM has been described so far which has a loose core consensus motif (V/I-X-V/I-V/I) (Song et al. 2004). This hydrophobic core takes on an extended conformation that embeds within a hydrophobic surface depression of SUMO. Flanking acidic residues can orient the binding of SIM to SUMO (Kerscher 2007; Hochstrasser 2009). Additionally, phosphorylation juxtaposed to the hydrophobic core can increase the number of charges and facilitate SUMO interaction (Stehmeier & Muller 2009).

Historically, SUMO has been proposed to antagonize ubiquitin conjugation on common substrates (Desterro et al. 1998). Recent evidence, however, revealed cross-talk between the ubiquitin and SUMO systems. Evidence is accumulating that SUMO chains can be sequentially modified by ubiquitin, forming hybrid SUMO-ubiquitin (ub) chains (Mullen & Brill 2008; Sun et al. 2007; Guzzo et al. 2012). Construction of these hybrid SUMO-ub chains is carried out by a newly defined group of enzymes known as SUMO-Targeted Ubiquitin Ligases (STUbLs) (Mullen & Brill 2008; Xie et al. 2007). STUbLs mediate hybrid chains’ function to direct modified proteins to the proteome and have been suggested to play a role in genome stability, transcriptional regulation, and DNA
stability (reviewed by Wang & Prelich 2009; Heideker et al. 2009). Adding to the complexity, emerging research has identified enzymes that recognize hybrid chains using a tandem SUMO- and Ubiquitin (tSIM-UIM) interacting motif (Guzzo & Matunis 2013). Tandem SIM-UIM containing proteins act on substrates previously targeted by STUbLs. For example, the BRCA1-A subunit RAP80 is a DNA repair factor and the first described tSIM-UIM containing protein. RAP80 interacts with ~80-fold higher affinity to hybrid SUMO-Ub chains in comparison to monomorphous SUMO or ubiquitin chains (Guzzo et al. 2012).

SUMO-Targeted Ubiquitin Ligases

STUbLs have been identified in multiple organisms and the STUbL family currently consists of: Human RNF4; Schizosaccharomyces pombe Rfp1, Rfp2 and Slx8; Dictyostelium discoideum Mip1; Drosophila melanogaster degringolade; and Saccharomyces cerevisiae Slx5-Slx8, Rad18, and potentially Uls1 (Sun et al. 2007; Parker & Ulrich 2012; Denuc & Marfany 2010; Alonso et al. 2012). To date, all known STUbLs share an N-terminal SUMO recognition region (with multiple SIMs) used in target identification as well as a C-terminal RING finger domain (Denuc & Marfany 2010). STUbLs are evolutionarily conserved and STUbL orthologs from other species can complement yeast STUbL mutants (Kosoy et al. 2007; Sun et al. 2007; Prudden et al. 2007). The best characterized members of the STUbL family are human RNF4 and budding yeast Slx5-Slx8.
**Slx5-Slx8: A Yeast SUMO-Targeted Ubiquitin Ligase**

Slx5 is one of the founding members of the STUbL class of enzymes. Originally identified in a synthetic lethal screen of cells lacking Sgs1, it functions as a heterodimer with Slx8 harboring RING-dependent ligase activity, and SIM containing Slx5 primarily responsible for SUMO recognition (Mullen et al. 2001).

Slx5-Slx8 controls the levels of cellular sumoylated proteins and high-molecular weight SUMO chains and is required for genome stability and DNA damage response (Uzunova et al. 2007; Zhang et al. 2006; Xie et al. 2007). Slx5 and Slx8 reside in the nucleus and Slx5 also forms distinct foci and localizes to sites of double stranded DNA breaks (Cook et al. 2009; Nagai et al. 2008). Loss of Slx5-Slx8 function leads to gross chromosomal rearrangements, spontaneous DNA damage, and sensitivity to genotoxic stress (Nagai et al. 2011; Heideker et al. 2009). Slx5-Slx8 appears to direct SUMO-conjugated proteins to the proteasome by mediating ubiquitylation (Uzunova et al. 2007; Mullen & Brill 2008). Consistent with this role, overexpression of Slx5 (in the presence of conjugation-competent SUMO) suppresses the lethality of *ulplts* cells (Xie et al. 2007).

Known substrates of Slx5-Slx8 include the transcriptional regulator Mot1, which regulates the DNA binding ability of TATA-binding protein, as well as the mating type transcription co-activators MATα1 and MATα2 that regulate transcription from mating type-specific genes (Wang & Prelich 2009; Xie et al. 2010; Nixon et al. 2010). Few *in vivo* targets of Slx5-Slx8 have been identified to
date largely due to the difficulty in identifying E3 targets. The small overall population of sumoylated proteins and the transient nature of ubiquitylated proteins hamper substrate identification. Previous work in the Kerscher lab has identified the E3 SUMO ligase known as Siz1 to be a potential target for the Slx5-Slx8 complex. Yeast two-hybrid assays performed in the Kerscher lab by previous master’s student Jason Westerbeck demonstrated a robust interaction between Siz1 and Slx5. Furthermore, Westerbeck and colleagues found that the interaction between Siz1 and Slx5 may be SUMO-dependent and requires a SIM located in the N-terminal domain of Slx5. They went on to show that Siz1 is an in vitro ubiquitylation substrate of Slx5-Slx8 (Westerbeck et al. 2013).

Aims

We hypothesize that Ulp1 desumoylates a key cell cycle regulator and loss of Ulp1 activity stalls the cell cycle during G2/M phase. Chapter 2 of this thesis describes the use of a novel substrate trapping mutant of Ulp1 created in the Kerscher lab by former master’s student Zac Elmore to identify and functionally characterize Ulp1 substrates involved in cell cycle progression (Elmore et al. 2011).

Chapter 3 of this thesis builds on previous data described above and will investigate the functional interplay between Siz1 with Slx5. Specifically, chapter 3 will show that Slx5 affects the steady state level of Siz1. Additionally, it will demonstrate that Slx5 affects the phosphorylation and sumoylation status of Siz1.
and will investigate the localization of GFP-tagged Siz1 in WT cells in comparison to \textit{slx5\Delta}, \textit{msn5\Delta}, and \textit{slx5\Delta/msn5\Delta} mutant strains.

