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An Exploration of Stress, Signaling, and Ubiquitin-like Modifiers

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A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

2014

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Program Authorized to Offer Degree:

Molecular and Cellular Biology

University of Washington

Abstract

An Exploration of Stress, Signaling, and Ubiquitin-like Modifiers

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Cells face a barrage of internal and external stresses that could inflict severe damage on the molecules essential for life. To cope with environmental challenges, cells have evolved complex stress responses. Posttranslational protein modifications, including ubiquitin-like modifiers, are a major means by through which cells respond to these changing environmental conditions. This thesis explores how the small ubiquitin-like modifier (SUMO) is used to modulate cellular outcomes via transcription, with a particular focus on cellular contexts that require rapid transcriptional changes, such as stress. I present evidence from studies in *Saccharomyces cerevisiae* that SUMO can be inhibitory to transcription both through direct signaling and non-signaling modes of function. First, I present my findings that SUMO can act as a signal to inhibit the transcription activation potential of a predicted SUMO-targeted ubiquitin ligase that also functions in transcriptional silencing. I discuss potential models for how SUMO might direct switching between transcriptional silencing and activation functions of this protein. Second, discuss independent work demonstrating that SUMO, a highly soluble protein, can promote transcriptional repression in a non-signaling manner by solubilizing transcriptional regulators at risk of misfolding during stress. I show that during hyperosmotic stress, two members of a transcriptional corepressor complex are rapidly and transiently SUMO-modified, and loss of this sumoylation drives accumulation of these proteins into inclusions and correlates with widespread loss of transcriptional repression. Finally, I discuss the broader implications of the signaling and non-signaling functions of SUMO in transcriptional modulation, with a focus on SUMO's potential to drive the adaptive potential of cells in changing environmental contexts.

ACKNOWLEDGEMENTS

So many people have helped me to become the person and the scientist that I am today. Your guidance, support, and encouragement over the years have made all of the difference to me. I can't thank you enough. There are a few people I'd especially like to acknowledge here.

Rich Gardner, my advisor and mentor. Your support, along with excellent training, has been so important to me. Thanks for giving me the freedom to try many different projects, for keeping your door open, and for advising me through the research ups and downs. Thanks for the time that you give to your students—it makes a difference.

All of the current and past members of the Gardner lab. You have made it fun! It has been a great experience to work with creative, smart, and hard-working people. Thank you to the high school students, undergraduates, and volunteers I've had the opportunity to work with—you have taught me so much about how to be a better mentor.

Terry Horton. You inspired me in the first place. You taught me that science is an active process, not just what we read in textbooks. Thank you for encouraging me to give research a try, for being such a wonderful mentor, and for advising me through some major challenges.

Jen Lachowiec. You know what you want and you go for it. Keep on going—I am cheering for you! Your tenacity and energy inspire me. I am so glad to call you my friend, and I look forward to many duck dinners, wherever the future takes us.

Mom and Dad. Thank you for your steadfast support. It means the world to me to have parents like you. Thanks for encouraging me play outside, to build things, to be a student *and* an athlete, to go after my dreams and goals. These things have shaped me in ways I am still discovering. Most importantly, thank you for setting the example of the person I want to be.

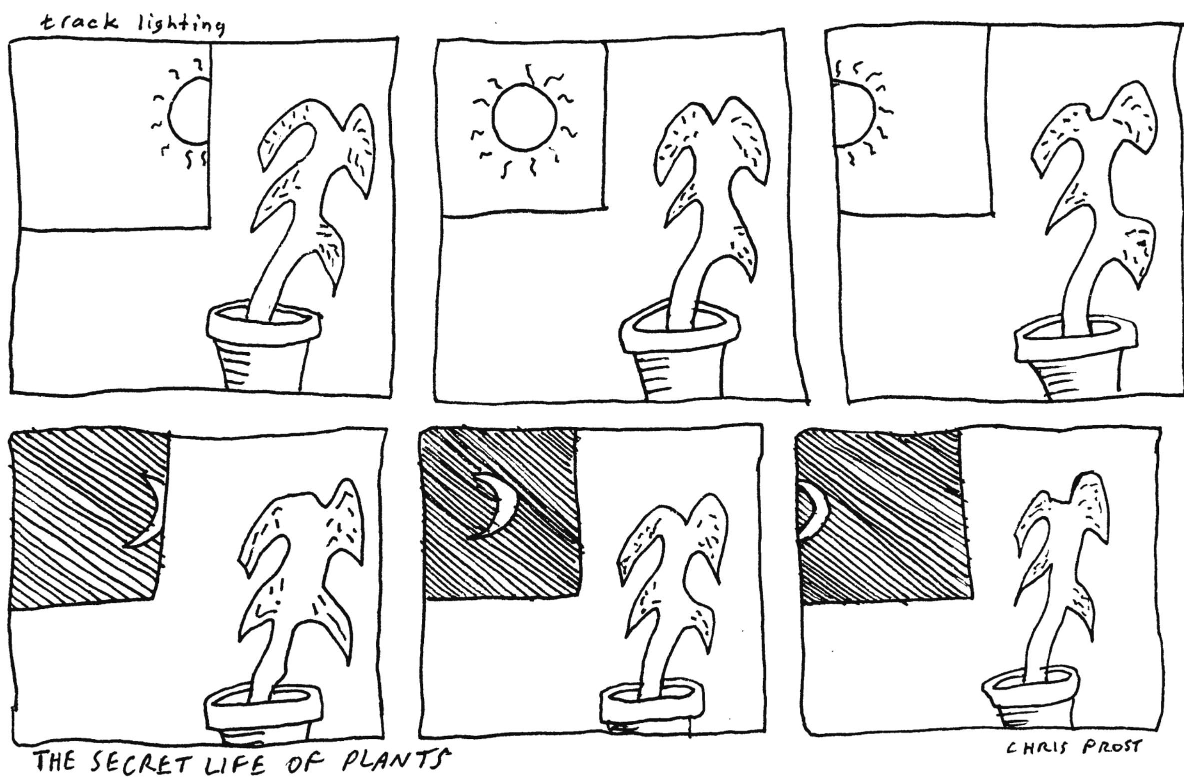
Steph. You inspire me! You have such a warm soul and creative mind. You are so lovely, in so many ways. I can't quite put how I feel into words. Thank you for being you.

Marilyn, Greg, and Audrey. You made me feel like family before I was family. Thank you for always being so welcoming and supportive.

Chris. I can't fully articulate how much your love and encouragement mean to me. You are the most wonderful husband, partner, and friend. My greatest inspiration is seeing how you take in the world and give back to it. You make life infinitely more amazing, and I am so excited for more adventures with you.

DEDICATION

Some people see things as they are on the surface, and others see something more.
This is dedicated to Chris, who always sees something more.



Credit: Chris Prost

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LIST OF ABBREVIATIONS

Atg5	Autophagy protein 5
Atg8	Autophagy protein 8
Atg12	Autophagy protein 12
GAD	Gal4 activation domain
GBD	Gal4 DNA-binding domain
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8
ISG15	Interferon-stimulated gene 15
PrD	Prion domain
PTM	Post-translational modification
RING	Really interesting new gene
SDD-AGE	Semi-denaturing detergent agarose gel electrophoresis
SIMs	SUMO interaction motifs, SUMO-interacting motifs
SUMO	Small ubiquitin-like modifier, small ubiquitin-related modifier
STUbL	SUMO-targeted ubiquitin ligase
TDG	Thymine DNA glycosylase
Ubl	Ubiquitin-like modifier
Uls1	Ubiquitin Ligase for SUMO conjugates 1
Urm1	Ubiquitin-related modifier 1

CHAPTER ONE:
BACKGROUND AND SIGNIFICANCE

Life is stressful. In their lifetimes, cells (and organisms) face a barrage of stressful events and environments that can inflict severe damage on the molecules essential for life—nucleic acids, proteins, and lipids. Cells have evolved complex stress responses in order to cope with environmental challenges such as physical or chemical stress, nutrient deprivation, or pathogenic attack. Stress responses must ameliorate the immediate damage caused by stress exposure and also adjust metabolic capacity, gene expression output, and other cellular systems to protect against further damage that could be incurred by prolonged exposure to stress. This dissertation explores the molecular underpinnings of how cells react and adapt to intra- and extracellular changes, focusing on the role of ubiquitin-like modifiers in signaling for these responses.

Posttranslational protein modifications (PTMs) are a major means through which cells respond to dynamic environmental conditions. These modifications add significant complexity to the cell signaling that can be achieved by a proteome. There are many types of PTMs, including small chemical modifications such as phosphorylation or acetylation, lipid and carbohydrate moieties, and modifications that are proteins themselves. Furthermore, many PTMs can be both added to and removed from target proteins, conferring even broader flexibility to PTM signaling.

Ubiquitin and ubiquitin signaling

First discovered in 1975, ubiquitin is a protein that is used as a PTM by all eukaryotes (Varshavsky, 2006). Ubiquitin is a highly conserved protein—yeast and human ubiquitin show 96% sequence similarity—and is so widely used in eukaryotic cell signaling that it plays a role in the regulation of nearly every cellular process. Ubiquitin adopts a compact, rapidly folding, and stable “beta-grasp fold” structure (Burroughs et al., 2007) in which five beta sheets appear to “grasp” a diagonal alpha helix. Ubiquitin’s C-terminal tail ends with a glycine residue, which becomes covalently attached to target proteins, also known as substrates. Ubiquitin is usually conjugated to lysine residues in substrates, but it can also be conjugated to the amino terminus of a protein (Ciechanover and Ben-Saadon, 2004) or to serine, threonine, and cysteine residues (Cadwell and Coscoy, 2005; McDowell and Philpott, 2013; Ravid and Hochstrasser, 2007; Wang et al., 2007). Ubiquitin can form chains on each of its 7 lysine residues and its N-terminal

methionine (Emmerich et al., 2011; Kirisako et al., 2006), vastly expanding the signaling possibilities that this PTM can achieve.

A multi-step enzymatic cascade is responsible for attaching ubiquitin to target proteins. First, the ubiquitin activating enzyme (also known as an E1) activates the C-terminus of ubiquitin in an ATP-dependent manner. Next, a ubiquitin conjugating enzyme (also known as an E2) coordinates the conjugation of ubiquitin to substrates by collaborating with a ubiquitin-protein ligase (also known as an E3), which confers substrate specificity. Underscoring ubiquitin's importance in cell signaling, there are 1000 predicted ubiquitin ligases encoded by the human genome (Ciechanover, 2006). Ubiquitination is reversible, and the removal of ubiquitin from substrates is catalyzed by deubiquitinating enzymes. An array of different binding domains and motifs interact with ubiquitin's signature fold (Husnjak and Dikic, 2012; Randles and Walters, 2012), enabling the proteins that contain these domains to read the information encoded by ubiquitin and its chains for cell signaling.

Ubiquitination of a substrate, especially in the form of lysine 48-linked chains, is widely known as a signal for degradation by a large multi-subunit proteolytic machine known as the proteasome (Hochstrasser, 1996; Varshavsky, 2006). This ubiquitin-proteasome system can be used for regulatory purposes, such as the regulated ubiquitination and degradation of the cyclin-dependent kinase inhibitor Sic1 to allow progression through the G1/S transition of the cell cycle (Feldman et al., 1997). Another major function of the ubiquitin-proteasome system is in protein quality control. Protein quality control machinery, including specialized ubiquitin ligases, specifically target the aberrant, misfolded, and/or toxic forms of proteins for ubiquitination and subsequent degradation by the proteasome (Fredrickson and Gardner, 2012). Ubiquitin signals for many non-proteolytic functions as well, and may control the function, localization, or protein-protein interactions of the substrate protein. Over the past several decades, ubiquitination has emerged as a key regulator of numerous cellular processes, a few of which include DNA repair, transcription, autophagy, and intracellular trafficking (Hochstrasser, 1996; Varshavsky, 2006). Perhaps not surprisingly, dysregulation of the ubiquitin-proteasome system (and other forms of ubiquitin signaling), has been implicated in numerous forms of cancer, neurodegenerative diseases, and other disorders, and many factors in ubiquitin-related signaling are being considered as therapeutic targets (Ciechanover, 2006).

A family of ubiquitin-like modifiers

The strategy of covalently attaching a modifier protein to other proteins appears to have radiated in eukaryotes, as there is a large family of ubiquitin-like modifiers (Ubls) that are used in cell signaling. Despite low sequence similarity to ubiquitin, Ubls exhibit high structural similarity to ubiquitin and are attached to substrates via their C-terminal tails (Hochstrasser, 2009; Kerscher et al., 2006; Vierstra, 2012). Many of these Ubls can also be detached and recycled. It seems that new functions for Ubls are discovered every year, and we are continuing to gain new insights into their accompanying enzymatic machinery, interaction motifs, and functional roles in the cell.

Some prominent examples of Ubls include ISG15, NEDD8, Atg12, and Atg8. ISG15 (Interferon stimulated gene 15) was the first Ubl discovered (Haas et al., 1987; Korant et al., 1984), and it appears to play a role in host responses to viral infections when induced by type 1 interferons (Morales and Lenschow, 2013). After interferon induction, many proteins become ISGylated (Giannakopoulos et al., 2005; Zhao et al., 2005), but it is still not clear what exact functional purpose ISGylation serves. NEDD8 (known as Rub1 and RUB in yeast and plants, respectively) exhibits relatively high sequence similarity to ubiquitin (60%) compared to other Ubls. NEDD8 is known to regulate the assembly, activity, and disassembly of Cullin-RING ligase complexes (Hotton and Callis, 2008), which are multi-subunit scaffolds of ubiquitination machinery. Modification by NEDD8 is therefore an excellent example of crosstalk between Ubl systems. Finally, Atg8 and Atg12 function in autophagy and share little sequence similarity with ubiquitin, but they have a beta-grasp fold structure. Atg8 is not conjugated to a protein, but instead becomes ligated to a lipid, which incorporates into a lipid bilayer. This conjugate helps form the structure that will become the autophagosome (Klionsky and Schulman, 2014; Metlagel et al., 2014). Atg8 also appears to function in coordinating various interacting proteins (via an Atg-interacting motif) at the membrane of the autophagosome (Kraft et al., 2012; Noda et al., 2010). Providing another interesting example of Ubl crosstalk, Atg12 has one main substrate, Atg5, and following ligation of Atg12 to Atg5, Atg12-Atg5 can function as an Atg8 ligase (Hanada et al., 2007).

One particularly interesting Ubl, Urm1, provides clues about an ancient origin of this family of modifiers. Urm1 shares sequence similarity with two bacterial proteins, MoaD and ThiS, which are involved in molybdenum cofactor biosynthesis and thiamine biosynthesis, respectively. The bacterial proteins do not serve as PTMs. Instead, they receive a sulfur atom at their C-termini via sulfurtransferase chemistry that echoes Ubl activation and conjugation but

involves single activating enzyme-like proteins (MoeB for MoaD and ThiF for ThiS, respectively) (Hochstrasser, 2009; Kerscher et al., 2006). Similarly, Urm1 also acts as a sulfur acceptor (through reactions catalyzed solely by the Urm1 activating enzyme Uba4) and functions in the thiolation of tRNA (Leidel et al., 2009). Urm1's function appears to be important for responses to nutrient starvation and oxidative stress (Leidel et al., 2009).

Perhaps the most thoroughly studied Ubl is the small ubiquitin-like modifier (SUMO). In most eukaryotic organisms, SUMO is essential for life and is a critical signal for many nuclear processes, including transcription, DNA repair, kinetochore function and chromosome stability (Gill, 2004; Johnson, 2004). Although SUMO shows little sequence similarity to ubiquitin (~18% in yeast), both proteins exhibit the beta-grasp fold. Similar to ubiquitination, sumoylation occurs via a multi-enzyme cascade (Johnson, 2004; Watts, 2013). Initially, a SUMO-activating enzyme activates SUMO in an ATP-dependent manner (Watts, 2013). A SUMO-conjugating enzyme then attaches SUMO to lysine residues of a target protein, either on its own or in collaboration with a SUMO ligase (Watts, 2013). Sumoylation is reversible and its removal is mediated by desumoylating enzymes (Watts, 2013). Unlike interaction with ubiquitin, which can occur via a wide array of different ubiquitin-interacting domains and motifs, SUMO interaction occurs through one primary mechanism: the SUMO-interaction motif, or SIM. SIMs are short hydrophobic sequences containing only a few residues (which are sometimes flanked by acidic residues or residues that can be phosphorylated) that interact with a groove in the SUMO structure (Kerscher, 2007).

Recently, a new form of Ubl crosstalk has emerged. SUMO-targeted ubiquitin ligases contain multiple short sequences that recognize SUMO, and they also have the enzymatic capability to attach ubiquitin to other proteins (Perry et al., 2008; Sriramachandran and Dohmen, 2014). The current model is that these enzymes use their SUMO-sensing capability to find proteins that have been tagged with SUMO, and then ligate ubiquitin to the target protein as well, resulting in dual modification of the target protein. We currently know very little about how broadly this mode of Ubl crosstalk is used in cell signaling.

SUMO: Not just for signaling?

SUMO has been found to modify many nuclear targets, including transcription factors, DNA repair machinery, and nuclear pore-associated proteins (Gill, 2004). In many cases, sumoylation of these proteins drives changes in function or activity, localization, and potential

interactions with other proteins (Gill, 2004; Johnson, 2004). An example of the precision in signaling that SUMO can confer is the sumoylation of human thymine DNA glycosylase (TDG), a base excision repair enzyme. The end product of TDG catalysis is an abasic site in DNA, and the rate-limiting step in the activity of TDG is its dissociation from this end product (Hardeland et al., 2002). Sumoylation (and interaction with an internal SIM) of TDG decreases binding of TDG to DNA and abasic sites by inducing a conformational change in TDG (Steinacher and Schar, 2005) which is thought to act as a release mechanism. TDG requires sumoylation and desumoylation for each round of its catalysis (Hardeland et al., 2002).

There are numerous other examples of how SUMO drives specific signaling outcomes for various cellular processes. Interestingly, in addition to targeting individual proteins, SUMO can modify multiple members of a complex or functional group of proteins at once. This has been shown for processes such as DNA repair and ribosome biogenesis (Jentsch and Psakhye, 2013). In the case of DNA double strand break repair, ablating sumoylation of individual proteins in this pathway does not seem to affect DNA repair outcomes, whereas blocking sumoylation of the group dramatically hinders double strain break repair (Psakhye and Jentsch, 2012). Thus it has been argued that SUMO's signaling effects can sometimes occur through "SUMO hubs" of modified groups of proteins, rather than acting at the level of individual proteins (Jentsch and Psakhye, 2013; Psakhye and Jentsch, 2012).