\textbf{Model 1. The ubiquitin and SUMO cycles.} Precursor SUMO and ubiquitin (blue rectangle) are initially processed by DUB/Ulp isopeptidases into conjugation competent form. Once processed, the modifier is covalently conjugated to a lysine side chain of a targeted substrate (red oval) in a three enzyme cascade consisting of E1 activating enzyme (light blue), E2 conjugating enzyme (green) and E3 ligase (yellow). After conjugation, additional monomers can be added to the substrate, or the process can be reversed by removing the modification using a DUB or Ulp isopeptidase (Kerscher et al. 2006).
Elmore et al. (2011) have previously reported the ability of a mutated form of Ulp1 to act as a substrate-trapping mutant. In their work, a truncation of Ulp1 comprising only the catalytic region (region 3) of the protein (Li & Hochstrasser 2003) was mutated by site directed mutagenesis. By switching the active cysteine residue at position 580 to a serine, the authors’ generated a substrate-trapping mutant designated Ulp1(III)\(^{C580S}\).

In this chapter we utilized the substrate-trapping mutant of Ulp1’s catalytic domain, Ulp1(III)\(^{C580S}\), to affinity purify and selectively enrich SUMO protease substrates. We identified 32 candid Ulp1 substrates through mass spectrometry analysis of U-Tag purified proteins.

**Materials and Methods**

**Yeast strain, plasmids, bacteria and growth conditions**

Yeast, plasmids, and bacterial strains used for this work are listed in table 1. Yeast media preparation and growth conditions were carried out as previously described (Amberg et al. 2005). All yeast strains were grown at 30°C in 2x media unless noted otherwise. Cells expressing a temperature-sensitive mutant of Ulp1 (\(ulp1ts\)) contain three mutations (I435V, N450S and I504T) in the catalytic domain, region 3 (Li & Hochstrasser 1999).

**Recombinant protein expression and bacterial protein extraction**
Induction of U-Tag overexpression – BOK 752 containing maltose binding protein fused Ulp1(III)\textsuperscript{C580S} (Elmore et al. 2011) was inoculated into 4mL of LB broth containing 60μg/mL carbenicillin (USABiological) and cultured overnight, rotating at 37°C. This 4mL culture was added to 200mL of SOC and grown at 37°C to OD\textsubscript{600} 0.3-0.5. A 1mL sample was harvested, washed once in 1x PBS, and resuspended in 1x LDS sample buffer containing 4% 2-mercaptoethanol (BME) (Invitrogen). This sample was boiled at 110°C for 3 minutes and then frozen at -80°C. This freeze then thaw process was done a total of three times.

To the remaining culture, 80μL of 1M IPTG was added to induce MBP-Ulp1(III)\textsuperscript{C580S} expression. The culture was shaken for 5 hours at 18°C. Another 1mL sample was taken as described above. To analyze protein induction, 10μL and 20μL of induced protein sample were subjected to SDS-PAGE on a pre-cast 4-12% Bis-Tris mini-gel (NuPAGE, Invitrogen). Samples ran for 55 minutes at 200 volts in commercial MOPS buffer (Invitrogen). Bands were analyzed by staining with Simply Blue SafeStain (Invitrogen) as per the manufacturer’s instructions.

The induced culture was harvested at 4°C for 15 minutes at 2,320x g (RCF). The supernatant was decanted away and residual media was removed by pipetting. Cell pellets were kept on ice and resuspended in 4mL of phosphate buffered saline (PBS) (10 mM phosphate, 150 mM sodium chloride, pH 7.4) containing 1x Halt Protease Inhibitor (PI) Cocktail, EDTA free (Thermo Fisher Scientific). These 4mL were spun down into a 2mL cryo-tube and snap frozen in liquid nitrogen. Pellets were kept at -80°C until use.
Bacterial protein extraction – Frozen induced cell pellets were thawed on ice and resuspended in 800µL 1x PBS containing 1x PI. Total volume was brought up to 2mL with 1x PBS. Cell suspensions were sonicated three times at 20% duty cycle for 20 seconds. Resulting lysates were clarified by centrifugation at 20879 x g (RCF) for 8 minutes in a refrigerated centrifuge. The supernatant or extracted protein was removed to a pre-chilled 15mL screw cap tube and the volume was adjusted to 4mL total using cold 1x PBS.

Affinity resin preparation – Gravity filtration columns were assembled as per the manufacturer’s instructions (Thermo Fisher Scientific). 500µL of amylose resin suspension (New England Biolabs) was equilibrated in ice cold 1x PBS. Then, equilibrated amylose resin was added to the column as a bed and washed three times with cold 1x PBS. Bacterial protein extractions (previous section) were added to the column to purify MBP tagged Ulp1(III)C580S. After three washes in ice cold 1x PBS, amylose resin with bound MBP tagged Ulp1(III)C580S was removed from the column and stored on ice in 1mL of 1x PBS containing 0.02% sodium azide. Amylose bound MBP-Ulp1(III)C580S is hereafter referred to as ‘U-Tag affinity resin.’

Yeast protein extraction

A single colony of YOK 428 was grown overnight in 40mL of selective media containing 2% dextrose and 200µg/mL G418. The next day, the 40mL starter culture was added to two liters of selective media containing 2% dextrose giving an OD₆₀₀ of about 0.2. The two liter culture was split into four flasks for
overnight growth. The third day, the logarithmically growing culture was diluted 1:2 in YPD to an \( \text{OD}_{600} \) of 0.8 and allowed to grow an additional 3 hours. The whole culture was harvested by centrifugation at 4,000xg for 10 minutes at 4°C. The pellet was washed first in 50mL wash buffer (table 2; appendix) and then in 10mL of extrusion buffer (table 2; appendix). Residual buffer was removed by micropipettor and the cell paste was scooped into a 10mL syringe with a sterile spatula. The cells were then extruded from the syringe into a 50mL centrifuge containing liquid nitrogen and snap-frozen as high density ‘noodles’ of cells.

Snap frozen cell noodles were added to a pre-cooled (-20°C) coffee grinder (Biospec Products) containing pre-powdered dry ice (enough to cover cells). The grinder was run for 5 minutes at -20°C to pulverize the cells. The resulting powder was placed at -80°C until the remaining dry ice is sublimated. Pulverized cell powder was resuspended in 20mL of extraction buffer + PI (table 2; appendix) then snap frozen.

**U-Tag affinity chromatography**

500μL of U-Tag affinity resin (preparation described above) was equilibrated in extraction buffer by washing 3 times with 1mL each. Equilibrated U-Tag resin was transferred to a gravity filtration column (Thermo Fisher Scientific), washed two additional times with one column volume of extraction buffer, and kept at 4°C.

Frozen yeast protein extract (described above) was thawed on ice. A 30μL aliquot was taken, mixed with an equal volume of 2x LDS sample buffer + 8%
BME (Invitrogen), and used as whole-cell extract in subsequent protein analysis (supplemental figure 6). To the chilled affinity column, 7.5mL of yeast protein extract was gravity filtered through the resin bed. The resin bed was then washed with three column volumes of extraction buffer + PI (table 2; appendix) containing 1% triton x-100. The substrate-bound U-Tag resin was transferred to a microcentrifuge tube and a 30µL sample was taken for subsequent protein analysis.

Proteins bound to U-Tag affinity resin were eluted from the resin using commercially available recombinant Ulp1-His6 (Invitrogen) to cleave captured substrates from the U-Tag. Cleavage and purification were carried out as per the manufacturer’s instructions. Briefly, substrate-bound U-Tag resin was collected by gentle centrifugation (5 seconds, 60 x g(RCF)) and resuspended in 200µl of the supplied 1x SUMO protease buffer. 10 units of SUMO protease were added. The tube was mixed gently and the reaction was incubated at 30°C for 6 hours. After incubation, the supernatant was collected and purified using a commercial PrepEase (affymetrix) Histidine-Tagged protein purification kit to remove Ulp1-His6. Column flow through containing SUMO conjugates and free SUMO were used in subsequent protein analysis.

Silver stain and mass spectrometry

Protein concentration of the purified SUMO conjugates solution (above section) was measure using commercial BCA assay kit (Sigma) as per the manufacturer’s protocol. Then, 50ng of total protein were separated on a precast
4-12% Bis-Tris mini-gel (NuPAGE, Invitrogen) for 55 minutes at 200 volts in 1x MOPS buffer. Protein bands were visualized in the gel using a commercial Pierce silver staining kit for mass spectrometry (Thermo Fisher Scientific) as per the manufacturer’s instructions.

The remaining solution of purified SUMO conjugates (~150µg) was sent to the Yale proteomics facility (Keck Biotechnology Resource Laboratory) for mass spectrometry analysis.

Results

Considering the important role of SUMO deconjugation to SUMO homeostasis, we sought to identify novel targets of Ulp1. Furthermore, given the essential nature of Ulp1 to the cell cycle, we reasoned that one or more proteins that co-purify with Ulp1(III)CS80S would be Ulp1 targets that are critically important for cell cycle progression. Here we report the use of Elmore and colleagues’ substrate-trapping mutant, known as U-Tag, to selectively purify Ulp1-interacting proteins (Elmore et al. 2011).

U-Tag affinity purifies sumoylated proteins from whole cell extracts

Cellular extracts fromulp1ts mutant YOK428 were subjected to U-Tag affinity purification (supplemental figure 6). Fifty nanograms of purified protein and an equal volume of mock purification eluate were separated by SDS-PAGE and visualized with silver staining to identify protein bands. Several distinct bands
were observed in the sample lane (figure 1. Lane 2) and were not present in the mock column control lane (figure 1. Lane 3) suggesting that these bands are U-Tag co-purifying proteins. Notably, we identified a single band at about 20kDa consistent with monomeric SUMO that was liberated from its substrate by recombinant Ulp1 (see methods; elution step). Several additional robust bands representing U-Tag purified proteins are indicated by arrows in figure 1.

**Mass spectrometry analysis identifies 32 U-Tag co-purifying proteins**

In order to identify U-Tag affinity purified proteins, mass spectrometry analysis was carried out by the Yale Proteomics facility (W.M. Keck Biotechnology Laboratory; New Haven, CT). Multiple dimension protein identification technique (MudPIT) was used to identify and sequence 69 peptide fragments belonging to 32 distinct proteins (table 3; appendix). Proteins represented by multiple fragments were present in relatively high concentration. Eight candidate sumoylated proteins were identified with more than three fragments; BMH1, SMT3, EF2 (ETF2), HSP82, KPYK1 (CDC19), PHSG (GPH1), RLA0 (RPP0), and VATE (VMA4) [table 3; parentheses represent gene names from Saccharomyces Genome Database]. In table 4, we have highlighted BMH1, VATE, and EF2 as candidates for future investigation as well as yeast SUMO which is our internal control. Vma4, Ef2, and Bmh1 were all previously identified as a sumoylated proteins in high-throughput proteomic studies (Denison et al. 2005; Sung et al. 2013). Bmh1 is of particular interest to us given the many regulatory functions of 14-3-3 proteins and its association with the anaphase promoting complex.
In summary, our data confirm that the U-Tag can effectively purify and enrich sumoylated proteins. Additionally, we have identified several candidate Ulp1 substrates including one regulator of the anaphase promoting complex.
Figure 1. U-Tag affinity resin selectively purifies sumoylated proteins from cellular extracts. Sumoylated proteins from YOK 428 were U-Tag affinity purified and eluted as described in Methods. 50ng of U-Tag purified proteins were loaded in lane 2 and an equal volume of mock eluate in lane 3. Lane 1 contains a molecular weight standard and lane 4 contains whole cell protein extraction (WCE) as a control for protein separation. Input proteins were run on a 4-12% Bis-Tris gel and Silver stained with Pierce silver staining kit for mass spectrometry (Thermo Scientific). Arrows indicate positions of select bands present in U-Tag lane but not in mock column control lane. Numbers indicate approximate molecular weight (left) and lane designation (bottom).
Discussion

This chapter reveals that the substrate-trapping mutant of Ulp1’s catalytic domain, Ulp1(III)C580S (Elmore et al. 2011), can be employed to affinity purify, selectively enrich, and identify candidate Ulp1 substrates. One interesting observation made from this screen for U-Tag interacting proteins is that Bmh1 carries the potential to be a Ulp1 substrate. Bmh1 is one of two proteins in Saccharomyces cerevisiae that represent the highly conserved 14-3-3 family of proteins. Previous proteomic studies have shown Bmh1 to be sumoylated (Denison et al. 2005) and revealed many putative interacting partners. Recent work in the Kerscher lab by Jeremy Wells (WM class of 2014) has confirmed Bmh1 sumoylation biochemically in a molecular weight shift assay, but the exact cellular consequences of Bmh1 sumoylation are unknown. Bmh1 has recently been shown to be a regulator of the cell cycle (Dial et al. 2007). Bmh1, in complex with the pseudosubstrate inhibitor Acm1, binds to a co-activator of the Anaphase-promoting complex (APC) known as Cdh1. This interaction sequesters Cdh1 and inactivates the APC; an event necessary for cell cycle progression through S-phase (Dial et al. 2007).