An intriguing and relatively new idea is that SUMO may also function in a manner that is potentially quite different from its signaling functions, using its distinctive physical properties. SUMO is known to be an extremely soluble protein. In fact, as an artificial fusion tag, it is a highly effective tool for enhancing the solubility of difficult-to-express recombinant proteins (Malakhov et al., 2004; Marblestone et al., 2006).

Evidence has emerged that SUMO may function as a solubilizing factor *in vivo*. SUMO has been found to modify a number of aggregation-prone proteins associated with neurodegenerative diseases (Krumova and Weishaupt, 2013). SUMOylation of α-synuclein, a major component of Lewy body inclusions (Goedert et al., 2013), has been shown to prevent α-synuclein fibril formation *in vitro,* and reduced SUMOylation of α-synuclein enhances its neurotoxicity *in vivo* (Krumova et al., 2011). However, the role of SUMO in protein aggregation diseases is not entirely straightforward and will likely depend upon the particular nature of the misfolded protein. For example, the modulation of huntingtin by SUMO is more complex: SUMOylation of polyQ-expanded huntingtin reduces its inclusion formation in mammalian cells but enhances neurodegeneration in flies (Steffan et al., 2004).

Interestingly, many stresses known to induce protein misfolding induce increased sumoylation of many proteins (Castro et al., 2012; Tempe et al., 2008). For example, after heat shock or oxidative stress, there is a general increase in the SUMOylation of predominantly nuclear proteins in yeast, plants, and mammalian cells (Golebiowski et al., 2009; Miller et al., 2010; Miller et al., 2013; Zhou et al., 2004). The majority of these nuclear SUMOylation targets are involved in transcription, chromatin regulation, RNA processing, and metabolism (Golebiowski et al., 2009; Miller et al., 2010; Miller et al., 2013; Zhou et al., 2004).

Perhaps some stress-induced sumoylation is directed at individual proteins or protein groups for directed signaling, so that they can function in response to a given stress, but there are also clues that SUMO may function in protein quality control during stress. It has been shown that overexpression of Hsp70 chaperones in plants results in reduced sumoylation during heat stress (Kurepa et al., 2003), suggesting that SUMO may have a chaperone-like role in maintaining protein homeostasis during stress. In addition, a subset of plant proteins targeted for stress-induced sumoylation also undergo increased ubiquitination (Miller et al., 2013). In addition, proteasome impairment in yeast has been shown to increase the global levels of high molecular weight SUMO conjugates that are also ubiquitinated (Uzunova et al., 2007). Furthermore, proteasome inhibition of mammalian cells has been found to increase protein sumoylation (Tatham et al., 2011; Uzunova et al., 2007), to an extent similar to what is observed during heat shock (Tatham et al., 2011). Interestingly, the proteasome inhibition-dependent increase in SUMOylation in mammalian cells requires active protein synthesis (Tatham et al., 2011), suggesting that sumoylation could target nascent misfolded proteins. It is important to better resolve the functions of SUMO during stress, particularly the role that SUMO might play in solubilizing its substrates rather than directing specific signaling outcomes.

This thesis explores the theme of multiple roles for SUMO in the cell, with a particular focus on SUMO's involvement in transcription. First, I describe tools that I have built to facilitate our lab's investigations of ubiquitin-like modifiers. I then present my findings of an unexpected transcriptional function of a predicted SUMO-targeted ubiquitin ligase. Finally, I discuss my work investigating transient protein sumoylation during hyperosmotic stress. I present evidence that SUMO modulates the transcriptional response to hyperosmotic stress by promoting solubility of its substrates. My conclusions and reflections explore the potential implications for SUMO-mediated transcriptional control in dynamic environments.

Acknowledgements

A portion of the text in this introduction is derived the following publication:

Gallagher, P.S., Oeser, M.L., Abraham, A.C., Kaganovich, D., and Gardner, R.G. (2014). Cellular maintenance of nuclear protein homeostasis. Cell Mol Life Sci *71*, 1865-1879.

CHAPTER TWO:

TOOLS FOR STUDYING UBIQUITIN-LIKE MODIFIERS

In addition to the research efforts that I describe in subsequent chapters, I have spent a significant portion of my graduate time developing tools to meet various experimental and technical needs for our lab's study of ubiquitin and other Ubls. This chapter discusses the development of these tools and explores their potential applications.

Transplantation of ubiquitination into a prokaryote

A major goal of the Gardner lab's research is to understand how cells handle aberrant, misfolded proteins. We are particularly interested in studying protein quality control mechanisms of the nucleus, where the major players and processes defining nuclear protein quality control are less well understood than in other cellular compartments (Fredrickson and Gardner, 2012). Rich previously identified the yeast protein San1 as protein quality control ubiquitin ligase that functions specifically in the nucleus (Gardner et al., 2005a).

While delving into the mechanism of how San1 recognizes its substrates for ubiquitination and subsequent degradation, our lab needed to answer the following question: Can San1 target misfolded/aberrant proteins on its own, or does it need the action of other factors, such as chaperones? This is an important question, considering that chaperones are known to target misfolded proteins, and there is some evidence that San1 can function collaboratively with some yeast chaperones (Guerriero et al., 2013; Heck et al., 2010; Prasad et al., 2010). We wanted to determine if a minimal ubiquitination cascade, without yeast chaperones or other factors, can be sufficient for ubiquitination of a San1 substrate.

The most direct way to test the hypothesis that San1 can target its substrates on its own is with an *in vitro* assay combining recombinant forms of ubiquitin, ubiquitin activating enzyme (Uba1), conjugating enzyme (Cdc34), ligase (San1), and substrate in the presence of ATP. However, we faced two technical challenges: 1) the San1 substrates did not express and/or purify well, likely due to misfolding issues, resulting in low yield (Eric Fredrickson, Joel Rosenbaum, and Richard Gardner, unpublished observations), and 2) full length San1 was difficult to purify, likely due to its high degree of intrinsic disorder (Rosenbaum et al., 2011), rendering it susceptible to proteolysis. With the addition of affinity tags at both the N- and C-

termini of San1 and optimization of the purification process, Eric and Joel were able to obtain enough recombinant San1 for use in *in vitro* systems such as ubiquitination assays using luciferase as the test substrate (Rosenbaum et al., 2011). However, if we were to determine if San1 is able to directly target its known yeast protein substrates, we would still need to address the issue of combining San1 with its yeast substrates in isolation from the rest of the yeast proteome.

Ubiquitination, while crucial for nearly every eukaryotic cell process, is completely absent from prokaryotic cell signaling. We used this to our advantage to address the technical challenge at hand. We designed an "*in coli*" ubiquitination system in which the components of the ubiquitination cascade, including San1, along with a yeast substrate protein, were co-expressed (Figure 2.1). This would enable us to test the minimal ubiquitination cascade in a living but non-eukaryotic system, in which San1 would be present while misfolding events of substrate proteins were actively occurring. Furthermore, we reasoned, the lack of a prokaryotic ubiquitin-proteasome system should result in the stable accumulation of ubiquitinated proteins at much higher levels than we might observe in a eukaryote.

I cloned San1's ubiquitination enzymatic pathway (ubiquitin, *UBA1, CDC34, SAN1*, and various test substrates) into three Duet vectors (Novagen), each of which contains two identical sets of T7*lac* promoters and ribosome binding sites. I co-transformed and selected for these plasmids in *T7* Express cells (New England Biolabs), and I induced co-expression of all components of the pathway by addition of IPTG to the growth medium. This strategy was similar to that used in a previous study that reconstituted the ubiquitination cascade to screen for substrates of a mammalian ubiquitin ligase (Su et al., 2006), and to earlier work reconstituting the conjugation pathway for the small ubiquitin-like modifier SUMO (Mencia and de Lorenzo, 2004; Uchimura et al., 2004a; Uchimura et al., 2004b).

I assayed for ubiquitination of San1 substrates, testing the hypothesis that San1 is capable of ubiquitinating its known yeast quality control substrates on its own. When I performed western blots, blotting for epitope-tagged substrates in total cell lysates, I was not able to observe higher molecular weight species of any substrate. Considering the high degree of San1-dependent ubiquitination that occurs in *E. coli*, we suspected that San1 targets many endogenous *E. coli* proteins. San1 recognizes its targets by sensing exposed hydrophobicity (Fredrickson et al., 2011). Normally San1 is localized to the nucleus, separate from active protein production, but in *E. coli*, this would not be the case. San1 would potentially be surrounded by an abundance of exposed hydrophobicity in nascent, not-yet-folded proteins.

To facilitate our ability to observe only the ubiquitination that occurs on our substrates of interest, I performed immunoprecipitations of the substrate and performed anti-ubiquitin western blots on the enriched substrate. I observed ubiquitination of several San1 yeast substrates (Figure 2.2), demonstrating that San1 is indeed capable of targeting substrates for ubiquitination on its own.

While performing ubiquitin blots of these cell lysates, I observed some laddering of ubiquitin signal when all components except for San1 were present, suggesting either that Cdc34 is capable of ubiquitinating itself or other proteins in the absence of a ubiquitin ligase or that charged Cdc34 can accumulate ubiquitin chains. Both of these possible scenarios have been shown to occur *in vitro* (Banerjee et al., 1993). Because this laddering still occurred when the loading buffer contained a reducing agent (Anupam Garg and Michelle Oeser, unpublished observations), we concluded that Cdc34 must be covalently modifying proteins in *E. coli* on its own. We noted that there was much more ubiquitination occurring when San1 was present versus when San1 was absent, suggesting that when San1 is present in *E. coli*, we are observing predominantly San1-dependent ubiquitination. Our observations of Cdc34-dependent ubiquitination prompted us to include a control strain in which Cdc34 is present but San1 is absent in our experiments. We only observed ubiquitin modification of San1 substrates when San1 was present (for example, in Figure 2.2).

The finding that San1 can ubiquitinate misfolded proteins its own was further supported by our *in vitro* observations that San1 preferentially ubiquitinates denatured luciferase over natively folded luciferase (Rosenbaum et al., 2011).

Potential applications of *in coli* Ubl systems

Taking advantage of the absence of ubiquitin and its conjugation machinery in prokaryotes, we were able to successfully build an *in vivo* ubiquitination system in isolation from its eukaryotic host environment. In our lab, the *in coli* ubiquitination system was a valuable tool that allowed us to bypass the technical challenges of *in vitro* experiments and successfully determine that the protein quality control ubiquitin ligase San1 can target its substrates on its own.

The *in coli* ubiquitination approach has potentially broad utility for the study of ubiquitin and other ubiquitin-like modifiers. Other groups have also shown that the enzymatic cascades for protein modification with ubiquitin, SUMO, and other Ubls can be reconstituted in prokaryotic

systems (Cerda-Maira et al., 2011; Ichimura et al., 2004; Keren-Kaplan et al., 2011; Mencia and de Lorenzo, 2004; O'Brien and DeLisa, 2012; Su et al., 2006; Uchimura et al., 2004a; Uchimura et al., 2004b). These studies serve as effective proofs of principle that functional Ubl cascades can be reconstituted in prokaryotes, but future work should go further to harness the full potential of *in coli* Ubl systems.

In our lab, a particularly daunting challenge is the search for a functional homolog of San1 in humans. If we could identify a human ubiquitin ligase that targets aberrant nuclear proteins, we would gain valuable insight into how nuclear protein quality control functions in humans. Furthermore, we might better understand what goes wrong during the development of the numerous protein aggregation diseases that involve misfolding and aggregation of nuclear proteins, such as Alzheimer's and Huntington's diseases.

Using sequence-based searches, we have not been able to identify any human homologs of San1. We think this is because San1 contains a large amount of intrinsic disorder; intrinsically disordered protein families have been found to exhibit high rates of divergence in sequence over evolutionary time (Marsh and Teichmann, 2014; Xue et al., 2013). To circumvent this challenge, we will need to develop functional assays to search for San1 homologs.

With its short generation time and abundance of genetic tools, *E. coli* is an excellent organism for high-throughput screening. The currently low-throughput *in coli* Ubl systems could easily be converted into high-throughput screening systems to tackle challenges like the search for a San1 homolog. Any step of the enzymatic cascade (activation, conjugation, ligation) could be converted into an event that determines the survival of the host cell. This would enable survival-based screening assays to investigate any step of the pathway. For example, one could build a split-protein system in which the attachment of ubiquitin (fused to one half of the dihydrofolate reductase or DHFR enzyme) to a substrate (fused to the other half of DHFR) enables survival in the presence of trimethoprim selection due to reconstitution of functional DHFR. This system could screen a library of potential human ligases for a given San1 substrate (or other quality control substrate) and identify a set of candidate San1 homologs. A bacterial two-hybrid approach, in which the split protein is a transcription factor driving transcription of a selectable marker, could also be effective for screening for ubiquitination events (or similarly, conjugation of other Ubls). Non-survival-based approaches could also serve as valuable screening tools, such as split fluorescent protein systems in which complementation events are evaluated with flow cytometry techniques. Importantly, these and many other protein complementation approaches have been widely used in *E. coli* for various screening purposes

(Michnick et al., 2010, 2011; Remy et al., 2007), and several can take advantage of commercially available libraries of fusion proteins.

In addition to its high-throughput potential, another key aspect of *in coli* Ubl systems is that the Ubl does not encode information that is relevant to *E. coli*. In the case of ubiquitin, ubiquitination of a substrate will not encode for degradation by the prokaryotic proteasome; instead, levels of ubiquitinated proteins will stably accumulate with time. *In coli* Ubl systems could be used to purify large amounts of Ubl-modified substrates (or activating or conjugating enzymes charged with the Ubl) for structural or functional studies.

Construction of a yeast strain expressing endogenously tagged ubiquitin

The ability to enrich for ubiquitin and other Ubls is important for both discovery-based proteomics approaches and specific biochemical inquiries about the modification of individual proteins. While some reagents to enrich for native ubiquitin (Hjerpe et al., 2009) or proteolytic remnants of ubiquitin modification (Xu et al., 2010) are commercially available, they remain relatively costly. In addition, there are no reagents available for the enrichment of many other Ubls. To achieve inexpensive enrichment of Ubls of interest, many investigators take advantage of the fact that many Ubls can be tagged at their N-termini with epitope or affinity tags and remain functional (Ellison and Hochstrasser, 1991; Li et al., 2006). The tagged Ubl can be expressed in addition to, or place of, the endogenous Ubl in yeast or mammalian cells.

Many studies of ubiquitin biology in yeast have made use of a strain in which all four ubiquitin loci have been disrupted and are covered by a plasmid encoding ubiquitin (Finley et al., 1994) (Figure 2.3, top). This strain, while very useful, has drawbacks that are important to consider. First, ubiquitin is not expressed from its endogenous genomic locus, nor is it under the control of the endogenous promoter (expression is under the control of a copper-inducible promoter, although copper induction is not required for sufficient expression of ubiquitin (Finley et al., 1994)). This is potentially problematic because *UBI4* in particular is highly regulated: this gene is strongly induced during, and is required for survival of, cellular stress (Finley et al., 1987; Seufert and Jentsch, 1990). Ubiquitin under a non-native promoter will not be similarly in tune with environmental fluctuations. A second drawback of this yeast strain is that few auxotrophic markers remain available for manipulation because of the number of genetic steps that were required to disrupt each of the native ubiquitin loci. This means that the genetic manipulations that are possible with the strain are limited.

We decided to address these issues by building our own yeast strain in which all endogenous loci of the yeast genome encode ubiquitin N-terminally tagged with an 8His (8xhistidine) metal affinity tag. We chose the 8His tag because it can be enriched by metal affinity in both native and denaturing conditions. Performing enrichments under strong denaturing conditions greatly enhances our ability to determine that proteins are truly covalently modified by ubiquitin and not simply associating with ubiquitin noncovalently. We chose the 8His tag in particular, rather than shorter poly-histidine tags, because we reasoned that the additional histidines could enhance binding to the metal affinity column even in the presence of strong denaturants.

Adding the 8His tag to all ubiquitin loci would require a number of genetic manipulations. The four genes that encode ubiquitin in the yeast (*UBI1/RPL40A*, *UB12/RPL40B*, *UBI3/RPS31*, and *UBI4*) are all expressed as protein fusions: *UBI1, UBI2,* and *UBI3* encode ubiquitin fused to ribosomal proteins, while *UBI4* is a polyubiquitin precursor consisting of five tandem repeats of the ubiquitin sequence (Ozkaynak et al., 1987). These fusions are proteolytically processed by to yield free, mature ubiquitin. To build our strain, we would need to add a tag sequence 5' to ubiquitin in *UBI1, UBI2,* and *UBI3* and a tag sequence 5' to each of the five repeats of the ubiquitin sequence in *UBI4* (and avoid disrupting any sequence required for processing) (Figure 2.3, bottom).

To construct this strain while maintaining the availability of as many auxotrophic markers as possible, we opted for a strategy in which an N-terminally 8His-tagged version of the ubiquitin sequence was integrated at *UBI1, UBI2,* and *UBI3* loci along with a *URA3* selection marker, resulting in one tagged and one untagged copy. Then the *URA3* marker was selected against by growing cells on 5-FOA, driving recombination and loss of the untagged copy of the ubiquitin sequence at that locus. For *UBI4*, the entire endogenous locus was knocked out with *URA3*. Then a version of *UBI4,* which encoded all five 8His-tagged ubiquitin repeats, was transformed into the strain and targeted to the *ubi4*Δ*::URA3* locus with some 5' and 3' UTR sequence of *UBI4.* Replacement of the *URA3* marker with the 8His-tagged *UBI4* gene was selected for with 5-FOA.

The final version of the strain had the same auxotrophic and drug selection markers available as the original parent strain; only the *UBI* loci (now tagged) were different from the parent. We confirmed tagging of each of the *UBI* loci by amplifying the tagged regions of ubiquitin off of genomic DNA from this strain. Our tags sequences included new restriction sites,

so we digested each PCR product, looking for a molecular weight shift due to digestion at the given site (Figure 2.4).