We propose that SUMO may play a role in Bmh1’s cooperative regulation of the APC and that interactions with Ulp1 regulate its sumoylation (model 2). Specifically, we propose that sumoylation of Bmh1 facilitates the interaction between Bmh1/Acm1/Cdh1 (CAB complex) and mediates APC deactivation at the onset of S-phase. Furthermore, we hypothesize that Ulp1 mediates
desumoylation of Bmh1 after G2, which initiates the dissociation of the CAB complex and reactivates the APC$^{Cdh1}$. This hypothesis is supported by previous findings that $ulp1ts$ cells grown at non-permissive temperatures arrest as large budded cells, presumably late in the cell cycle. This late-stage arrest could be caused by a failure of Ulp1 to desumoylate cell cycle regulators, such as Bmh1. Additionally, strains expressing $ulp1ts$ have artificially stabilized Clb2 levels. Clb2 is a B-type cyclin and APC substrate that promotes transition from G2 to M phase in yeast cells. Clb2 is targeted for destruction via a destruction box motif during G1 when the CAB complex is dissociated and the APC$^{Cdh1}$ is active. This buildup of Clb2 could be due to reduced APC activity and prolonged sequestration of CDH1 in the CAB complex, potentially because Ulp1 fails to desumoylate Bmh1.

To this end, our future work will investigate the consequences of Bmh1 sumoylation. Using a SUMO deficient mutant of Bmh1 Ulp1 created in the Kerscher lab by Jeremy Wells (Bmh1 sumo-no-more or SMN; figure 2) we will conduct a top-down investigation of the role of Bmh1-SUMO on the cell division cycle. We will use a $bmh1\Delta bmh2\Delta$ double mutant strain expressing either wild-type Bmh1 or Bmh1(SNM) from a plasmid to look for growth defects incurred from loss of Bmh1 sumoylation. Preliminary experiments performed in the Kerscher lab by Jeremy Wells have revealed a temperature sensitive phenotype associated with deficient Bmh1 sumoylation.

Next, we will verify Bmh1-SUMO as a U-Tag interacting protein and a Ulp1 substrate in vivo. By co-expressing affinity tagged Ulp1(III)$^{C580S}$ and epitope
tagged Bmh1 we will pull down Ulp1(III)\textsuperscript{C580S} and co-purify sumoylated Bmh1 from yeast extracts. From there, we will express tagged Bmh1 in \textit{aulp1ts} strain coexpressing conjugation competent SUMO to show that desumoylation of Bmh1 is abolished at non-permissive temperatures.

Finally, we plan to investigate the molecular consequences of Bmh1 sumoylation. Again, using the SUMO-deficient mutant we will determine what, if any, role sumoylation plays in the regulation of APC\textsuperscript{Cdhl} activity. Specifically, we will determine if CAB complex formation and dissociation is facilitated by Bmh1-SUMO dynamics.
Model 2. Proposed role for SUMO dynamics in regulation of APC<sup>Cdh1</sup> activity.

Sumoylation of Bmh1 during late G1 (bottom arrow) facilitates the association of Bmh1, Acm1, and the APC co-activator Cdh1 (CAB complex) (Dial et al. 2007). Sequestration of Cdh1 in the CAB complex throughout S-phase and G2 maintains the APC in an inactive state. Ulp1 mediated desumoylation of Bmh1 in late G2 or early M phase (top arrow) causes dissociation of the CAB complex and APC<sup>Cdh1</sup> reactivation. The APC<sup>Cdh1</sup> then triggers mitotic exit through degradation of the cyclin Clb2.
Figure 2. Sumoylation site prediction and mutagenesis of Bmh1. We used SUMOplot™ Analysis Program to predict sumoylation sites in Bmh1. A SUMO-no-more mutant was constructed by Jeremy Wells (WM class of 2014) mutating six high probability lysine residues into arginine. Colored residues on the right represent mutated amino acids. SUMOplot™ Analysis Program (http://www.abgent.com/sumoplot.html)
Chapter 3. A SUMO-targeted ubiquitin ligase is involved in the cell cycle-specific degradation of the SUMO E3 ligase Siz1

Chapter 3 builds on previous data collected by Jason Westerback described above and reported in the Kerscher lab’s most recent publication (Westerbeck 2011). This chapter reports our findings on the functional interplay between Siz1 with Slx5. Specifically; the data presented here shows that Slx5 affects the steady state level of Siz1. Additionally, it demonstrates that Slx5 influences the phosphorylation and sumoylation status of Siz1. Finally, this chapter reports the localization of GFP-tagged Siz1 in WT cells in comparison to slx5Δ, msn5Δ, and slx5Δ/msn5Δ mutant strains.

Materials and Methods

Yeast strains, Media and Plasmids

Yeast strains and plasmids used in this study are listed in Table 1. Unless otherwise noted, yeast media preparation and manipulation of yeast cells was performed as previously reported (Guthrie and Fink, 2002). All strains were grown at 30°C unless otherwise noted.

Cell synchronization and drug treatments

Where indicated yeast cells were synchronized in G2/M phase by
incubating logarithmically grown cells in 15 μg/ml nocodazole (Acros Organics 358240500) for 3 hrs at 30°C. Cells were arrested in S-phase by addition of 0.1M hydroxyurea (Sigma H8627) and incubation at 30°C for 3 hours. For cycloheximide chase experiments, 25 μg/ml cycloheximide (Sigma C7698) was added to G2/M arrested cells. 2.5 ODs of cells were harvested at the indicated time-points.

Cloning and epitope-tagging of yeast genes

Chromosomal tagging and gene deletions in yeast were carried out by PCR-based homologous recombination (Longtine et al., 1998). Strain YOK821 (s/Δx5) was used to epitope tag SIZ1 with a 13myc epitope tag. Briefly, primers OOK663 and OOK662 were used to amplify the 13xmyc-ADH1-His3MX6 cassette with 40bp of SIZ1 sequence homology from the plasmid pFA6a-13myc-His3MX6 (Longtine). PCR amplification was carried out using Phusion High-Fidelity PCR kit (NEB E0553S) with DMSO and high GC buffer as recommended by the manufacturer. For transformation, 6.5 μg of purified SIZ1-13myc-His3MX6 PCR product was combined with 4 ODs of competent s/Δx5 (YOK821) cells, incubated for 30 minutes at 30°C, heat shocked at 42°C for 30 minutes and plated on SD–His dropout. Resulting colonies were screened by western blotting using an anti-myc antibody. Subsequently, the SIZ1-myc s/Δx5 strain (YOK2264) was backcrossed to YOK819 to obtain SIZ1-myc SLX5(WT) progeny (YOK2286). An amplicon of SIZ1-13myc was also cloned into a gateway compatible pRS315 plasmid.
The *msn5* null mutant was constructed in the Kerscher lab by Nagesh Parsupala, who introduced a Hygromycin deletion cassette with 78 bp flanking sequence homology to *msn5* gene upstream and downstream region.

**Preparation of yeast extracts, gel electrophoresis, and western blotting**

Whole cell yeast extracts were prepared by TCA glass bead lysis. Briefly, ~4 ODs of pelleted yeast cells were washed once in 800 µl of 20% TCA and resuspended in 400 µl of 20% TCA. 200 µl of glass beads (Sigma G8772) were added and the samples were vortexed at 4°C for 4 minutes. The beads were allowed to settle and the supernatant was transferred to a new tube and pelleted by centrifugation at 15K rpm for 8 minutes. Pellets were washed once using 800 µl of ice-cold 2% TCA. The supernatant was removed and pellets were resuspended in 200 µl of TCA-sample buffer (15% glycerol, 80 mM Tris base, 3.5% SDS, Bromophenol Blue, and BME [40 µl/ml]). The samples were boiled at 100°C for 2 minutes, and whole cell protein extracts corresponding to ~0.2 ODs were separated on a pre-cast NuPAGE Novex 4-12% Bis-Tris gels (NP0321 Life Technologies) or home-made 8% Tris-Glycine gels. After separation proteins were transferred to polyvinylidene difluoride (PVDF) membrane (IPVH00010 - Millipore) for 30 minutes at 19 V. Blots were blocked in TBS (150 mM NaCl, 50 mM Tris-HCl at pH 7.4) containing 4% milk for an hour and then incubated in 4% milk containing primary antibody overnight at 4°C followed by incubation with secondary antibodies for 1-3
hours at ambient temperature. After antibody incubations blots were extensively washed in TBS + 0.1% Tween 20 (TBST). Antibodies were used at the following concentrations; anti-myc (1:5000; Covance MMS-150R), anti-Pgk (1:10000; Life Technologies A6457), anti-mouse(HRP) (1:15,000; abcam ab9740). Proteins were visualized on film using ECL substrate (Millipore Immobilon Western ECL substrate WBKL S0 100).

**Fluorescent Microscopy**

Images of live cells were collected using a Zeiss Axioskop fitted with a Retiga SRV camera (Q-imaging), i-Vision software (BioVision Technologies), and a Uniblitz shutter assembly (Rochester, NY). Pertinent filter for the above application was CZ909 (GFP) (Chroma Technology Group). Where applicable, images were normalized using i-Vision software.

**Results**

**Slx5 affects the steady-state level, phosphorylation, and sumoylation of Siz1 in vivo**

In order to understand the role of Slx5 in Siz1 stability, we compared endogenous Siz1 levels from wild-type (WT) and slx5 null (Δ) strains. Briefly, YOK 2264 containing 13X Myc-tagged Siz1 was mated with isogenic WT strain YOK 819. In meiotic progeny expressing Myc-tagged Siz1, immuno-blotting detected the protein running as a distinct band just below the 150kDa marker
Intriguingly, an increased level of high molecular weight adducts of Siz1 were also observed in slx5Δ cells. These slower moving adducts are consistent with sumoylated forms Siz1. Siz1 is located in the nucleus during interphase, but is exported to the cytoplasm during mitosis. As mentioned above, cytosolic Siz1 enriches at the bud neck of dividing yeast cells where it sumoylates the septin proteins Cdc3, Cdc11, and Shs1 (Johnson and Gupta, 2001). Nuclear egress of Siz1 is mediated by the karyopherin Msn5 which is known to export phosphorylated proteins (Makhnevych et al., 2007). At a point prior to anaphase, Siz1 becomes phosphorylated by an unknown kinase, which may be linked to its export by Msn5 (Johnson and Gupta, 2001). We investigated the phosphorylation status of Siz1 by comparing both WT and slx5Δ cells growing logarithmically, arrested in S-phase with hydroxyurea (HU), and arrested in G2/M with nocodazole (NZ) (Figure 3B). The levels of unmodified Siz1 as well as two phosphorylated forms were markedly enhanced in slx5Δ cells. The effect was less pronounced after S-phase and G2/M arrest with almost complete phosphorylation of Siz1 in G2/M arrested cells. Phosphorylation was confirmed by phos-tag SDS-PAGE (Wako Pure Chemical Industries) and immune-blotting (Parsupala, N., 2013; data not shown).

**Slx5 is required to modulate the levels of Siz1 in the nucleus during mitosis**

As shown in Figure 3, Slx5 influences the steady state level of Siz1 by an undetermined mechanism. This increase in steady state is coupled with an
increase in sumoylated abducts of Siz1. Given the historic role of ubiquitylation in protein turnover, as well as the specificity of STUbLs for sumoylated proteins, we believe that Slx5 mediated ubiquitylation tags Siz1 for degradation. In order to test this hypothesis we conducted a series of stability assays comparing the half-life of Siz1 from WT and slx5d cells. To our surprise, we were unable to detect a change in Siz1 turnover when assaying the whole cellular pool of Siz1 (data not shown).

As discussed previously, Slx5 resides in the nucleus while Siz1 localization is dynamic and cell cycle-dependent. As a result, we reasoned that Slx5 could be modulating the degradation of a nuclear subpopulation of Siz1 that is not detectable when immune-blotting the whole cellular pool of Siz1. Therefore, we have devised a strategy that examines the interaction between Slx5 and nuclear Siz1. By mutating MSN5 we were able to block the nuclear egress of Siz1 during mitosis thereby enriching nuclear targets of Slx5. Using this model, we first observed GFP-tagged Siz1 in wild type cells and compared it to slx5Δ, msn5Δ and the msn5Δslx5Δ double mutant in logarithmically growing and G2/M-arrested cells. Consistent with previous results (Makhnevych et al., 2007), Siz1-GFP resides in the nucleus for most of the cell cycle, but re-localizes to the septin ring during mitosis (Figure 4A). However, in both the msn5Δ and the msn5Δslx5Δ double mutant, Siz1 was retained in the nucleus during mitosis and could not be detected at the septin ring (Figure 4A).

Next we investigated the effect of Msn5 on the steady state level and phosphorylation state of Siz1. We compared a 4 mutant panel of WT, slx5Δ,
and strains expressing Myc-tagged Siz1 in logarithmic growth and G2/M arrest. In comparison to WT, no reduction in steady state level of Siz1 was detected in msn5Δ cells; however, we did observe an increase in steady state from logarithmically growing slx5Δ cells consistent with figure 3. The phosphorylation state of Siz1 was dramatically influenced by SLX5, MSN5, and cell cycle stage (Figure 5). In comparison to WT, mutating msn5Δ reduced the phosphorylation state and abolished the doubly phosphorylated adduct of Siz1 running at the highest molecular weight. In contrast, mutating slx5Δ in both the single and double mutants increases the level of Siz1 phosphorylation. Similar to Figure 3, G2/M arrest equalizes phosphorylation state and levels of Siz1 in WT, slx5Δ and msn5Δslx5Δ strains. Intriguingly, the phosphorylation of Siz1 from msn5Δ cells remains reduced during mitosis arrest implying that some degree of Siz1 phosphorylation is linked to nuclear export.