We next tested how the tagged strain grew under various stress conditions: normal media at high temperature (37ºC), media plus canavanine (a toxic arginine analog that can cause protein misfolding), and media plus high ethanol (10% ethanol). We found that multiple versions of strains expressing 8His-tagged ubiquitin grew more slowly than an untagged strain at 37ºC and on media containing 1ug/ml canavanine (Figure 2.5). We wondered whether this growth deficit was due to our choice of tag (8His versus another affinity tag) or due to tagging of the endogenous *UBI* genes, so we assayed the growth of strains expressing untagged, 6His-myc-tagged, or 8His-tagged ubiquitin over the disrupted *UBI* genes (Finley et al., 1994). We found that with this genetic background, the strain expressing 8His-tagged ubiquitin grew more poorly at 37ºC and on canavanine than the other two strains, though not as poorly as our endogenous 8His-tagged ubiquitin strains (Figure 2.5). These data suggest that while 8His-ubiquitin does seem to disrupt cells' ability to grow under protein misfolding conditions, endogenous tagging of ubiquitin with an 8His tag further reduces growth under these stresses. Growth on high ethanol, on the other hand, did not appear to strongly hamper growth of strains expressing endogenously tagged ubiquitin, which grew nearly as well as their wild type parent strain. In this case, a strain expressing 8His-tagged ubiquitin from all endogenous loci except for *UBI4*, and in which *UBI4* had been deleted, also grew well (while for other stresses it grew poorly) (Figure 2.5) suggesting growth deficits in strains expressing 8His-tagged ubiquitin may be due to issues relating to *UBI4*.

UBI4 is the ubiquitin gene that is required for growth during stress (Finley et al., 1987). To further verify that *UBI4* function is deficient in these strains, we could replace wild type *UBI4* either on a plasmid or by integration into the genome in our current strain and determine if *UBI4* replacement rescues growth under stresses like high temperature or canavanine. Then we will need to determine the exact source of the issue with *UBI4*, which will help us build a better strain in the future. It is possible that adding tag sequences to *UBI4* could affect *UBI4* expression at the transcript and/or protein levels. We will need to determine if there are differences in *UBI4* mRNA levels and in steady state levels of total ubiquitin during stress. Another explanation for slow growth, if untagged and tagged *UBI4* expression levels are equal, is inefficient processing of the tagged ubiquitin repeats of Ubi4 by deubiquitinases. We could assay this by adding additional sequence for an epitope tag to the N-terminus of the first ubiquitin repeat of *UBI4*, looking for the unprocessed and cleaved forms of otherwise wild type

or 8His-tagged Ubi4 over a time course of stres. Alternatively, we could assay processing of recombinant wild type or 8His-tagged Ubi4 by a deubiquitinase *in vitro*. However, these analyses should be coupled with testing of other tags for genomic tagging of *UBI4* and the other *UBI* loci, such as the 6His-myc tag, which seems to function well (and, in fact, grows better than its parent strain) during stress in the *UBI*-null background strain (Figure 2.5).

Because we did observe normal growth when cells were grown on complete media, we reasoned that our current version of the strain could still be useful for growth under normal growth conditions. Our lab successfully used a version of the strain to identify ubiquitinated forms of the RNA polymerase I subunit Rpa190, a substrate of the deubiquitinating enzyme Ubp10 (Richardson et al., 2012). Ben Reed has observed the same ubiquitination patterns when enriching for untagged, wild type ubiquitin with tandem ubiquitin-binding entity reagents (unpublished observations).

Construction of a yeast strain expressing endogenously tagged SUMO

The yeast genome contains one gene that encodes SUMO: *SMT3*. Several previous studies have made use yeast strains that express epitope or affinity tagged *SMT3* under an inducible promoter on a plasmid (Denison et al., 2005; Hannich et al., 2005; Johnson and Blobel, 1999). These investigations successfully identified numerous sumoylated proteins. However, because I was interested in the dynamics of sumoylation during stress (see Chapters 4 and 5), I wanted to make a strain with *SMT3* tagged at its genomic locus, maintaining endogenous control of its expression.

As with ubiquitin, I wanted to add an N-terminal poly-histidine tag to Smt3 so that I could identify true SUMO-modified proteins by enriching for SUMO under strong denaturing conditions. I also needed to add an epitope tag for western blotting, because I had found out that several commercial antibodies against yeast SUMO (Smt3) are much more effective at identifying free SUMO than SUMO conjugated to substrates. In addition, although a few excellent homemade anti-Smt3 antibodies have been made by different labs (Kolesar et al., 2012; Montpetit et al., 2006), these antibodies are in limited supply.

I chose to generate a strain that expressed 6His-FLAG-Smt3. This tagged form of Smt3 (under the control of the *GAL1* promoter on a high-copy plasmid) was previously used to identify sumoylated proteins (Johnson and Blobel, 1999). I made a cassette that combined the tagged *SMT3* sequence with the *HIS3MX6* selection marker, plus some additional *SMT3* 3’ UTR

sequence to target the and replace the endogenous *SMT3* gene by homologous recombination. After integrating the cassette, I verified the strain by colony PCR and western blotting. Importantly, the tagged SUMO appears functional in yeast. I found that strains expressing tagged and untagged Smt3 exhibit the same sumoylation dynamics under normal growth conditions and in stress (Figure 2.6). There appears to be more free (unconjugated) SUMO in the tagged than in the untagged strain (Figure 2.6). This could indicate that one or more of the steps in the enzymatic cascade for conjugation of SUMO to substrates (activation, conjugation, and ligation) is less efficient when SUMO is tagged. Alternatively, there could be a defect in processing of SUMO to its mature form by Ulp1. Despite the difference in levels of unconjugated SUMO in tagged versus untagged strains, both strains grow equally well in both normal and stress conditions (Figure 2.7). Because *SMT3* is an essential gene (Giaever et al., 2002), any major disruption of function should result in an altered growth phenotype. We concluded that the tagged SUMO appears functional under the conditions we would use in our experiments.

We successfully used the strain in proteomics and targeted biochemical experiments to identify proteins that are sumoylated during stress and to assay their requirements for sumoylation (see Chapters 4 and 5).

Acknowledgements

Anupam Garg made the observation that Cdc34 is capable of ubiquitinating proteins in *E. coli* in the absence of a ubiquitin ligase. Construction of the 8His-tagged ubiquitin strain was done in collaboration with two other members of the Gardner lab, Sara Simonson and Ben Reed.

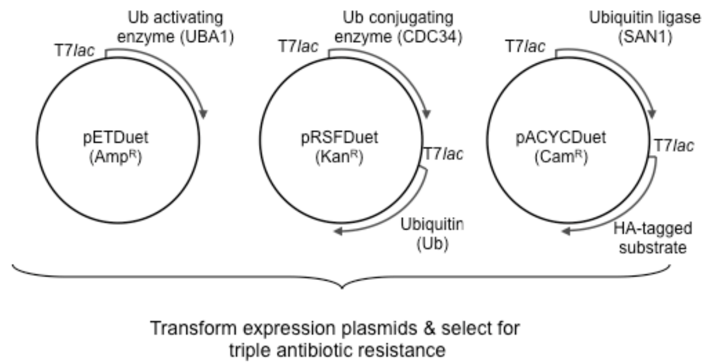
The plasmids and constructs used to make the *6His-FLAG-SMT3* strain were generated from a plasmid generously shared by Erica Johnson.

Figure 2.2 is derived from the following publication:

Rosenbaum J.C.*, Fredrickson E.K.*, Oeser M.L., Garrett-Engele C.M., Locke M.N., Richardson L.A., Nelson Z.W., Hetrick E.D., Milac T.I., Gottschling D.E., Gardner R.G. (2011) Disorder targets misorder in nuclear quality control degradation: a disordered ubiquitin ligase directly recognizes its misfolded substrates. *Molecular Cell* 41:93-106.

Figures 2.6 and 2.7 are derived from the following manuscript:

Oeser M.L. and Gardner R.G. Sumoylation of a prionogenic transcription corepressor prevents its inclusion formation during hyperosmotic stress. *Manuscript submitted for review*.



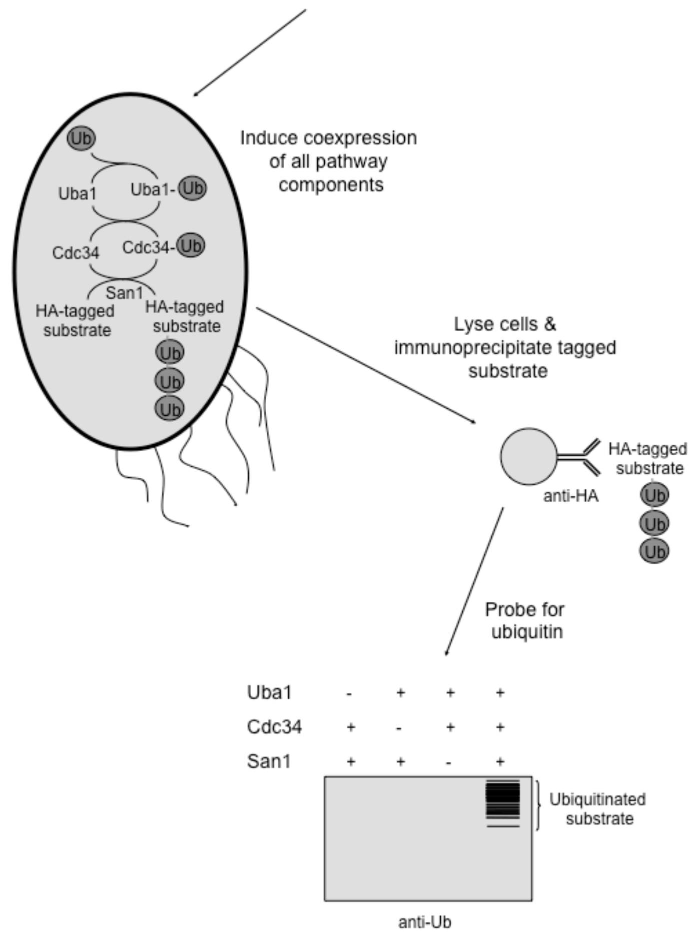


Figure 2.1. Schematic representation of an "*in coli*" ubiquitination system. Essential components of the ubiquitin cascade (*UBA1, CDC34, SAN1*, ubiquitin, and the test substrate) were cloned into Duet vectors (Novagen) and co-transformed into *T7* Express cells (New England Biolabs). Expression of all components was induced by addition of 800uM IPTG. Cells were lysed, the HA-tagged substrate was immunoprecipitated, and western blotting with anti-ubiquitin antibodies was performed to detect ubiquitinated forms of the substrate.

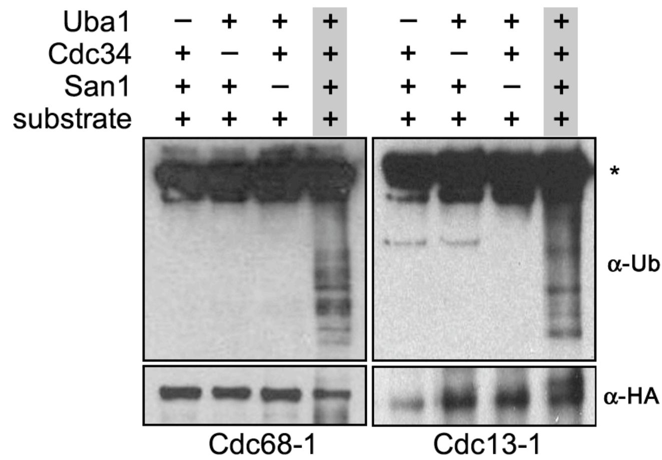
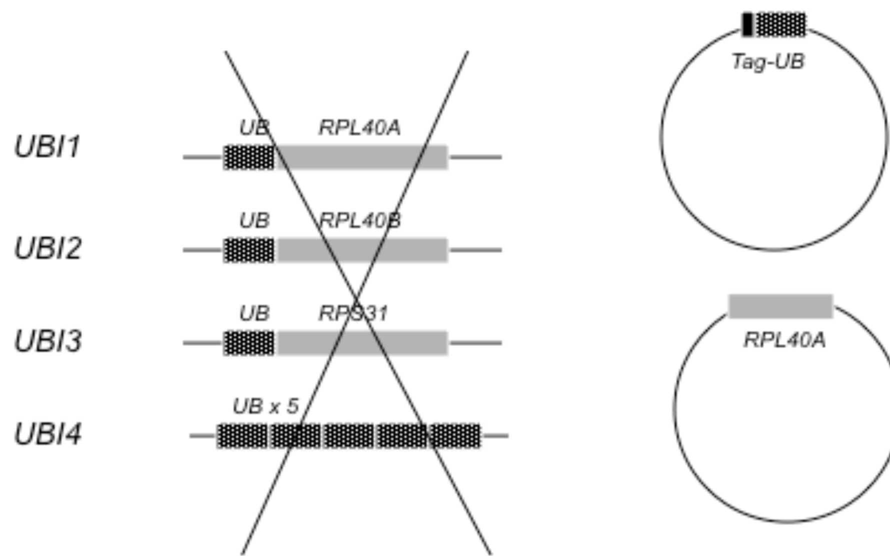


Figure 2.2. The *in coli* ubiquitination assay demonstrates that San1 can ubiquitinate substrates directly. 2xHA-tagged substrates (Cdc68-1 and Cdc13-1) were immunoprepcipated from *E. coli* cells expressing the San1 ubiquitination cascade and analyzed by western blot. Blots were probed with anti-ubiquitin antibodies to assess substrate ubiquitination and anti-HA antibodies to assess substrate immunoprecipitation. Annotations above the lanes indicate components expressed. The antibody band is marked by an asterisk.



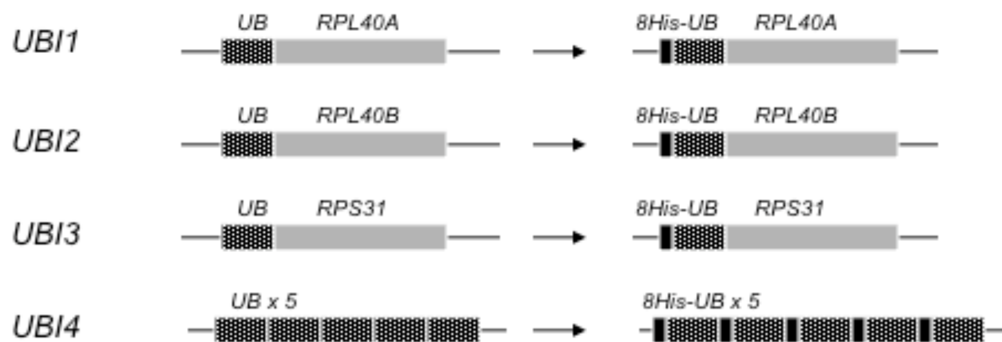


Figure 2.3. Schematic representation of tagged ubiquitin strains. Yeast contain four genes that express ubiquitin, either as fusions to ribosomal proteins or as tandem fusions. Top: Previous studies have used a strain in which all *UBI* loci were deleted and were covered by high-copy plasmid expressing tagged ubiquitin and the tail of *UBI1* (Finley et al., 1994). The most commonly used strain is one in which the high-copy ubiquitin is 6His-myc-tagged and is shown in the growth assays in Figure 2.5. Bottom: We aimed to make a strain in which all four *UBI* loci were tagged in the genome and no auxotrophic changes had been introduced.

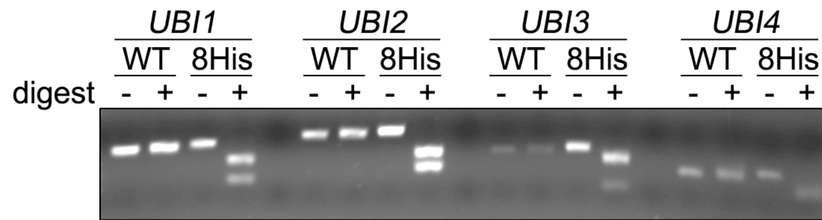


Figure 2.4. Verification of 8His tagging of all *UBI* loci in yeast. Each of the 8His tags we added to the *UBI* loci included unique restriction sites. To verify tagging at each locus, we PCR amplified around the 5' end of each *UBI* gene using oligos that would generate a product for both wild type and tagged versions of the locus. We then digested the PCR products with the appropriate restriction enzymes to verify tagging at each *UBI* locus. This verified tagged strain is shown in the bottom row of the growth assays in Figure 2.5.

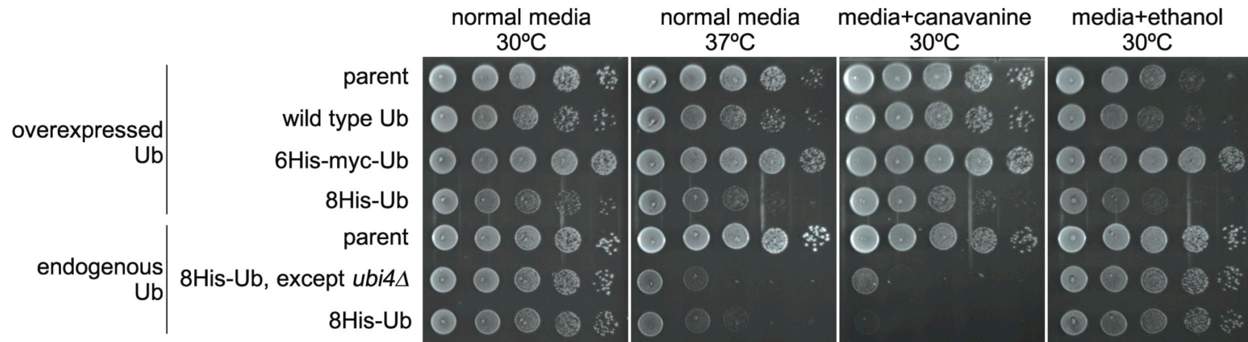


Figure 2.5. Assessing the growth of strains expressing tagged ubiquitin. Ten-fold serial dilutions of cells were spotted on normal media (yeast complete), media plus canavanine (yeast complete + 1ug/ml canavanine), or media plus ethanol (yeast complete +10% ethanol) and incubated at 30ºC for 3 days, or were spotted onto yeast complete media and were incubated at 37ºC for 3 days. The strain in the topmost row is the parent for the subsequent three strains in which all *UBI* loci have been deleted and are covered by a high-copy plasmid overexpressing ubiquitin. The strain in the fifth row from the top is the parent strain for the subsequent two strains, in which endogenous *UBI* loci are tagged.