In order to demonstrate that nuclear accumulation of Siz1 leads to its degradation, we performed a cycloheximide chase of Siz1 in mitotically arrested WT and msn5Δ cells (Parsupala, N., 2013; Figure 4B). As predicted, we found that the half-life of Siz1 was dramatically reduced in the msn5Δ strain. In comparison, Siz1 was only slightly modulated in the WT strain. Finally, to show that Slx5 contributes to Siz1 stability, we performed a cycloheximide chase of nuclear retained Siz1 in msn5Δ and msn5Δslx5Δ strains (Figure 4C). Consistent with our prediction, Siz1 was stabilized in the slx5Δmsn5Δ double mutant, but degraded rapidly in the msn5Δ mutant. Furthermore, sumoylated adducts of Siz1 accumulated in the double mutant, while all forms of Siz1 were unstable in the
msn5Δ mutant. This effect was specific to Siz1 retained in the nucleus during mitosis as a similar experiment conducted in S-phase arrest showed no change in Siz1 stability or sumoylation (Figure 6).

In conclusion, our data reveals for the first time the functional interplay between SUMO E3 ligases and SUMO-targeted ubiquitin ligases and demonstrates that at least three post-translational modifications (phosphorylation, sumoylation, and ubiquitylation) are involved in this process. This Slx5-dependent regulation of nuclear localized Siz1 may work in cooperation with other pathways to prevent the accumulation of specific nuclear SUMO conjugates that interfere with cell cycle progression or other vital processes (Figure 4D).

Discussion

In this chapter we present new data on the regulation of the E3 SUMO ligase Siz1 by the SUMO-targeted ubiquitin ligase Slx5. Siz1 resides in the nucleus throughout interphase, but becomes phosphorylated in G2/M concurrent with its nuclear export (Johnson and Gupta, 2001). Recent work by Makhnevych et al. (2007) has shown that Siz1 is exported from the nucleus by the karyopherin Msn5 during mitosis where it enriches at the bud neck and participates in septin sumoylation. In addition to mitotic phosphorylation, Siz1 is modified by SUMO chains in vivo (Takahashi and Kikuchi, 2005). Previous studies in our lab have identified a physical interaction between sumoylated Siz1 and Slx5 as well as identified Siz1 as a STUbL substrate in vivo (Westerbeck et al., 2013). Building
upon this work, this chapter explores the functional role of Slx5 mediated
ubiquitylation and sheds light on the fate of nuclear Siz1 during mitosis. Our data
raises the possibility of tripartite regulation of Siz1 involving phosphorylation,
sumoylation, and Slx5-mediated ubiquitylation. Specifically, I found that both
phosphorylated and sumoylated Siz1 accumulate in slx5Δ mutant cells (Figures 3
and 5). When Siz1 is retained in the nucleus of an msn5Δ mutant,
phosphorylated and sumoylated species are rapidly degraded (Figure 4B). In an
msn5Δslx5Δ double mutant, however, phosphorylated and sumoylated Siz1 are
stabilized in G2/M, suggesting that STUbLs play a role in modulating SUMO E3
ligases in the nucleus (Figure 4C).

The cross-talk between phosphorylation and sumoylation is poorly
understood; however, a link between sumoylation and phosphorylation has
previously been established. The Siz1 ortholog PIAS1 contains a phospho-
regulated SIM which is required to modulate its interactions with specific
transcription factors (Stehmeier and Muller, 2009). Siz1 contains a bona fide
SIM but we have not determined whether it constitutes a phospho-regulated SIM
(Uzunova et al., 2007). It would be beneficial to determine how phosphorylation
and sumoylation work together in governing Siz1 interactions, but our current
understanding of Siz1 phosphorylation is in its infancy and many aspects of Siz1
modification remain unclear. Future research in the lab will identify both the
modifying kinase and phosphorylation sites in order to expand upon research
initiated with this chapter and further characterize the complex cross-talk of
modifications that regulate Siz1.
In addition to describing the interplay between phosphorylation, sumoylation, and STUbL mediated ubiquitylation, this work begins to decode the link between Siz1 phosphorylation and its nuclear shuttling. Msn5 is known to export phosphorylated cargoes, but it is currently unknown whether or not Siz1 export depends upon phosphorylation (Takahashi et al., 2008; Makhnevych et al., 2007). Our data shows that Msn5 can itself influence phosphorylation of Siz1. Specifically, mutating Msn5 abolishes a slow moving phosphorylated adduct we believe to be a doubly phosphorylated species of Siz1 (Figure 5). From this preliminary data, we could speculate that a phosphorylation event regulated by Msn5 facilitates Siz1 export during mitosis. Alternatively, this second phosphate addition could be acquired in the cytosol after export and influence Siz1 interactions with septin proteins. Further work in this direction will explore the timing and regulation of Siz1 nuclear shuttling in order to further our understanding of cytoplasmic sumoylation.

In conclusion, our data describes the first account of functional interaction between SUMO ligases and SUMO-targeted ubiquitin ligases. We believe that the cell cycle-specific degradation of Siz1 by Slx5/Slx8 plays an important physiological role in reducing nuclear SUMO E3 ligase activity as the cell enters mitosis. This process likely works in conjunction with the nuclear export pathway of Siz1 and may prevent the accumulation of nuclear SUMO conjugates that interfere with cell cycle progression. Considering that Slx5 and Siz1 are evolutionarily conserved proteins, we predict that the STUbL-mediated regulation of SUMO-E3 ligases extends to RNF4 and PIAS proteins in mammalian cells. It
will be interesting to determine whether RNF4 affects the turn-over of phosphorylated and sumoylated PIAS1 in mammalian cells. This would further support the emerging theme of a cross-talk between phosphorylation, sumoylation, and ubiquitylation in SUMO-targeted degradation.