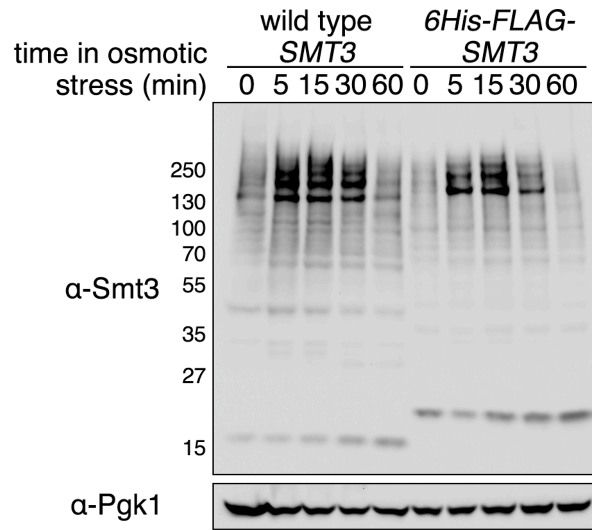


Figure 2.6. Total sumoylation in wild type and *6His-FLAG-SMT3* strains. Comparison of global sumoylation changes in wild type *SMT3* and *6His-FLAG-SMT3* cells during a time course of hyperosmotic stress (1.2M sorbitol). Cell lysates were prepared and proteins were separated by SDS-PAGE. Changes in sumoylation patterns were analyzed by western analysis using an anti-Smt3 antibody.

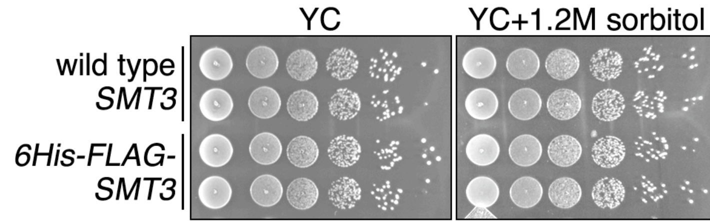


Figure 2.7. Assessing the growth of strains expressing tagged SUMO. Addition of the *6His-FLAG* sequence to endogenous *SMT3* does not alter growth under non-stressed and hyperosmotic stress conditions. Wild type *SMT3* and *6His-FLAG-SMT3* cells were 10-fold serially diluted onto YC plates with and without 1.2M sorbitol and incubated at 30˚C for 3 days.

CHAPTER THREE:

TRANSCRIPTIONAL SILENCING AND ACTIVATING FUNCTIONS OF A PREDICTED SUMO-TARGETED UBIQUITIN LIGASE

SUMO-targeted ubiquitin ligases: a special class of ubiquitin ligase

Until recently, it was thought that SUMO and ubiquitin act independently or in opposition to one another (e.g. by competing for the same lysine on substrates (Desterro et al., 1998; Hoege et al., 2002)). A new class of ubiquitin ligases has been found to mediate crosstalk between ubiquitin and SUMO. Researchers have identified SUMO-targeted ubiquitin ligases (STUbLs), which are able to preferentially target sumoylated proteins via short hydrophobic SUMO interaction motifs (SIMs) (Geoffroy and Hay, 2009; Sriramachandran and Dohmen, 2014). There is also some evidence that STUbLs may ubiquitinate proteins in a SUMO-independent manner, possibly by recognizing SUMO-like domains in the substrate protein (Xie et al., 2010). Upon deletion of the yeast STUbL genes, there is an accumulation of high molecular weight sumoylated proteins in a proteasome-dependent manner (Uzunova et al., 2007), suggesting that these ligases many substrates of these ubiquitin ligases are ubiquitinated for degradation by the proteasome. The discovery of STUbLs therefore establishes a new mode through which ubiquitin and SUMO interact.

There are three known STUbLs in budding yeast (Parker and Ulrich, 2012; Uzunova et al., 2007), one in fission yeast (Kosoy et al., 2007; Sun et al., 2007), one in *Drosophila* (Barry et al., 2011), and two in mammals (Erker et al., 2013; Lallemand-Breitenbach et al., 2008; Poulsen et al., 2013; Tatham et al., 2008). In addition, six potential STUbLs have been identified in *Arabidopsis* (Elrouby et al., 2013). All known STUbLs contain multiple N-terminal SUMO interaction motifs and a C-terminal RING domain (Perry et al., 2008), a domain that confers ubiquitin ligase activity (Deshaies and Joazeiro, 2009). Thus far, STUbLs appear to be involved in nuclear processes (Prudden et al., 2007), which is consistent with SUMO's known role in regulating many nuclear functions. The yeast heterodimer STUbL Slx5-Slx8 has been shown to be involved in responses to DNA damage (Cook et al., 2009; Ii et al., 2007; Nagai et al., 2008), turnover of transcription factors (Wang and Prelich, 2009; Xie et al., 2010), and regulation of the nuclear pool of the SUMO ligase Siz1 via ubiquitination and degradation (Westerbeck et al., 2014). The extensively studied mammalian STUbL RNF4, which dimerizes and is activated as a ubiquitin ligase in the presence of SUMO chains (Rojas-Fernandez et al., 2014), has been

shown to function in double-stranded DNA break repair (Galanty et al., 2012; Groocock et al., 2014; Vyas et al., 2013; Yin et al., 2012). In addition, RNF4 ubiquitinates the sumoylated form of PML and disrupts PML nuclear bodies in response to arsenic treatment. Interestingly, RNF4 appears to be a functional homolog of the Slx5-Slx8, as it can suppress slow growth phenotypes associated with mutations in cells lacking the *SLX5* or *SLX8* genes (Uzunova et al., 2007).

Unique roles for a predicted yeast STUbL

In this study, we focused on a less characterized STUbL, the yeast predicted ubiquitin ligase Uls1 (Ubiquitin Ligase for SUMO conjugates 1), to learn more about how its ability to interact with SUMO contributes to its function. In addition to four N-terminal SIMs and a C-terminal RING domain, Uls1 also contains predicted helicase domains similar to those found in members of the Swi2/Snf2 family of chromatin remodeling enzymes (Figure 3.1). These additional domains are not found in any other STUbL, suggesting the potential for unique functions of Uls1.

So far, it is known that when *ULS1* is deleted along with *RAD54* and *RDH54*, two genes with Swi2/Snf2 homology important for homologous recombination, the damage repair protein Rad51 accumulates inappropriately (at non-double strand break sites) along DNA (Shah et al., 2010). It has also been shown that Uls1 can facilitate the clearing of Rad51 filaments when both the *RAD54* and *RDH54* genes have been deleted (Chi et al., 2011), further suggesting that Uls1 might have overlapping function with Rad54 and Rdh54. However, no connection between Uls1 STUbL activity and DNA repair has been established.

Uls1 also appears to play a role in telomere biology. Deletion of *ULS1* results in a loss of inhibition of non-homologous end joining of telomeres and increased telomere-telomere fusions (Lescasse et al., 2013). Loss of Uls1 also results in increased levels of sumoylated Rap1, an important regulator of transcription, chromatin silencing, and telomere function (Lescasse et al., 2013). This suggests that sumoylated Rap1 may be a substrate for ubiquitination by Uls1 (and subsequent degradation), but no direct evidence of Uls1 ubiquitinating Rap1 has been shown.

Interestingly, a fragment of Uls1 was identified many years ago as a strong disruptor of chromatin silencing (Zhang and Buchman, 1997). In yeast, silent chromatin is a chromatin state characterized by regional gene repression, decreased accessibility to restriction endonucleases and other DNA-modifying enzymes, and persistence through cell divisions, equivalent to heterochromatin in other eukaryotes (Moazed, 2001). Yeast silent chromatin is established and

maintained at three genomic loci: the telomeres, silent mating type loci and the repetitive rDNA array. Silencing primarily involves the action of the Sir (silent information regulator) proteins, the repressor-activator protein Rap1, and at telomeres, the Ku proteins (Rusche et al., 2003).

It is interesting that a predicted SUMO-targeted ubiquitin ligase is implicated in silencing regulation, because both ubiquitin and SUMO are important regulators of chromatin silencing. Ubiquitin is known to be an important signal for distinguishing silent and active chromatin, as ubiquitinated histone H2B is associated with methylation of histone H3 lysine 4 and lysine 79, marks associated with active chromatin (Noma and Grewal, 2002; Roguev et al., 2001; van Leeuwen et al., 2002). Deubiquitination of H2B is important for silencing, as deletion of the deubiquitinating enzyme Ubp10 that targets H2B results in loss of telomere silencing (Gardner et al., 2005b). SUMO is also important in silent chromatin: deletion of the nonessential SUMO gene in fission yeast results in loss of silencing at the silent mating type loci (Shin et al., 2005). Furthermore, SUMO has been found by chromatin immunoprecipitation to be present in relatively high levels at telomeres, and these levels decrease with greater distance from telomeres (Nathan et al., 2006).

It was previously found that an open reading frame encoding the N-terminus of Uls1 disrupts chromatin silencing in yeast when overexpressed and interacts by two-hybrid with Sir4 (silent information regulator 4) (Zhang and Buchman, 1997), a protein required for chromatin silencing (Moazed, 2001). It is not clear, however, if endogenous Uls1 is a negative regulator of silencing. It has been shown that fragments of Uls1 can restore silencing to mutated silent mating type loci via tethering to the nuclear periphery (Andrulis et al., 2004), suggesting that Uls1 may contribute to, rather than disrupt, chromatin silencing in yeast.

Much remains to be discovered about the cellular roles of Uls1, the loss of which has been shown to result in accumulation of sumoylated proteins (Uzunova et al., 2007) in a manner that is distinct from the loss of another yeast STUbL, Slx5-Slx8 (Tan et al., 2013). Because no substrates for ubiquitination or actual ubiquitin ligase activity of Uls1 have yet been confirmed, we do not actually know if Uls1 is a true STUbL. Our current understanding of this protein's function in the cell remains limited. Here we present evidence that Uls1 has additional roles in chromatin and transcription that strongly depend on its interaction with SUMO but are independent of STUbL function.

Uls1 contributes to chromatin silencing at multiple endogenous silent loci in yeast

Uls1 was previously shown to interact with SUMO (Hannich et al., 2005; Uzunova et al., 2007), and its SIMs were identified by sequence searching (Uzunova et al., 2007). We first wanted to confirm that each predicted SIM of Uls1 interacts with SUMO. I performed yeast two-hybrid experiments in which Uls1 was fused to the Gal4 activation domain (GAD) and SUMO lacking its C-terminal glycines (so that it cannot be conjugated to other proteins) was fused to the Gal4 binding domain (GBD). Wild type Uls1 interacted with SUMO, but a mutant lacking all four predicted SIMs did not (Figure 3.2), indicating that there are no additional sequences in Uls1 that interact with SUMO. Furthermore, mutant versions of Uls1 containing only one of each SIM also showed interaction with SUMO (Figure 3.2), demonstrating that each predicted SIM does promote interaction with SUMO.

Uls1 was previously reported to be a disruptor of chromatin silencing in yeast (Zhang and Buchman, 1997). In that study, the disrupting fragment of Uls1 was overexpressed and required a wild type copy of *ULS1* in the background for loss of silencing. To better understand Uls1's role in chromatin silencing, we deleted *ULS1* in strains containing silencing reporters at telomere VII-L, at the *HML* silent mating type locus, and at the rDNA repeats. Deletion of *ULS1* resulted in a slight loss of silencing at both the telomere and *HML*, suggesting that Uls1 normally contributes to, rather than disrupts, silencing at these loci (Figure 3.3). Loss of *ULS1* did not affect silencing at the rDNA repeats (Figure 3.3).

We were interested in the role of Uls1 in transcription globally. We performed RNA sequencing on total RNA from *ULS1* and *uls1*Δ cells and found that loss of *ULS1* resulted in altered transcription by at least 1.5-fold in 23 genes (Table 3.1). The majority (21 out of 23) of these loci showed increased expression. Eight of the genes whose transcript levels were altered (seven increased, one decreased) are located within 10kb of a chromosome end (Table 3.1), further suggesting that *ULS1* normally contributes to chromatin silencing at subtelomeric regions.

The disruption of silencing observed by Zhang & Buchman depends on the overexpression of Uls1's N-terminus, which contains four predicted SIMs, and requires a wild type copy of *ULS1*. Using a strain in which a *URA3* reporter gene has been inserted near *TEL VII-L*, we found that overexpression of the N-terminus (residues 1 to 895), but not the full-length version, of Uls1 results in a loss of silencing of the reporter gene when a wild type copy of *ULS1* is present in the background (Figure 3.4).

We wondered if overexpression of the SIM-containing Uls1 fragment drives a gain of transcription activation function for the background wild type copy of Uls1, perhaps by titrating away a SUMO-dependent signal that regulates Uls1. Deletion of all four predicted SIMs abolishes Uls1 interaction with SUMO, suggesting that there are no additional SUMO-interacting motifs present in Uls1. When we removed all four of Uls1's SIMs from the N-terminal fragment of Uls1, we found that its overexpression leads to a far weaker disruption of silencing (Figure 3.5). This suggests a connection between the SUMO-sensing ability of Uls1 and a potential transcription activation function of this protein. We therefore designed a system to more directly assess if Uls1 is capable of transcription activation.

Mutant Uls1 that cannot interact with SUMO is a strong transcriptional activator

To test if mutant Uls1 that is not able to interact with SUMO can act as a transcriptional activator, we fused Uls1 to the GBD and tested its activation of a *GAL1*-driven reporter gene in the absence of a GAD. We found that wild type Uls1 fused to GBD (GBD-Uls1) does not activate transcription, but GBD-fused mutant Uls1 lacking all four SIMs (GBD-Uls1$^{\Delta SIMs}$) is a strong transcriptional activator (Figure 3.6), suggesting that Uls1's ability to interact with SUMO inhibits transcription activation activity of Uls1. To examine the role of Uls1's individual SUMO interaction motifs in inhibiting transcriptional activation, we made all possible combinations of SIM deletions in GBD-Uls1. We observed weak transcriptional activation when certain combinations of two or three SIMs were deleted, but only when all four SIMs are deleted does GBD-Uls1 mediate strong transcriptional activation (Figure 3.6). These data suggest that Uls1's SIMs act in combination and that partial SUMO-interacting capacity of Uls1 is at least partially sufficient to suppress its transcription activation function. Therefore, GBD-Uls1 is a strong activator only when it is completely unable to interact with SUMO.

Interestingly, transcriptional activation by GBD-Uls1$^{\Delta SIMs}$ is maintained when the C-terminal half of the Uls1 protein is absent (Figure 3.7), indicating that the activating function of GBD-Uls1$^{\Delta SIMs}$ is independent of catalytic activity by the RING or helicase-like domains of Uls1. These data indicate that transcription activation by GBD-Uls1$^{\Delta SIMs}$ is mediated by protein-protein interactions that recruit activating factors to the *GAL1* promoter. The finding that transcriptional activation occurs when Uls1's SUMO interaction motifs are deleted suggests that SUMO may be inhibitory to such an interaction.

To better understand which regions of Uls1 contribute to its transcriptional activation phenotype, we made further C-terminal truncations of the GBD-Uls1 construct. To our surprise, we found that removal of Uls1 sequence C-terminal to residue 580 resulted in some activation by GBD-Uls1 when the SIMs were still intact, and removal of sequence C-terminal to residue 555 gave strong transcriptional activation whether or not Uls1's SIMs were intact (Figure 3.8). These data suggest that there may be additional sequence of Uls1 that inhibits an activating function of Uls1 in addition to its SIMs. Curiously, this strong activation disappeared from SIM-intact Uls1 when we further truncated Uls1, removing all sequence C-terminal to residue 530 (Figure 3.8), and we will need further refine the regions involved in and required for transcriptional activation by Uls1. Altogether, these findings are consistent with a model in which Uls1 has the potential to activate transcription when targeted to a promoter, and that this activation function is normally masked. The ability to interact with SUMO appears to be one repressive feature of Uls1. We wondered what, in addition to removal of SUMO interaction motifs, could result in activation of Uls1?

Uls1 is sumoylated

Uls1 has been identified as a sumoylated protein in a previous SUMO proteomics study (Hannich et al., 2005), so we wondered if Uls1's sumoylation, which could potentially induce intramolecular conformational changes due to interaction of Uls1's SIMs with its conjugated SUMO, might relate to its function in transcription. Loss of sumoylation of Uls1 might therefore also result in strong transcriptional activation of Uls1.

To first determine if Uls1 is indeed sumoylated, I constructed a strain in which 6His-FLAG-tagged SUMO is integrated at its genomic locus and under the control of its own promoter (described in Chapter 2). I performed metal affinity pulldowns of SUMO in highly denaturing conditions to enrich for SUMO-conjugated proteins queried the eluates for epitope-tagged Uls1. I observed a higher molecular weight Uls1 band in the eluates from the SUMO pulldown, suggesting that Uls1 is indeed sumoylated (Figure 3.9). Interestingly, this higher molecular weight form of Uls1 was absent for mutant Uls1 lacking all four SIMs (Figure 3.9), suggesting that Uls1's SIMs are required for its sumoylation. It has previously been shown that proteins containing SIMs can use these motifs to interact with SUMO-charged Ubc9, thereby facilitating their own sumoylation (Kolesar et al., 2012; Parker and Ulrich, 2012). Our data indicate the Uls1 may also use its SIMs in this manner.

Our future experiments will need to determine if Uls1's sumoylation is related to its ability to activate transcription. Ideally, we would like to identify the exact sites of sumoylation, but Uls1 is a ~1600 amino acid protein with many lysine residues. We might first learn a great deal from our truncation mutants that we have shown to alter Uls1's ability to activate transcription: which of these, if any, show altered sumoylation when we perform SUMO pulldowns and probe for Uls1? Does sumoylation (or lack of sumoylation) correlate with transcription activation by Uls1?

We are especially eager to determine if a mutant Uls1 lacking the additional repressive region, which when deleted results in transcription activation independent of the SIMs, shows altered sumoylation. This region may contain the site of Uls1 sumoylation. If transcriptional activation of Uls1 depends on its ability to sense its own conjugated SUMO, we would expect to see transcriptional activation with loss of the SIMs and with loss of the site of sumoylation. There are multiple lysines that lie in and around this region, and we could mutate them individually (or in combination, as I describe for Cyc8 in Chapter 4).

Are we observing physiological features and functions of Uls1?

Our data show that Uls1, which contributes to chromatin silencing at endogenous silent loci, is also capable of activating transcription. Our current working model is that Uls1's ability to sense SUMO, and perhaps to become sumoylated, inhibits an ability to strongly activate transcription. We predicted that in specific physiological contexts, Uls1's interaction with SUMO and/or its own sumoylation is altered in a way that releases inhibition of its transcriptional activation. However, we still do not know if the mutations we have introduced actually reflect a normal function of Uls1. Our experiments examining transcriptional activation by mutant Uls1 have used constructs that artificially tether Uls1 to the *GAL1* promoter via the fused GBD, and there are several important questions to address.

First, does Uls1 interact with endogenous promoters on its own? An alternative explanation for transcriptional activation by mutant Uls1 is that Uls1 can interact with transcriptional machinery or other chromatin factors, but when fused to a GBD now recruits these factors to a promoter. One could use chromatin immunoprecipitation followed by microarray or sequencing to determine if Uls1 resides at promoter regions. If Uls1 does localize to promoters, the next question is if Uls1 functions in modulating transcription at those loci. Initial studies could assess if mutant Uls1 lacking the SIMs (Uls1$^{\Delta SIMs}$) exhibits altered transcription and combine these data with chromatin IP studies to better evaluate direct effects

on transcription by mutant Uls1. Finally, given that the transcriptional function of Uls1 appears to be regulated, are there specific cellular contexts in which Uls1 acts to regulate transcription? This is the most difficult question to answer. It will likely require initial studies of Uls1 tethered to a specific promoter, driving expression of a gene whose readout is easy to assay in a relatively high-throughput manner (e.g. expression of a fluorescent protein to be measured by flow cytometry, or an enzyme like beta-galactosidase to be measured by a colorimetric assay). With such a system, we could test wild type Uls1's transcriptional effects under an array of different conditions to identify specific contexts (if there are any) that can drive changes in Uls1's transcriptional roles.

SUMO as an inhibitor of transcription

Our findings that Uls1 is capable of promoting silencing as well as transcriptional activation are reminiscent of another investigation in which SUMO was found to oppose transcription by altering the function of a protein that both activates and represses transcription. Sumoylation of the mammalian transcription factor Sp3 was previously found to act as a switch between activating and repressive functions (Ross et al., 2002). Sumoylation of Sp3 (or artificial SUMO fusion to Sp3) was found to turn Sp3 into a repressor, while loss of sumoylation promoted activating function of Sp3 (Ross et al., 2002). Furthermore, the sumoylation status of Sp3 directed its nuclear localization: unsumoylated Sp3 localized to puncta and to the nuclear periphery, while sumoylated Sp3 was diffuse throughout the nucleus (Ross et al., 2002).

The most interesting aspect of transcriptional activation by Uls1, which appears to be regulated by its ability to interact with SUMO through its SIMs, is that Uls1 itself is modified by SUMO. What do Uls1's SIMs interact with? Its own conjugated SUMO? Or SUMO conjugated to other chromatin-associated proteins, or even histones? It has been shown that all four of the core histones in yeast are sumoylated, and that this modification negatively affects transcription (Nathan et al., 2006). Notably, sumoylation of histones increases near telomeres (Nathan et al., 2006), and during activation of inducible genes, histone sumoylation transiently decreases (Nathan et al., 2006). Another interesting study found that there is an accumulation of Ubc9 at promoters of inducible genes during activation but that loss of Ubc9 (and sumoylation at promoters) results in even higher induction of expression of these genes (Rosonina et al., 2010), suggesting that SUMO is indeed a key regulator during gene activation but acts to keep transcription in check.

Is there a switch between different SUMO-interacting modes, in which Uls1's SIMs alternate between interacting with its conjugated SUMO and with other sumoylated proteins, depending on the chromatin context? If this occurs, it is possible that during switching events, Uls1 might transiently exist in its transcriptional activation mode.

Future work needs to elucidate the molecular interactors of Uls1 to better understand its actual mechanism for transcriptional activation. Thinking more broadly, it will be interesting to determine if other STUbLs (and perhaps other proteins with multiple SIMs) share this capacity for transcriptional activation. Only a few STUbLs have been confirmed so far, and many appear to function in DNA repair, suggesting that they do interact with chromatin. It will be important to determine if transcriptional control is general feature among STUbLs, or if this function is unique to Uls1.

Acknowledgements

Some of the mutagenesis and transcriptional activation assays were performed by Emily Engelhart and Janani Gopalan. Emily Engelhart first made the observation that Uls1 does not require any C-terminal enzymatic domains for its transcriptional activation (Figure 3.7). Janani Gopalan generated a series of truncations in Uls1 that revealed SIM-independent transcriptional regulatory regions of Uls1 (Figure 3.8).

Much of the data and some of text of this chapter will be included in the following manuscript in preparation:

Oeser M.L., Gopalan J., Engelhart E., Gardner R.G. Mutational analysis reveals a transcription activation domain in a SUMO-targeted ubiquitin ligase with silencing function. *In preparation*.

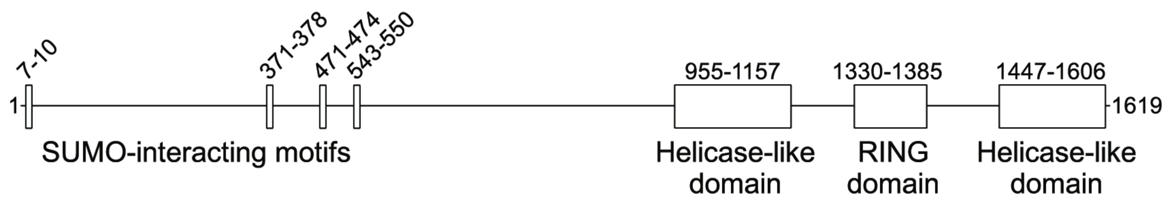


Figure 3.1. Schematic depiction of the topology of Uls1. Uls1 contains four N-terminal SUMO interaction motifs (SIMs) and a C-terminal RING domain that is predicted to confer ubiquitin ligase activity. Uls1 also contains C-terminal helicase-like domains found in some chromatin remodeling enzymes.

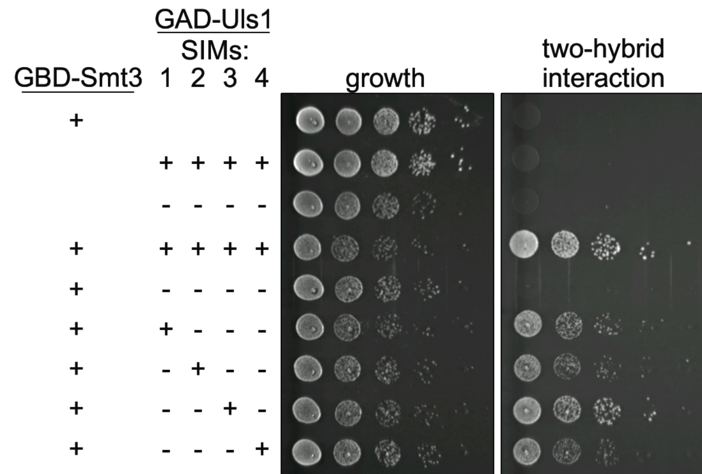


Figure 3.2. Yeast two-hybrid assay to test for interaction between Uls1 and SUMO. Different combinations of Uls1 fused to the Gal4 activating domain (GAD) were co-expressed with Gal4 DNA binding domain (GBD)-fused Smt3 (the yeast SUMO). The "+" and "-" symbols indicate presence of the construct and presence/absence of SUMO interaction motifs (SIMs) in Uls1; blank labeling indicates omission of the construct from that strain. Shown are sets of ten-fold serial dilutions of cells spotted onto media in which all strains can grow (yeast complete media lacking tryptophan and leucine; left panel) and media that tests for two-hybrid interaction via activation of a *HIS3* reporter gene under control of a *GAL1* promoter (yeast complete lacking tryptophan, leucine, and histidine; right panel). Plates were incubated at 30˚C for 3 days.

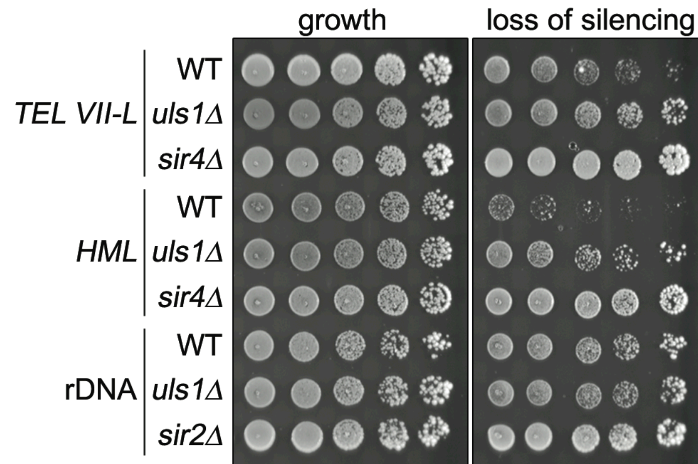


Figure 3.3. Uls1 contributes to chromatin silencing at multiple endogenous silent loci. Ten-fold serial dilutions of cells spotted onto media assessing normal growth (yeast complete media) or loss of silencing (yeast complete media lacking uracil) via expression of a *URA3* reporter at the telomere *TEL VII-L*, the silent mating type locus *HML*, or at an rDNA repeat. The *sir4Δ* deletion serves as a positive control for loss of silencing at the telomere and silent mating type locus; the *sir2Δ* strain serves as control for loss of silencing at the rDNA repeats. Plates were incubated at 30˚C for 3 days.

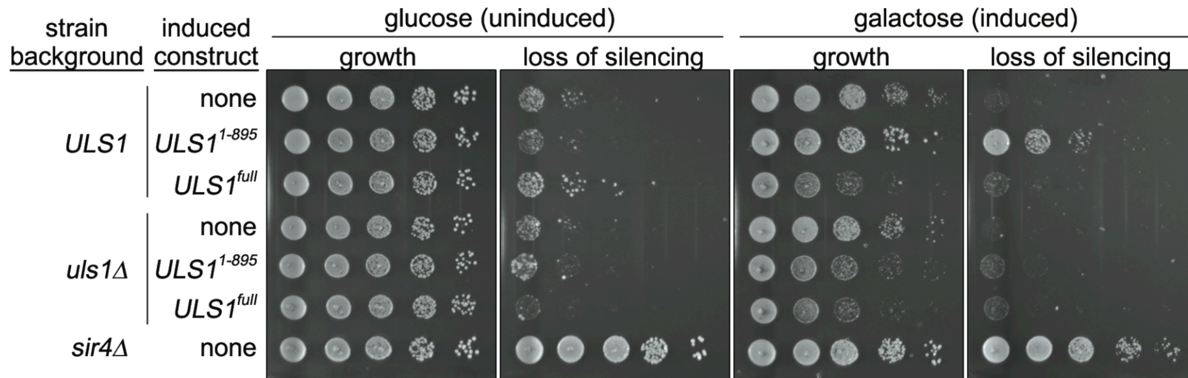


Figure 3.4. Overexpression of the N-terminus of Uls1 disrupts chromatin silencing at telomeres, if a wild type copy of *ULS1* is present. Ten-fold serial dilutions of cells spotted onto media assessing normal growth (yeast complete media) or loss of silencing (yeast complete media lacking uracil) via expression of a *URA3* reporter at the telomere *TEL VII-L.* In these strains, the endogenous copy of *ULS1* is either present or deleted, and various constructs of Uls1 are under the control of galactose-inducible promoters. In the two leftmost panels, the media contains 2% glucose and the constructs are not overexpressed; in the two rightmost panels, the media contains 3% galactose and the constructs are overexpressed. The *sir4Δ* deletion serves as a positive control for loss of telomere silencing. Plates were incubated at 30˚C for 3 days.

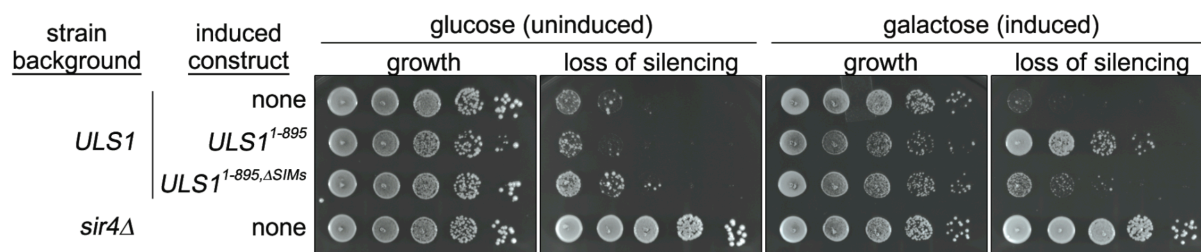


Figure 3.5. The SUMO interacting motifs of Uls1 contribute to disruption of chromatin silencing at telomeres. Ten-fold serial dilutions of cells spotted onto media assessing normal growth (yeast complete media) or loss of silencing (yeast complete media lacking uracil) via expression of a *URA3* reporter at the telomere *TEL VII-L.* In these strains, an endogenous copy of *ULS1* is present, and various constructs of Uls1 are under galactose-inducible promoters. In the two leftmost panels, the media contains 2% glucose and the constructs are not overexpressed; in the two rightmost panels, the media contains 3% galactose and the constructs are overexpressed. The *sir4Δ* deletion serves as a positive control for loss of telomere silencing. Plates were incubated at 30˚C for 3 days.

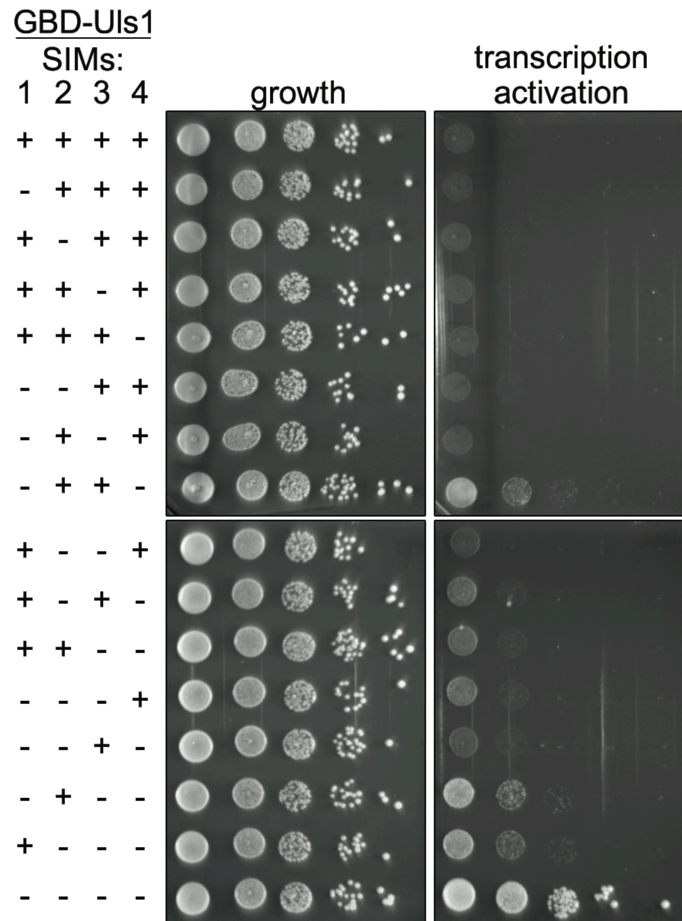


Figure 3.6. Mutant Uls1 that cannot interact with SUMO is a strong transcriptional activator. Ten-fold serial dilutions of cells were spotted to assess normal growth (yeast complete media lacking tryptophan) or transcriptional activation of a *HIS3* reporter gene under the control of a *GAL1* promoter (yeast complete media lacking tryptophan and histidine). The strains are expressing various versions of Uls1 fused to the Gal4 binding domain (GBD). The "+" and "-" symbols indicate presence/absence of each of the four SUMO interaction motifs (SIMs) in Uls1. The top and bottom sets of spots to assay "growth" and "transcription activation" were each imaged from the same plate, and those images were arranged such that all spots aligned vertically. Plates were incubated at 30˚C for 3 days.

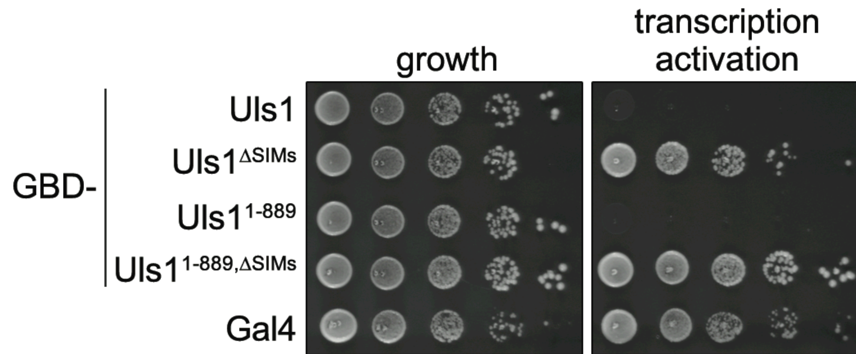


Figure 3.7. Uls1 does not require its C-terminal catalytic domains for transcriptional activation. Ten-fold serial dilutions of cells were spotted to assess normal growth (yeast complete media lacking tryptophan) or transcriptional activation of a *HIS3* reporter gene under the control of a *GAL1* promoter (yeast complete media lacking tryptophan and histidine). The strains are expressing various versions of Uls1 fused to the Gal4 binding domain (GBD). Plates were incubated at 30˚C for 3 days.

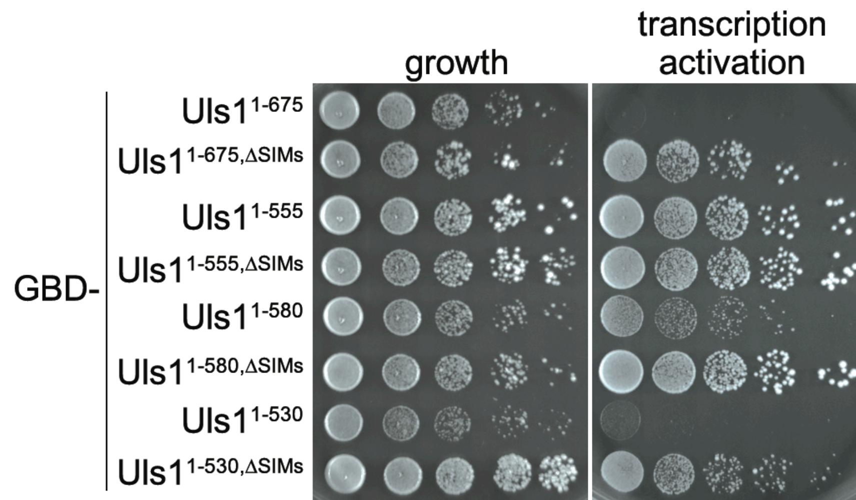


Figure 3.8. An additional region of Uls1 is inhibitory to transcription activation. Ten-fold serial dilutions of cells were spotted to assess normal growth (yeast complete media lacking tryptophan) or transcriptional activation of a *HIS3* reporter gene under the control of a *GAL1* promoter (yeast complete media lacking tryptophan and histidine). The strains are expressing various versions of Uls1 fused to the Gal4 binding domain (GBD). Plates were incubated at 30˚C for 3 days.