STUbL regulation of sumoylation pathway likely extends far beyond Siz1. Recent work by Albuquerque et al. (2013) shows an antagonistic interaction between Slx5 and the SUMO E3 ligase Mms21. Using quantitative mass spectrometry they assessed the abundance of sumoylated proteins in a wild-type strain in comparison to slx5A mutant strain. They found that deleting SLX5 increased the abundance of most sumoylated proteins, with Mms21-specific targets being substantially elevated. It would be interesting to determine if Slx5 influences the degradation of Mms21 in a manner similar to Siz1. The antagonistic relationship between Slx5 and Mms21 fits with our finding and supports a role for STUbLs in regulating cellular SUMO homeostasis.
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Figure 3. Slx5 affects the steady-state level and phosphorylation status of Siz1. (A) Altered steady-state level of Siz1 in slx5Δ cells. A heterozygous diploid SLX5/slx5Δ SIZ1/SIZ1-myc/HIS3 strain was sporulated and the resulting haploid progeny of two tetrads (tetrad 7 (YOK 2279-2282) and tetrad 10 (YOK 2283-2286) were genotyped (WT and Δ). Proteins were extracted from the indicated haploid strains to determine the steady-state levels of the myc-tagged Siz1 protein in WT and slx5Δ progeny. An anti- myc antibody was used to detect Siz1 on immunoblots of SDS-PAGE separated proteins. Note the increased steady-state levels and modifications of Siz1 in slx5Δ strains (tetrad 7-4 and tetrad 10-3) in comparison to Siz1 levels in SLX5 WT strains (tetrad 7-1 and tetrad 10-4). Equal protein loading was confirmed by immunoblot for the housekeeping protein 3-phosphoglycerate kinase (PGK1). (B) Siz1 is differentially phosphorylated under various growth conditions in WT (YOK 2286) versus slx5Δ (YOK 2264) cells. Log: untreated, logarithmically growing cells; HU: hydroxyurea treatment to arrest in S-phase; NZ: nocodazole treatment to arrest in G2/M. Endogenous, myc-tagged Siz1 protein in WT and slx5Δ cells was detected after immunoblotting of SDS-PAGE separated proteins using an anti-myc antibody. (*) and **) denotes differentially phosphorylated forms of Siz1.
A

![Image of protein expression analysis](image)

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D

- Siz1
- Pgk1

**nucleus**
- Siz1
- Siz1 (m)
- Siz1 (p)
- STUbL
- Siz1 (p)

**cytosol**
- Siz1
- kinase
- Siz1 (p)
Figure 4. Slx5 modulates the levels of Siz1 in the nucleus. (A) WT (YOK2738), slx5Δ (YOK2751), msn5Δ (YOK2624), and msn5Δslx5Δ (YOK2735) strains expressing Siz1-GFP as the only copy of this SUMO ligase were imaged during logarithmic growth (log - left panel) or after nocodazole induced G2/M arrest (Noc - right panel). The localization of Siz1-GFP at septins is indicated with yellow arrows and the localization of nuclei in msn5Δ and msn5Δslx5Δ strains is indicated with white arrow heads. (B) Siz1 is rapidly degraded in an msn5Δ mutant: Isogenic WT (YOK 2397) and msn5Δ (YOK 2514) strains expressing endogenous full-length Siz1-myc were grown overnight in YPD medium. Cells in logarithmically grown cultures were arrested with nocodazole. 10 ODs of G2/M-arrested cells were pelleted, washed and resuspended in fresh YPD medium without nocodazole containing 25 µg/ml of cycloheximide. Subsequently, protein extracts of 2.5 ODs of cells were prepared at the indicated time points (0, 10, 30, 60 min) prior to western blotting to detect Siz1-myc. (C) A deletion of SLX5 stabilizes Siz1 in an msn5Δ mutant: Isogenic msn5Δ and msn5Δslx5Δ strains expressing Siz1-myc from LEU2/CEN plasmid pRS315 were grown overnight in selective media. Cells in logarithmically grown cultures were arrested with nocodazole and benomyl. 22 ODs of G2/M-arrested cells were pelleted, washed and resuspended in fresh YPD medium without nocodazole containing 25µg/ml of cycloheximide. Subsequently, protein extracts of 2 ODs of cells were prepared at the indicated time points (0, 40, 60, 90, 120 minutes) prior to western blotting to detect Siz1-myc and Pgk1 proteins. The first two lanes, msn5Δ (log) and msn5Δslx5Δ (log), are overloaded to show SUMO adducts of Siz1 in these strains. (D) Model of a STUbL-dependent nuclear degradation pathway of sumoylated Siz1: At the onset of mitosis nuclear Siz1 becomes auto-sumoylated (green circles) and phosphorylated (p) via an unknown kinase. Phosphorylated Siz1 is then subject to Msn5-mediated nuclear export to facilitate septin sumoylation in the cytosol. Sumoylated Siz1 that remains in the nucleus as the cell enters mitosis (in our experiments this was accomplished through deletion of MSN5) is subject to STUbL-mediated ubiquitylation (circle labeled Ub) and degradation. Other non-STUbL-dependent pathways for the regulation of Siz1 activity and levels may exist.
Figure 5. Slx5, Msn5 and cell cycle stage affect the phosphorylation state of Siz1. Strains 2397 (WT) 2396 (slx5Δ) 2514 (msn5Δ) and 2513(slx5Δ/msn5Δ) expressing endogenous Siz1-Myc were grown up overnight in YPD. Proteins were extracted from 4 ODs of Log and G2/M arrested cells. Phosphorylation state of Siz1Myc from treated and untreated cells was detected on immunoblots of SDS-PAGE separated proteins. Two levels of phosphorylation as well as unmodified Siz1 were detected. (Siz1-P and P-Siz1-P) denote the two phosphorylation states of Siz1. Siz1 phosphorylation is decreased in msn5Δ versus WT cells, but increased when SLX5 is also deleted. Siz1 is differentially phosphorylated in WT versus slx5Δ cells and overall phosphorylation increases during G2/M arrest confirming the findings from Figure 8B. Log: untreated, logarithmically growing cells; Nocodazole: cells treated with 25 μg/mL nocodazole to arrest in G2/M; WT: Wild type; Δ/Δ: slx5Δ and msn5Δ strain YOK 2513
Figure 6. Cycloheximide chase of S-Phase arrested cells. Strains YOK 2757 (WT), 2759 (msn5Δ), and 2761 (slx5Δ/msn5Δ) expressing Siz1Myc from a LEU2/CEN plasmid pRS315 were grown overnight in selective media to log phase. Cultures that had not reached stationary phase after overnight growth were arrested in S-phase with 0.1M hydroxyurea. 22 ODs of S-phase arrested cells were pelleted, washed and resuspended in fresh YPD medium without hydroxyurea containing 25μg/ml of cycloheximide. Subsequently, protein extracts of 2 ODs of cells were prepared at the indicated time points (0, 30, 60, 90, 120 minutes) prior to western blotting to detect Siz1-myc. MPT; Minutes post-treatment with cycloheximide; WT: Wild type; -Siz1 denotes unmodified Siz1 and Siz1Myc SUMO® denotes sumoylated forms of Siz1.
Appendix