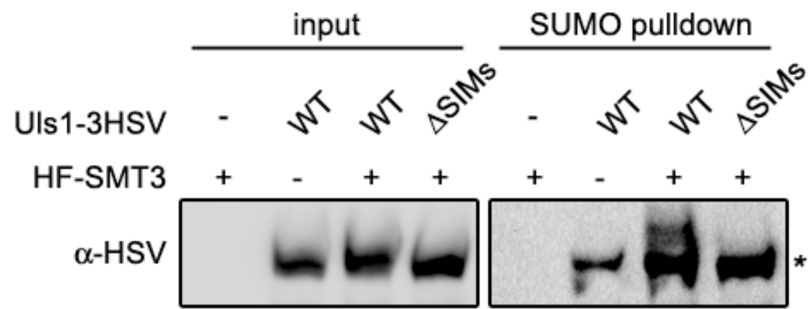


Figure 3.9. Uls1 is modified by SUMO. Cells expressing 6His-FLAG-Smt3 (HF-Smt3) and 3xHSV epitope-tagged Uls1 (Uls1-3HSV) collected at equivalent cell densities. Cell lysates (input) and purified sumoylated proteins (SUMO pulldown) were subject to western analyses using anti-HSV antibody to detect Uls1. The asterisk marks Uls1 that was nonspecifically bound to the metal affinity resin.

Systematic name	Common name	Distance from end (kb)	Log_2(fold change)	Fold change
YIR043C	**YIR043C**	**2.2**	**1.26**	**2.39**
YGL259W	**YPS5**	**8.5**	**1.07**	**2.10**
YGL263W	**COS12**	**2.8**	**0.99**	**1.99**
YOL164W-A	**YOL164W-A**	**4.1**	**0.98**	**1.98**
YMR323W	**ERR3**	**4.3**	**0.98**	**1.97**
YFR023W	PES4	70.3	0.93	1.91
YIR042C	**YIR042C**	**4.6**	**0.92**	**1.89**
YPR007C	REC8	378.7	0.82	1.77
YBL093C	ROX3	44.3	0.78	1.72
YGR117C	YGR117C	365.9	0.78	1.71
YPL198W	RPL7B	173.2	0.77	1.70
YER187W	YER187W	10.6	0.75	1.68
YKL178C	STE3	113.2	0.73	1.66
YLR054C	OSW2	248.7	0.73	1.65
YCL048W-A	YCL048W-A	41.5	0.66	1.58
YBR188C	NTC20	209.5	0.64	1.56
YPR145C-A	YPR145C-A	123.4	0.62	1.54
YBR049C	REB1	334.4	0.61	1.53
YIR039C	**YPS6**	**9.4**	**0.60**	**1.51**
YLR414C	PUN1	124.8	0.59	1.51
YHL012W	YHL012W	78.9	-0.66	0.63
YBL113C	**YBL113C**	**0.3**	**-0.94**	**0.52**

Table 3.1. Genes with altered expression in cells lacking *ULS1*. RNA-sequencing was performed to assess differences between wild type and *uls1Δ* cells. Genes with at least a 1.5-fold change in gene expression are shown here, with loci that lie within 10kb of the nearest chromosome end shown in bold.

CHAPTER FOUR:

HYPEROSMOTIC STRESS INDUCES RAPID AND TRANSIENT CHANGES IN PROTEIN SUMOYLATION

A cell's ability to sense, react, and adapt to adverse environmental conditions is critical for survival. As a consequence, cells have evolved stress responses that rapidly cope with immediate damage caused by acute exposure to stress. Stress responses also adjust metabolic capacity and gene expression output to react against the initial damage of stress and to protect against further damage that can be incurred by prolonged exposure to stress. Given the importance of stress responses to cell physiology, stress-response mechanisms are well conserved and are often altered in human disease.

Posttranslational protein modification by SUMO has emerged as a key signal in eukaryotic stress responses. Much of what we currently know about SUMO's role in stress comes from proteomic studies in yeast, plants, and mammalian cells. It has been found that following heat shock (Castoralova et al., 2012; Golebiowski et al., 2009; Kurepa et al., 2003; Miller et al., 2010; Miller et al., 2013; Miller and Vierstra, 2011; Tatham et al., 2011; Zhou et al., 2004), oxidative stress (Miller et al., 2013; Zhou et al., 2004), and ethanol stress (Miller et al., 2013; Zhou et al., 2004), dramatic increases in protein sumoylation occur, suggesting that SUMO may be an important and broadly used signal in the response to stress. The pool of proteins sumoylated during stress is enriched for proteins known to function in RNA-related processes: numerous transcription factors and chromatin modifiers, as well as proteins involved RNA metabolism, processing, and export undergo sumoylation in stress (Castoralova et al., 2012; Golebiowski et al., 2009; Kurepa et al., 2003; Miller et al., 2010; Miller et al., 2013; Miller and Vierstra, 2011; Zhou et al., 2004), linking stress-induced protein sumoylation to gene expression. However, we still don't fully understand the function that massive stress-induced sumoylation serves. Sumoylation can direct changes in protein function, localization, and interactions (Geiss-Friedlander and Melchior, 2007; Johnson, 2004). Sumoylation can also play a role in protein folding and quality control (Ahner et al., 2013; Castoralova et al., 2012; Gomes et al., 2012; Tatham et al., 2011; Wang and Prelich, 2009). Does the SUMO modification convey regulatory information for its protein targets to coordinate the proper cellular response to stress? Or does SUMO serve a more general function in protecting the proteome from stress? These outcomes are not mutually exclusive and their involvement will depend upon the specific proteins sumoylated during stress.

We were interested in gaining deeper understanding of the immediate, short-term changes in protein sumoylation that occur following stress events. We found that hyperosmotic stress triggered a uniquely rapid and transient sumoylation pattern in which both components of a transcription corepressor complex—Tup1 and Cyc8—are highly sumoylated and then desumoylated in the minutes following hyperosmotic shock. Through gene expression and cell biological analysis of SUMO-deficient mutants of Tup1 and Cyc8 during osmotic stress, we found that loss of sumoylation of Tup1 and of Cyc8 have distinct outcomes, and that loss of Cyc8 sumoylation, specifically, results in widespread increases in gene expression as well as stress-induced formation of punctate inclusions of Cyc8. We propose a model in which SUMO acts as a solubilizing agent to protect against misfolding and loss of function of Cyc8 during hyperosmotic stress in yeast.

Rapid, transient sumoylation changes occur during hyperosmotic stress

To examine changes in protein sumoylation that occur during stress, I constructed a yeast strain in which we altered the endogenous SUMO gene, *SMT3*, by adding a *6His-FLAG* sequence to the 5' end (described in Chapter 2). This construct facilitates the ability to enrich for sumoylated proteins using metal affinity chromatography and to detect sumoylated proteins by western analysis using anti-FLAG antibodies.

Using the *6His-FLAG-SMT3* strain, I investigated changes in protein sumoylation during a time course following several stresses: hyperosmotic stress (1.2M sorbitol), heat shock (42°C), and high ethanol (10% v/v). While heat shock and high ethanol resulted in a steady accumulation of sumoylated proteins over time, hyperosmotic stress caused a uniquely rapid and transient sumoylation pattern (Figure 4.1). I also observed similar sumoylation patterns (albiet with faster kinetics and reduced amplitude) when I exposed *6His-FLAG-SMT3* cells were to hyperosmotic stresses of 0.6M NaCl or 0.6M KCl (Figure 4.2). This indicates that the change in sumoylation induced by 1.2M sorbitol is a general response to hyperosmotic stress. Based on this generality, I continued to use 1.2M sorbitol as the hyperosmotic stress condition for further experiments.

Tup1 and Cyc8 are the main proteins sumoylated during hyperosmotic stress

We wanted to identify the proteins sumoylated during hyperosmotic stress. To do this, we adopted a mass spectrometry approach. The striking changes in sumoylation seen 15

minutes after addition of 1.2M sorbitol led us to opt for a label-free strategy, which is a common measure to gauge enrichment of significant changes in the absence of isotopic labeling (Bantscheff et al., 2007). Using metal affinity chromatography, I enriched for sumoylated proteins from *6His-FLAG-SMT3* cell lysates generated under denaturing condtions from cultures obtained before and after 15 minutes of hyperosmotic stress (Figure 4.3). Upon analyzing the purified sumoylated proteins by mass spectrometry, we found that two proteins—Tup1 and Cyc8—were enriched in the 15 minute samples. This result was interesting because Tup1 and Cyc8 form a transcription corepressor complex that regulates the transcription of genes involved in carbon metabolism, mating-type switching, and responses to stress (Malave and Dent, 2006).

I verified that Tup1 and Cyc8 are sumoylated during hyperosmotic stress by enriching for sumoylated proteins using metal affinity chromatography from *6His-FLAG-SMT3* cells expressing epitope-tagged versions Tup1 and Cyc8. Both proteins displayed multiple transient sumoylated species after hyperosmotic stress (Figure 4.4). I also performed these pulldowns from cells collected during heat and high ethanol stress, and it appears that the sumoylation of Tup1 and Cyc8 is specific to hyperosmotic stress; neither protein showed strong sumoylation at the time points assessed in the other stresses (Figure 4.5).

I confirmed that Tup1 and Cyc8 were the primary proteins sumoylated during hyperosmotic stress by deleting the *TUP1* or *CYC8* genes and examining total sumoylation after hyperosmotic stress. Loss of either gene abolished the sumoylation signal normally observed (Figure 4.6). This result indicated that Tup1 and Cyc8 might need to be in a complex together for their individual sumoylation. To address this possibility, I generated mutant forms of Tup1 and Cyc8 that cannot interact with each other. In Tup1, residues 1-72 mediate the interaction with Cyc8 (Tzamarias and Struhl, 1994). In Cyc8, the Tup1-interaction region comprises residues 1-175 (Tzamarias and Struhl, 1995). We expressed epitope-tagged versions of either wild type Tup1 or Tup1$^{\Delta 1\text{-}72}$ and wild type Cyc8 or Cyc8$^{\Delta 1\text{-}175}$ in a strain in which endogenous untagged Tup1 and Cyc8 were both present. This configuration allowed for Tup1-Cyc8 complexes to form normally in the cell. Using this strategy, we found that only the wild type epitope-tagged forms of Tup1 and Cyc8, which can incorporate into Tup1-Cyc8 complexes, were sumoylated after 15 minutes of hyperosmotic stress (Figure 4.7). Neither Tup$^{\Delta 1\text{-}72}$ nor Cyc8$^{\Delta 1\text{-}175}$ were sumoylated during hyperosmotic stress (Figure 4.7), demonstrating that complex formation is required for Tup1 and Cyc8 sumoylation.

Siz1 and Ulp1 are involved in dynamic Tup1 and Cyc8 sumoylation

Both the increase and reduction in the sumoylated forms of Tup1 and Cyc8 occur rapidly following osmotic stress, suggesting that both phases of the pattern are regulated and may be important for cells' response to osmotic stress. We therefore wanted to identify the enzymes that mediate the sumoylation of Tup1 and Cyc8. We did so by testing the requirement for known components of the yeast SUMO modification pathway. There are four yeast SUMO ligases: Siz1, Siz2, Cst9, and Mms21 (Watts, 2013). Siz1, Siz2, Cst9 are nonessential (Cheng et al., 2006; Johnson and Gupta, 2001; Strunnikov et al., 2001; Takahashi et al., 2001; Takahashi et al., 2003), whereas Mms21 is essential (Zhao and Blobel, 2005). There are two yeast SUMO proteases: Ulp1 and Ulp2 (Watts, 2013). Ulp1 is essential and Ulp2 is important for normal cell growth (Li and Hochstrasser, 2000; Schwienhorst et al., 2000). For the nonessential genes *CST9*, *SIZ1*, and *SIZ2*, I constructed strains with the genes deleted. For *MMS21*, *ULP1*, and *ULP2*, I constructed strains in which each of the individual proteins could be depleted by regulated degradation.

To accomplish the depletion experiments, we adopted a previously developed method whereby the degradation of a target protein can be selectively induced by adding a degron to the protein that renders it sensitive to the plant hormone auxin (Havens et al., 2012; Nishimura et al., 2009). I fused an optimized auxin-sensitive degron (generously provided by Britney Moss and Jennifer Nemhauser, Department of Biology, University of Washington) to Mms21, Ulp1, or Ulp2. I then expressed the degron-fused proteins in cells also expressing an auxin receptor that functions as part of yeast's endogenous Cullin-RING degradation machinery. In all cases, 60 minutes of treatment with auxin resulted in >90% reduction of each protein (Figure 4.8). Auxin treatment did not alter sumoylation patterns over time in control strains (Figure 4.8), indicating that auxin by itself has no effect on sumoylation.

Using the deletion and depletion strains, I examined if loss of function for SUMO pathway enzymes affected the total protein sumoylation during hyperosmotic stress. Of the SUMO ligases, only loss of Siz1 resulted in an appreciable reduction of hyperosmotic-dependent sumoylation (Figure 4.9, top), as has been recently reported (Abu Irqeba et al., 2014). Of the SUMO proteases, only loss of Ulp1 function showed sustained sumoylation during hyperosmotic stress at a time point (30 minutes) when the induced sumoylation normally began to decrease in wild type cells (Figure 4.10, top). These results were recapitulated when I focused specifically on the sumoylation of Tup1 and Cyc8 by performing SUMO pulldowns and probing for each protein by western blot (Figure 4.9, bottom and Figure 4.10, bottom).

Together, these data indicate that Siz1 is the main SUMO ligase and Ulp1 is the main SUMO protease for both Tup1 and Cyc8 during hyperosmotic stress. That the loss of Siz1 does not result in a complete loss of sumoylation of Tup1 and Cyc8 could indicate that Ubc9 alone is able to sumoylate of these proteins during hyperosmotic stress, or it could mean that additional SUMO ligases contribute to Tup1 and Cyc8 sumoylation (or can compensate in the absence of Siz1). To determine this, we will need delete or deplete other ligases in addition to Siz1. Similarly, loss of Ulp1 does not fully stabilize sumoylated Tup1 and Cyc8: at 60 minutes, there is a decrease in sumoylation of both proteins. Perhaps Ulp2 contributes to their desumoylation or can compensate in the absence of Ulp1, which we could determine by depleting both SUMO proteases prior to hyperosmotic stress. Alternatively, there may be reasons other than desumoylation for the decrease in sumoylated Tup1 and Cyc8; degradation by the proteasome is one possibility, which we could test by inhibiting the proteasome prior to hyperosmotic stress.

Identification of sumoylation sites in Tup1 and Cyc8

To be able to investigate the function of Tup1 and Cyc8 sumoylation, we needed to generate mutant versions of these proteins that cannot be sumoylated. Therefore, we needed to identify the sites of sumoylation on both Tup1 and Cyc8.

For Tup1, it was previously reported that Tup1 is sumoylated at lysine 270 during normal growth conditions (Wykoff and O'Shea, 2005). I generated an epitope-tagged Tup1 with the corresponding K270R mutation ($Tup1^{K270R}$). Mutation of lysine 270 completely abolished Tup1 sumoylation during hyperosmotic stress (Figure 4.11), indicating that lysine 270 is the sole site modified by SUMO in response to hyperosmotic stress. For Cyc8, sumoylation has been demonstrated (Panse et al., 2004; Texari et al., 2013), but the exact sites of Cyc8 sumoylation are unknown. Cyc8 contains 32 lysines, and we found that 3 lysine residues C-terminal to position 735 were predicted to be SUMO consensus sites using the SUMO prediction algorithm http://sumosp.biocuckoo.org (Xue et al., 2006). However, individual mutation of these residues did not alter Cyc8 sumoylation (our unpublished observations). To provide clues as to the location of Cyc8's sumoylation sites, I generated C-terminal truncation mutants of Cyc8. I found Cyc8 sumoylation was greatly reduced when the protein was truncated from residues 745 to 715 (Figure 4.12), indicating that most of Cyc8 sumoylation occurs within this region. I mutated the lysine residues in this region (lysines 735, 736, and 738) and a proximal lysine residue at 748 to arginine (now called $Cyc8^{4KtoR}$). After doing so, I observed a dramatic reduction in the hyperosmotic stress-induced sumoylation of Cyc8 (Figure 4.13).

My previous experiment that showed a loss of hyperosmotic stress-dependent sumoylation in strains where either *TUP1* or *CYC8* was deleted. This could indicate that most of the strong SUMO signal we observe around 15 minutes of hyperosmotic stress is due to sumoylated Tup1 and Cyc8, or it could be due secondary effects related to the loss of the Tup1-Cyc8 corepressor complex. To revisit this question, I used the Tup1 and Cyc sumoylation-deficient mutants instead of strains where genes were deleted. I found that mutation of the Tup1 and Cyc8 sumoylation sites completely abrogated the global sumoylation pattern observed during hyperosmotic stress (Figure 4.14). Based on this observation, we inferred that the main SUMO conjugates formed in wild type cells are indeed sumoylated Tup1 and Cyc8.

The transience of Tup1 and Cyc8 sumoylation bears a striking resemblance to the transient gene expression changes that occur with hyperosmotic stress (O'Rourke and Herskowitz, 2004). Because Tup1 and Cyc8 form a transcriptional corepressor complex, we wondered if sumoylation of Tup1 and/or Cyc8 contributes to gene expression in hyperosmotic stress. A key question to resolve is whether the combined sumoylation of Tup1 and Cyc8 serves a single purpose or if the individual sumoylation of Tup1 and Cyc8 each directs distinct outcomes. This is especially important to consider because our data suggest that it is the intact Tup1-Cyc8 complex that becomes sumoylated.

Acknowledgements

Britney Moss and Jennifer Nemhauser generously shared unpublished plasmids and suggestions for the auxin-dependent protein depletion strategy.

We also received plasmids and antibodies from Erica Johnson, Jeff Laney, Dennis Wykoff, and Xiaolan Zhao.

Much of the data and text of this chapter are derived from the following manuscript:

Oeser M.L. and Gardner R.G. Sumoylation of a prionogenic transcription corepressor prevents its inclusion formation during hyperosmotic stress. *Manuscript submitted for review*.