PCR-based gene modification; Myc tagging Siz1 (Modified from Longtine et al., 1998)

Primes OOK662 and 663 (table 5) were used to PCR amplify 13xmyc-ADH1-His3MX6 cassette with 40bp of SIZ1 sequence homology from the plasmid pFA6a-13myc-His3MX6. Primer overhangs are homologous to 40 bases upstream of SIZ1 stop codon and 40 bases downstream from SIZ1 stop codon, but do not include the stop codon itself. 8x 50μL PCR reactions were pooled and extracted with phenol/chloroform/isoamylalcohol. The aqueous extract was ethanol precipitated and resuspended in 25μl of 1x Tris-EDTA (TE) by vortexing and pipetting. Transformation mixture was made by combining the 12.5μl of pure PCR product with 160μl of sterile 50% glycerol, 20μl of 10X TE, 20μl of 1M lithium acetate and 5 μl of denature herring sperm DNA (ssDNA).

5mL of YOK 821 (s/α5A) was grown overnight in YPD. In the morning, the stationary culture was diluted to OD600 of 0.3 and allowed to grow for 2 generations. 1OD of the recovered culture was harvested and suspended in 100mM lithium acetate + 1X TE then incubated at 30°C for 30 minutes.

After incubating for 30 minutes, the cells were collected by brief (5 second) centrifugation and gently resuspended in the transformation mixture from above. Cells in transformation mixture were incubated at 30°C for an additional 30 minutes before heatshocking at 42°C for 40 minutes. A long heat (30-40 minutes) is critical for successful transformation. After heat shocking, the cells were collected by brief centrifugation and washed once in pre-warmed 2x YEP and resuspended in pre-warmed 2x YPD. The cells were then incubated at 30°C, spinning for 1 hour and plated on a single selective plate. Transformant colonies were visible after 3 days.

Additional notes: Incubating cells overnight in 100mM lithium acetate + 1X TE before transformation increases efficiency. We have also had success with overnight recoveries in YPD after transformation and overnight growth in liquid selective media.

45
Preparation of high cell density yeast ‘noodles’ and cryogenic cell lysis.

A single colony was grown overnight in 40mL of selective media containing 2% dextrose. The next day, the 40mL starter culture was added to two liters of selective media containing 2% dextrose and split into four 1L flasks to allow for sufficient aeration. The two liter culture was grown to late log phase and the whole culture was harvested by centrifugation at 4,000 x gravity in the cold. The resulting cell pellet was washed first in 50mL wash buffer (table 2) and then in 10mL of extrusion buffer (table 2). Residual buffer was removed by micropipetor and the cell paste was scooped into a 10mL syringe with a sterile spatula. The cells were then extruded from the syringe into a 50mL centrifuge containing liquid nitrogen and snap-frozen as high density ‘noodles’ of cells. The centrifuge tube containing noodles and residual liquid nitrogen was placed at -80°C with holes punched in the cap to allow the nitrogen to vaporize.

Yeast noodles were lysed using a cryogenic tissue grinder (coffee grinder) to break open the cells. To do this, dry ice was powdered in a pre-chilled (-20°C) grinder. The amount of dry ice powder used is arbitrary, but was enough to take up about half the volume of the grinder and completely cover the yeast noodles when they are added. Yeast noodles were taken from the -80°C freezer and immediately placed into the dry ice. Cells were lysed by grinding for 5 minutes on the espresso setting at -20°C. The resulting powder of pulverized yeast cells and dry ice was placed at -80°C in a 50mL centrifuge tube with the top loose to allow the dry ice to sublimate. The powdered yeast cells were thawed on ice and resuspended in extraction buffer (table 2).
2 Liters of yeast expressing Smt3gg and a temperature sensitive mutant of Ulp1 (ulp1ts) that is deficient in SUMO removal was collected and snap frozen as high cell density 'noodles' by extruding cell paste from a 10ml syringe into liquid nitrogen.

Frozen cells are cold lysed by grinding in dry ice in a cryogenic tissue grinder (pictured). Dry ice was allowed to sublimate at -80° C. Disrupted cell powder was suspended in buffer + protease inhibitors, clarified, and used as the input for affinity purification in the next step.

Ulp1(3)\textsuperscript{C580S} expressed in bacteria was bound to amylose beads forming a Ulp1(3)\textsuperscript{C580S} affinity resin (U-Tag). 7.5ml of cell extract was gravity filtered through 450ul of Ulp1(3)\textsuperscript{C580S} affinity resin.

Sumo conjugates, as well as free SUMO, were liberated from the affinity resin by cleaving with catalytically active Ulp1-His6 (Invitrogen). Catalytic Ulp1 was then removed by metal affinity chromatography leaving purified SUMO and SUMO conjugates.

**Supplemental Figure 1.** Diagram of methodology used for U-Tag affinity purification.
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<th>Plasmids or construction</th>
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Table 2. Buffers used in Chapter 2.

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2% Dextrose                  |                                                                | Chapter 2; From Dr. Kamakaka, Rohinton T. Personal communication |
| Extrusion | 50mM HEPES pH 7.8  
150mM NaCl  
1mM EDTA  
14mM 2-mercaptoethanol  
5mM MgCl₂ | Halts inhibitor cocktail (Sigma) | Chapter 2; From Dr. Kamakaka, Rohinton T. Personal communication |
| Extraction | 50mM HEPES pH 7.8  
325mM NaCl  
14mM 2-mercaptoethanol  
5mM MgCl₂  
10% glycerol | Halts inhibitor cocktail (Sigma) to 1x  
4mM Benzamidine  
50μM TPCK  
5-μM TLCK  
1mM PMSF  
10mM NEM | Chapter 2; From Dr. Kamakaka, Rohinton T. Personal communication |
Table 3. Raw data from MudPIT analysis of U-Tag affinity purified proteins. Parentheses represent gene names from *Saccharomyces* Genome Database (yeastgenome.org)

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**Table 4. Selected results from MudPIT analysis of U-Tag affinity purified proteins.** Full results from the mass spectrometry analysis are in table 3.

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Table 5. Primers used in PCR-based Myc tagging of SIZ1. Red letters represent SIZ1 sequence homology.

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<td>5'-ATGGAAAACGCAAGATTATGGAAAGAAATACAACAGTGGTCGATCCCGGGTTAATTA-3'</td>
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References:


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SUMOplot™ Analysis Program (http://www.abgent.com/sumoplot.html)