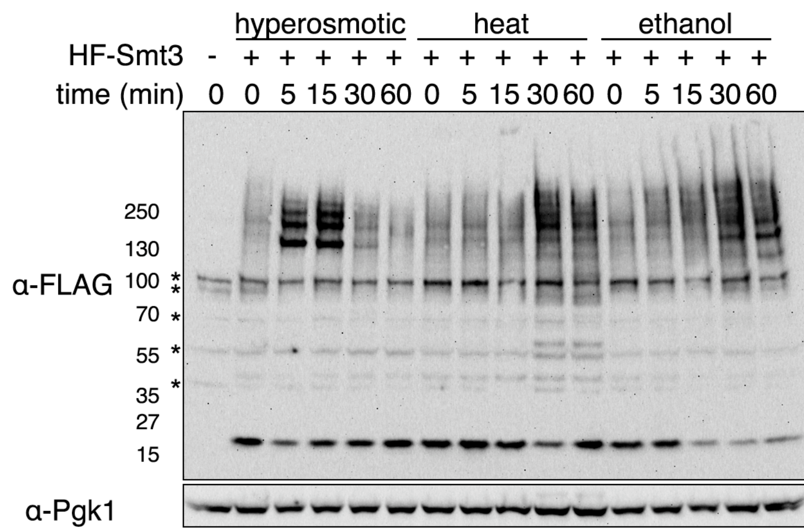


Figure 4.1. Different stresses induce rapid changes in protein sumoylation. Comparison of global sumoylation changes that occur during a time course of hyperosmotic stress (1.2M sorbitol), heat shock (42˚C), or high ethanol (10% v/v). Cells expressing 6His-FLAG-Smt3 (HF-Smt3) were subjected to each stress over a time course of 60 minutes. Changes in sumoylation patterns were examined by western analysis using an anti-FLAG antibody. Cells expressing endogenous Smt3 were used as a control for nonspecific bands, indicated by asterisks on the left. Pgk1 was detected as a loading control.

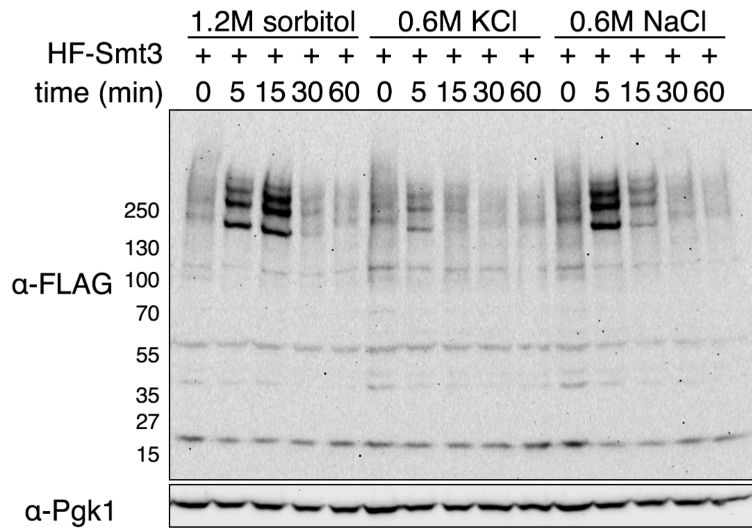


Figure 4.2. Hyperosmotic stress induced rapid, transient changes in protein sumoylation. Examination of global sumoylation changes during different hyperosmotic stresses. Changes in sumoylation patterns were examined by western analysis using an anti-FLAG antibody. Pgk1 was detected as a loading control.

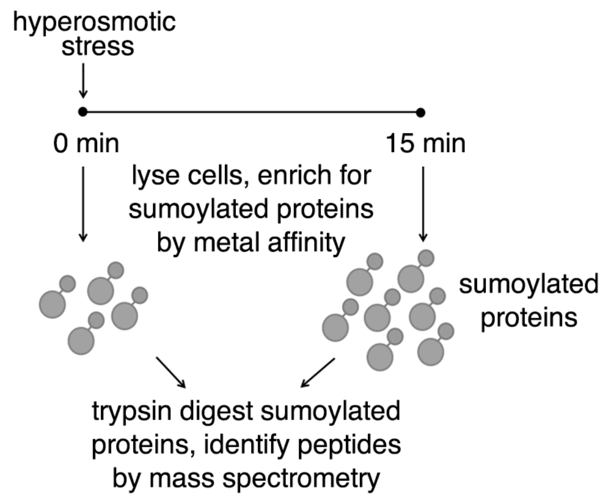


Figure 4.3. Schematic representation of mass spectrometry strategy to identify proteins sumoylated during hyperosmotic stress.

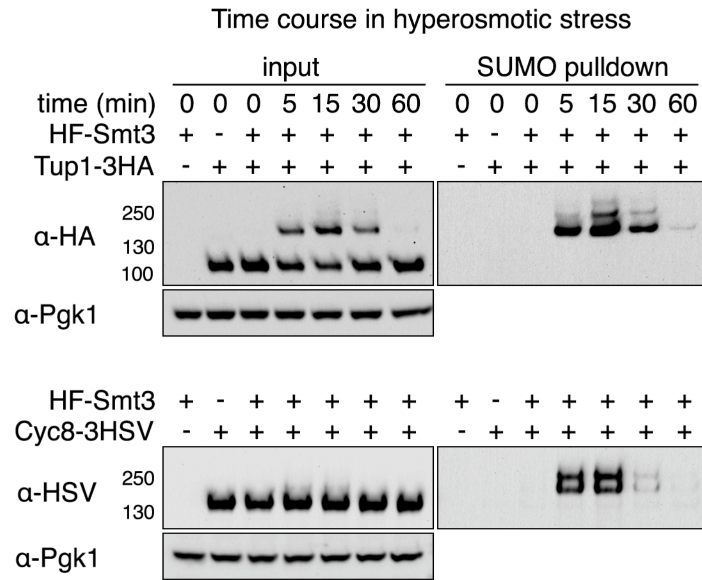


Figure 4.4. Tup1 and Cyc8 are sumoylated during hyperosmotic stress. Cells expressing 6His-FLAG-Smt3 (HF-Smt3) and either a 3xHA epitope-tagged Tup1 (Tup1-3HA) or a 3xHSV epitope-tagged Cyc8 (Cyc8-3HSV) were subject to hyperosmotic stress (1.2M sorbitol) over a 60-minute time course. Cell lysates (input) and purified sumoylated proteins (SUMO pulldown) were subject to western analyses using anti-HA, anti-HSV, or anti-Pgk1 antibodies to detect Tup1, Cyc8, or Pgk1 respectively.

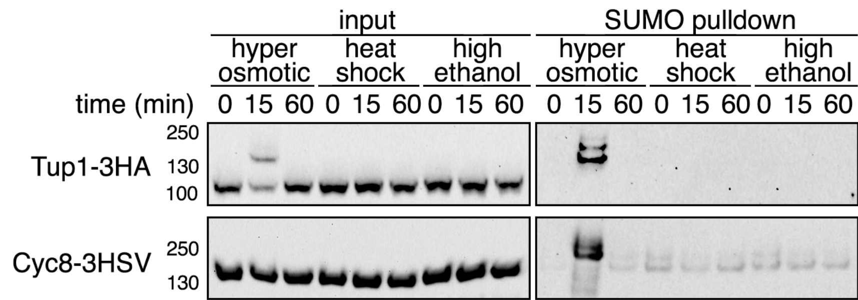


Figure 4.5. Tup1 and Cyc8 not sumoylated in heat or high ethanol stress. Cells expressing 6His-FLAG-Smt3 (HF-Smt3) and either a 3xHA epitope-tagged Tup1 (Tup1-3HA) or a 3xHSV epitope-tagged Cyc8 (Cyc8-3HSV) were subject to hyperosmotic stress (1.2M sorbitol), heat stress (42ºC), or high ethanol (10% v/v) over a 60-minute time course. Cell lysates (input) and purified sumoylated proteins (SUMO pulldown) were subject to western analyses using anti-HA and anti-HSV antibodies to detect Tup1 and Cyc8, respectively.

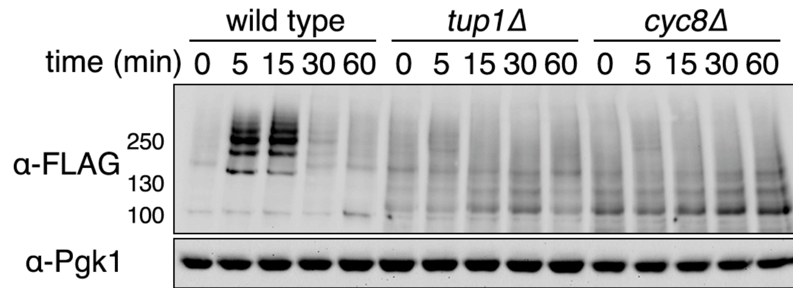


Figure 4.6. Deletion of *TUP1* or *CYC8* abolishes the rapid and transient sumoylation observed in wild type total cell lysates during hyperosmotic stress. *6His-FLAG-SMT3*-expressing wild type, *tup1*Δ, and *cyc8*Δ cells were subjected to hyperosmotic stress (1.2M sorbitol). Changes in sumoylation patterns were examined by western analysis using an anti-FLAG antibody. Pgk1 was detected as a loading control.

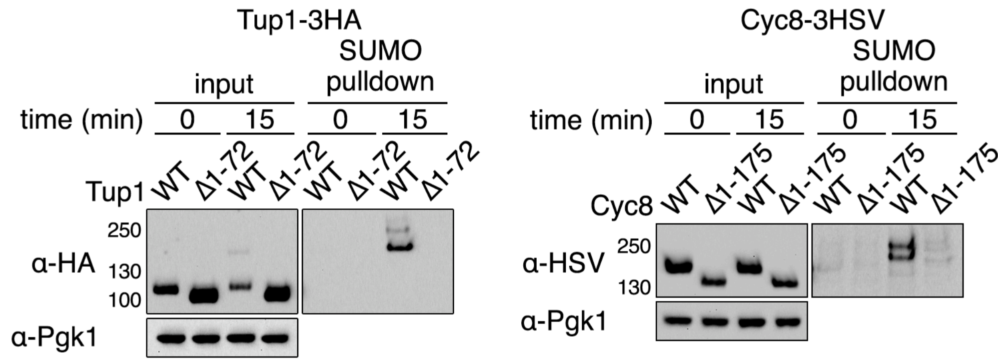


Figure 4.7. Tup1 and Cyc8 must be in the Tup1-Cyc8 complex to be sumoylated. Cells expressing 6His-FLAG-Smt3 (HF-Smt3) and full length or truncated Tup1-3HA or Cyc8-3HSV were lysed, and cell lysates (input) and purified sumoylated proteins (SUMO pulldown) were subject to western analyses using anti-HA, anti-HSV, or anti-Pgk1 antibodies to detect Tup1, Cyc8, or Pgk1 respectively.

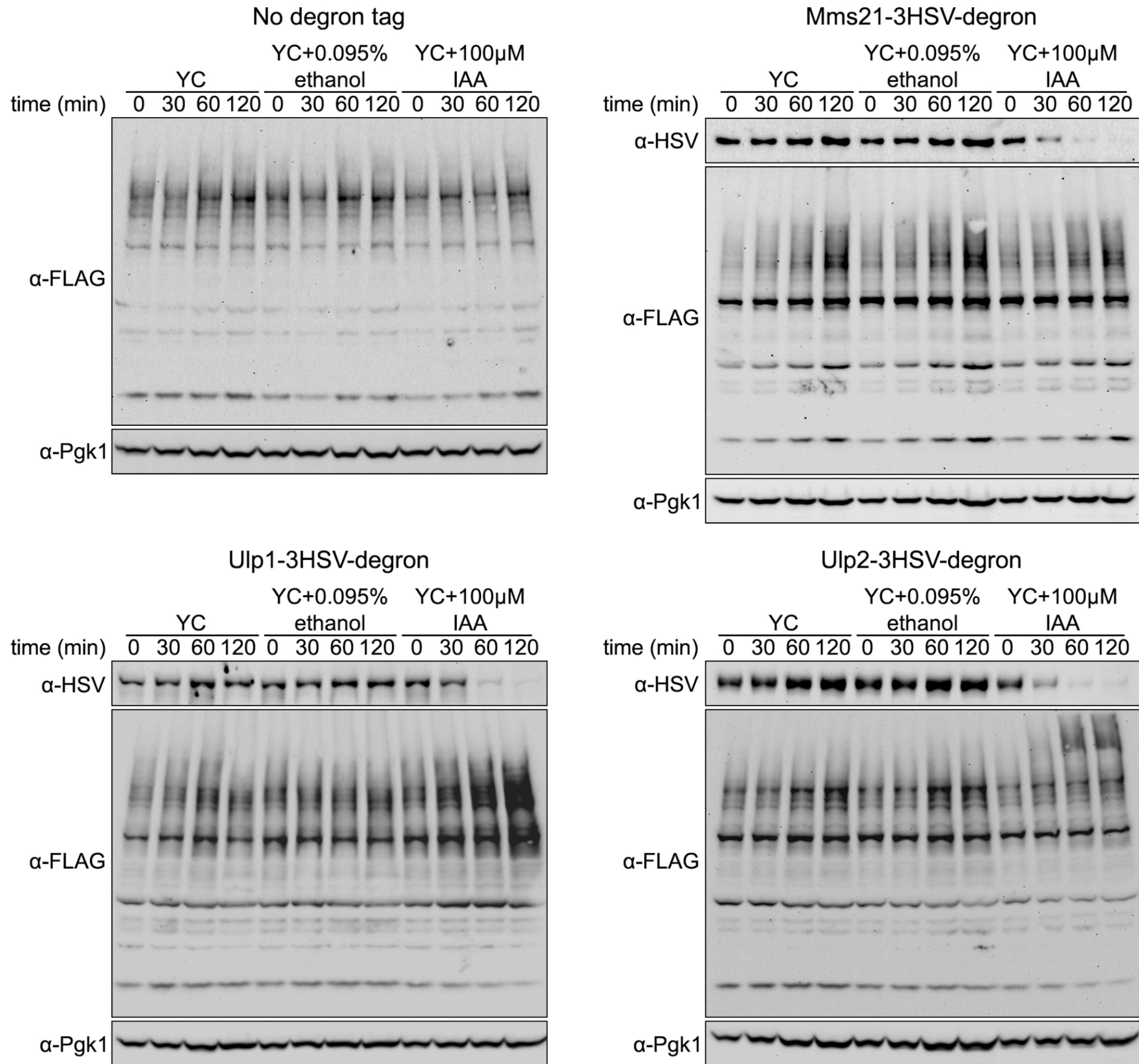


Figure 4.8. Mms21, Ulp1, and Ulp2 can be depleted with an auxin-degron depletion system. Time course of auxin-depletion experiments for cells expressing the Mms1-degron, Ulp1-degron, or Ulp2-degron constructs. Top left: *6His-FLAG-SMT3* cells were treated with no vehicle, vehicle control (0.095% ethanol), or 100μM indole-3-acetic acid (IAA, in 0.095% ethanol) for a 2-hour time course. Cell lysates were prepared and proteins separated by SDS-PAGE. Changes in sumoylation was analyzed by western analysis using anti-FLAG antibody. Anti-Pgk1 antibody detected Pgk1 as a loading control. Clockwise after top left: *6His-FLAG-SMT3* cells expressing either the Mms1-3HSV-degron, the Ulp1-3HSV-degron, or the Ulp2-3HSV-degron received the same treatment and analysis as the untagged strain, and anti-HSV antibodies detected changes in levels of the Mms21-, Ulp1-, and Ulp2-3HSV degron constructs.

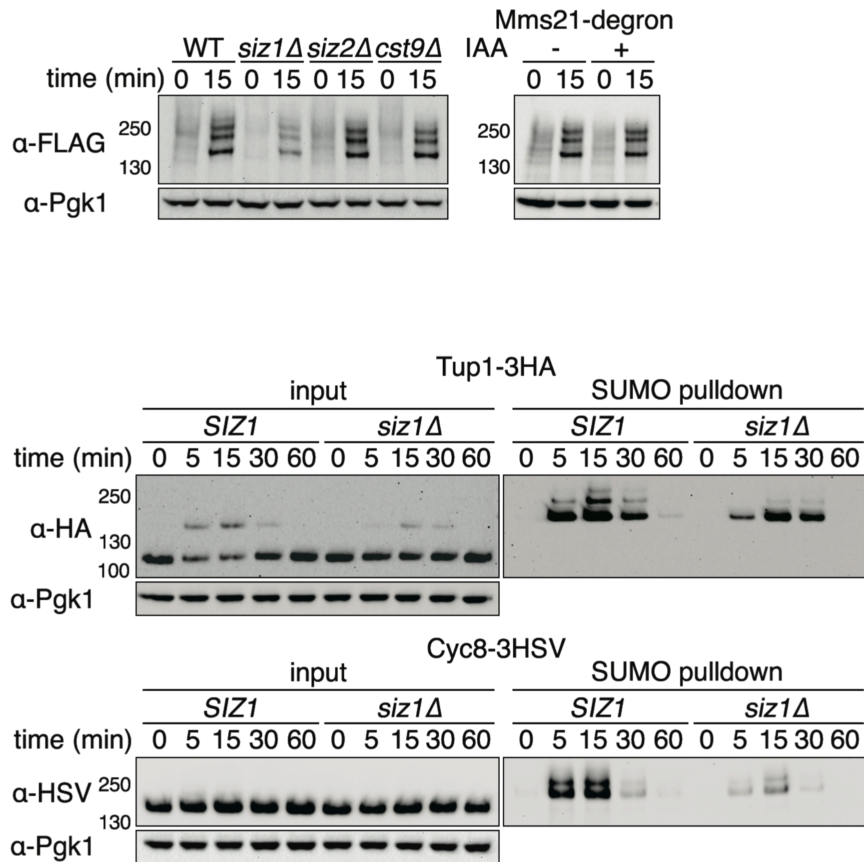


Figure 4.9. Siz1 is the main SUMO ligase acting on Tup1 and Cyc8 during hyperosmotic stress. Top: *6His-FLAG-SMT3* wild type, *siz1*Δ, *siz2*Δ, or *cst9*Δ cells (left panels), or *6His-FLAG-SMT3* Mms1-degron cells treated with (+IAA) or without (-IAA) auxin pretreatment (right panels) were subject to hyperosmotic stress (1.2M sorbitol) for 0 or 15 minutes. Changes in sumoylation patterns were examined by western analysis using an anti-FLAG antibody. Pgk1 was detected as a loading control. Bottom: *6His-FLAG-SMT3* wild type or *siz1*Δ cells expressing either Tup1-3HA or Cyc8-3HSV were subjected to hyperosmotic stress (1.2M sorbitol) over a 60-minute time course. Cell lysates (input) and purified sumoylated proteins (SUMO pulldown) were subject to western analyses using anti-HA, anti-HSV, or anti-Pgk1 antibodies to detect Tup1, Cyc8, or Pgk1, respectively.

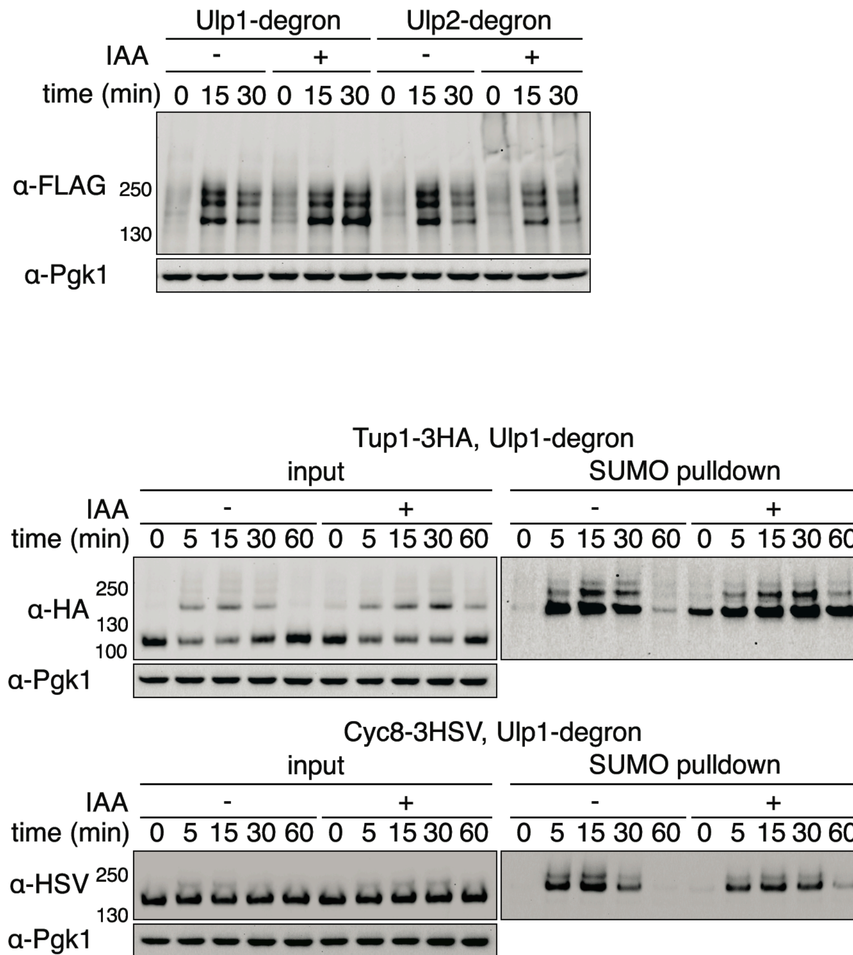


Figure 4.10. Ulp1 is the main SUMO protease acting on Tup1 and Cyc8 during hyperosmotic stress. Top: *6His-FLAG-SMT3* Ulp1-degron or Ulp2-degron cells with (+IAA) or without (-IAA) auxin pretreatment were subject to hyperosmotic stress (1.2M sorbitol) for 0, 15, or 30 minutes. Changes in sumoylation patterns were examined by western analysis using an anti-FLAG antibody. Pgk1 was detected as a loading control. Bottom: *6His-FLAG-SMT3* Ulp1-degron cells with (+IAA) or without (-IAA) auxin pretreatment were subjected to hyperosmotic stress (1.2M sorbitol) over a 60-minute time course. Cell lysates (input) and purified sumoylated proteins (SUMO pulldown) were subject to western analyses using anti-HA, anti-HSV, or anti-Pgk1 antibodies to detect Tup1, Cyc8, or Pgk1, respectively.

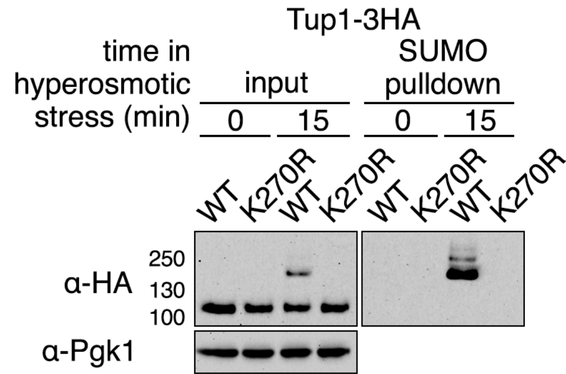


Figure 4.11. Mutation of lysine 270 abolishes Tup1 sumoylation during hyperosmotic stress. *6His-FLAG-SMT3* cells expressing either wild type Tup1-3HA or Tup1^{K270R}-3HA were examined for Tup1 sumoylation: cell lysates (input) and purified sumoylated proteins (SUMO pulldown) were subject to western analyses using anti-HA or anti-Pgk1 antibodies to detect Tup1 or Pgk1, respectively.

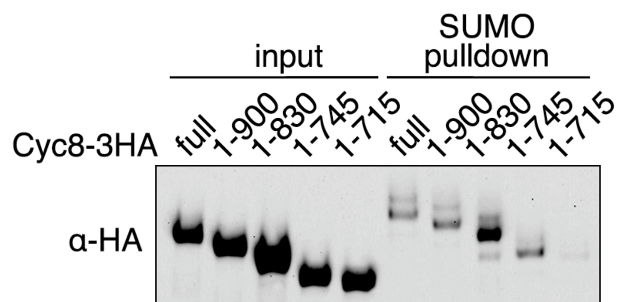


Figure 4.12. Truncations of Cyc8 to define the region of sumoylation. Cells expressing 6His-FLAG-Smt3 and the indicated Cyc8-3HSV mutant were subject to hyperosmotic stress (1.2M sorbitol) for 15 minutes. Cell lysates were generated and subject to metal affinity chromatography to purify sumoylated proteins. Cell lysates (input) and purified sumoylated proteins (SUMO pulldown) were subject to SDS-PAGE and western analyses using anti-HSV antibodies.

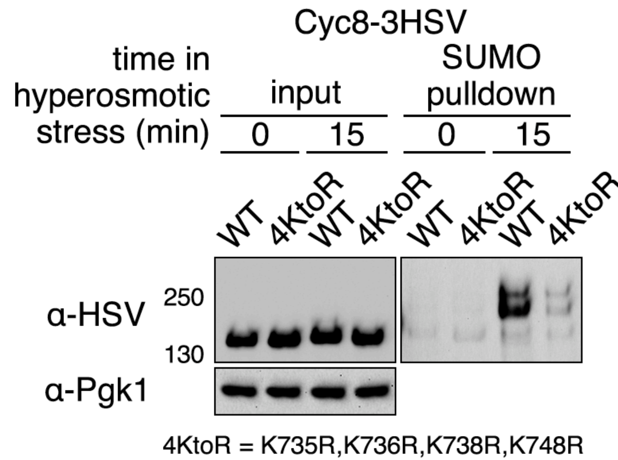


Figure 4.13. Mutation of multiple lysines in Cyc8 abolishes Cyc8 sumoylation during hyperosmotic stress. *6His-FLAG-SMT3* cells expressing either wild type Cyc8-3HSV or $Cyc8^{4KtoR}$-3HSV were examined for Cyc8 sumoylation: cell lysates (input) and purified sumoylated proteins (SUMO pulldown) were subject to western analyses using anti-HSV or anti-Pgk1 antibodies to detect Cyc8 or Pgk1, respectively.

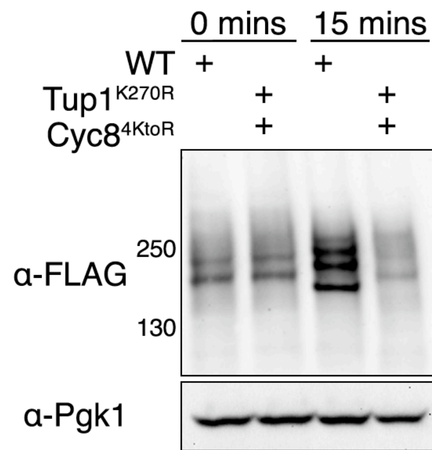


Figure 4.14. Mutation of lysines in Tup1 and Cyc8 abolishes the sumoylation observed in total cell lysates during hyperosmotic stress. *6His-FLAG-SMT3* wild type and *tup1*^{K270R}*cyc8*^{4KtoR} cells were subjected to hyperosmotic stress (1.2M sorbitol) for 0 or 15 minutes, and global sumoylation patterns examined by western analysis using an anti-FLAG antibody. Pgk1 was detected as a loading control.

CHAPTER FIVE:

PROTEIN SUMOYLATION DURING HYPEROSMOTIC STRESS PROTECTS AGAINST DYSREGULATION OF GENE REPRESSION AND INCLUSION FORMATION

In Chapter 4, I described our discovery that Tup1 and Cyc8 are rapidly but transiently sumoylated in response to hyperosmotic stress. Tup1 and Cyc8 are both members of the Tup1-Cyc8 transcriptional corepressor complex, which interacts with DNA-bound repressor proteins to repress the transcription of hundreds of genes in yeast (Malave and Dent, 2006). It is known that cells undergo massive transcriptional changes to respond to hyperosmotic shock, and most gene expression changes occur with kinetics similar to the sumoylation changes we observe: most genes that are up- or downregulated in hyperosmotic stress show the greatest change at 20-30 minutes following osmotic shock (O'Rourke and Herskowitz, 2004).

We hypothesized that the sumoylation of Tup1 and Cyc8 is involved in regulating the gene expression program during hyperosmotic stress. We wanted to assess the contributions of sumoylation on each protein, so we examined gene expression changes in a way that enabled us to identify outcomes of sumoylation of Tup1 and Cyc8 individually and in combination. I performed an mRNA transcript microarray experiment using strains expressing different combinations of wild type and sumoylation-deficient Tup1 and Cyc8: Tup1/Cyc8, Tup1^{K270R}/Cyc8, Tup1/Cyc8^{4KtoR}, or Tup1^{K270R}/Cyc8^{4KtoR}. I examined gene expression at 0, 30, and 60 minutes after 1.2M sorbitol addition to cover the times of known gene expression changes (O'Rourke and Herskowitz, 2004).

Here I present the data as both the gene expression changes (Figure 5.1, right panel) and the mutant effect ratios (Figure 5.1, left panel), the latter of which facilitates observation of changes in a mutant strain relative to the wild type strain. I calculated mutant effect ratios by subtracting the $\log_2$ values of the wild type strain from each mutant strain for every time point after application of the stress, similar to previous analyses performed (O'Rourke and Herskowitz, 2004). As shown in Figure 5.1, the sumoylation-deficient Tup1 mutant (Tup1^{K270R}) showed a distinct pattern of gene expression changes when compared with the sumoylation-deficient Cyc8 mutant (Cyc8^{4KtoR}). The gene expression changes in the double sumoylation-deficient mutant were very similar to those observed in the sumoylation-deficient Cyc8 mutant (Figure 5.2), suggesting that loss of Cyc8 sumoylation is epistatic to loss of Tup1 sumoylation, in terms of effects on gene expression.

Interestingly, the sumoylation-deficient Cyc8 strains predominantly showed increased gene expression during hyperosmotic stress (bracketed genes in Figure 5.1). This occurred for genes that are normally upregulated, downregulated, or unchanged by hyperosmotic stress. We hypothesized that the sumoylation of Cyc8 might not be a signal for regulation of a specific subset of genes. Rather, Cyc8 sumoylation might protect the general ability of Cyc8 to repress transcription during hyperosmotic stress.

Cyc8 sumoylation prevents inclusion formation during hyperosmotic stress

We wondered how sumoylation might protect Cyc8 repressive activity during hyperosmotic stress. It has been reported in the literature that hyperosmotic stress induces protein misfolding *in vivo* (Burkewitz et al., 2011; Burkewitz et al., 2012), particularly in glutamine-rich proteins that form amyloids (Moronetti Mazzeo et al., 2012). Cyc8 has a glutamine-rich prion domain (PrD) that forms intracellular amyloids when overexpressed by itself (Alberti et al., 2009; Patel et al., 2009). Based on this, we reasoned that hyperosmotic stress might induce misfolding of Cyc8, which in turn would reduce the functional levels of Cyc8-Tup1 complexes.

How would sumoylation function to prevent misfolding? It has been shown that SUMO is a highly soluble protein that promotes the solubility of recombinant proteins as a fusion (Malakhov et al., 2004; Marblestone et al., 2006). Furthermore, sumoylation prevents the aggregation of alpha-synuclein (Krumova et al., 2011), androgen receptor (Mukherjee et al., 2009; Rytinki et al., 2012), and polyQ-expanded ataxin-7 (Janer et al., 2010) in cell culture. Therefore, we hypothesized that sumoylation of Cyc8 might promote Cyc8 solubility during hyperosmotic stress, enabling the Cyc8-Tup1 complex to function properly in transcriptional repression.

We explored this hypothesis by determining if the sumoylation-deficient Cyc8^{4KtoR} protein forms inclusions *in vivo* during hyperosmotic stress. To do this, I made strains expressing GFP-tagged wild type Cyc8 or sumoylation-deficient Cyc8^{4KtoR} in combination with untagged wild type Tup1 or sumoylation-deficient Tup1^{K270R}. When I exposed cells to hyperosmotic stress over a time course of 60 minutes, I found that Cyc8^{4KtoR}-GFP formed transient inclusions that peaked in number during the first 15 minutes of hyperosmotic stress and disappeared by 60 minutes. (Figure 5.3). I did not observe any inclusion formation for wild type Cyc8-GFP. The inclusions

formed by Cyc8^{4KtoR}-GFP were independent of Tup1 sumoylation as the inclusions formed in cells with either Tup1 or sumoylation-deficient Tup1^{K270R} (Figure 5.3).

These results prompted us to query if sumoylation-deficient Tup1 also formed inclusions during hyperosmotic stress. I generated strains expressing GFP-tagged wild type Tup1 or sumoylation-deficient Tup1^{K270R} in combination with untagged wild type Cyc8 or sumoylation-deficient Cyc8^{4KtoR}. When I treated cells with hyperosmotic stress, we found that neither Tup1-GFP nor Tup1^{K270R}-GFP formed inclusions over the course of hyperosmotic stress in wild type Cyc8 cells (Figure 5.4). However, both Tup1-GFP and Tup1^{K270R}-GFP formed transient inclusions in sumoylation-deficient Cyc8^{4KtoR} cells, peaking at 15 minutes and disappearing by 60 minutes (Figure 5.4). Thus, loss of Cyc8 sumoylation caused both Cyc8 and Tup1 to form transient inclusions during hyperosmotic stress.

Cyc8 inclusions are SDS sensitive

Cyc8 has a prion domain (PrD) (Alberti et al., 2009; Patel et al., 2009), which can form SDS-resistant amyloids when the domain is overexpressed by itself (Alberti et al., 2009). Because we examined full-length Cyc8 under endogenous expression conditions, we wanted to determine if the hyperosmotic stress-dependent Cyc8^{4KtoR}-GFP inclusions are in an SDS-resistant amyloid state. One common technique to assess the amyloid nature of yeast prions is semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) (Alberti et al., 2009; Halfmann and Lindquist, 2008; Kryndushkin et al., 2003). Using SDD-AGE, we found Cyc8^{4KtoR}-GFP did not form SDS-resistant amyloids during hyperosmotic stress (Figure 5.5). As controls, overexpression of the Cyc8 PrD and the known prion Rnq1 did form SDS-resistant amyloids (Figure 5.5). In a second comparison to amyloid prions, I tested the ability of Cyc8^{4KtoR}-GFP inclusions to be cured by passaging cells on medium containing guanidine hydrochloride, which is a treatment that can suppress amyloid prion formation in yeast (Derkatch et al., 1997; Santoso et al., 2000; Sondheimer and Lindquist, 2000; Tuite et al., 1981; Wickner, 1994). After passaging cells on guanidine hydrochloride, Cyc8^{4KtoR}-GFP still formed identical inclusions (Figure 5.6). Altogether, transient Cyc8^{4KtoR}-GFP inclusions do not appear to be amyloid in nature.

One way to more directly test if SUMO alters the solubility of Cyc8 during hyperosmotic stress is to compare the solubility of wild type and sumoylation-deficient Cyc8 during stress. When I attempted solubility assays in which the insoluble fraction is pelleted by

ultracentrifugation, I did not observe differences in the solubility between wild type and mutant Cyc8. This could be because only a small fraction of Cyc8 undergoes changes that will alter its solubility (after all, only a small fraction of the pool of Cyc8 is sumoylated during hyperosmotic stress), or because the inclusions we observe reflect a protein misfolding transition state that will not show major changes in solubility. It will be important to address this issue with future experiments, perhaps with different techniques to assay solubility (e.g. sedimentation through a gradient) or with *in vitro* studies.

The prion domain of Cyc8 is involved in sumoylation-deficient inclusion formation

Because Cyc8 has a PrD, we wanted to explore the extent to which the PrD was involved in the transient formation of $Cyc8^{4KtoR}$-GFP inclusions during hyperosmotic stress. The Cyc8 PrD spans residues 441-677 ((Alberti et al., 2009) and Figure 5.7). I deleted the PrD in $Cyc8^{4KtoR}$-GFP to yield the variant $Cyc8^{4KtoR, \Delta PrD}$-GFP. I then expressed either $Cyc8^{4KtoR}$-GFP or $Cyc8^{4KtoR, \Delta PrD}$-GFP as the only version of Cyc8 in cells, and examined inclusion formation at 0, 5, and 15 minutes after initiation of hyperosmotic stress. The number of cells with at least one inclusion at 15 minutes was reduced by ~75% in $Cyc8^{4KtoR, \Delta PrD}$-GFP cells compared with $Cyc8^{4KtoR}$-GFP cells (Figure 5.8). This finding is consistent with the hypothesis that the PrD is involved in sumoylation-deficient Cyc8 inclusion formation. Interestingly, we also saw a modest, but statistically significant, reduction in the number of $Cyc8^{4KtoR, \Delta PrD}$-GFP cells containing at least one inclusion compared with $Cyc8^{4KtoR}$-GFP cells at 5 minutes (Figure 5.8). At this time point, the inclusions in $Cyc8^{4KtoR, \Delta PrD}$-GFP cells didn't qualitatively appear as intense or abundant as those in $Cyc8^{4KtoR}$-GFP cells. From these results, we concluded that the Cyc8 PrD is important but not required for the formation of inclusions.

We next wanted to know if deletion of the PrD would alter the sumoylation of Cyc8. We wondered if loss of this domain would result in reduced sumoylation. I performed SUMO pulldowns and blotted for wild type Cyc8 or $Cyc8^{\Delta PrD}$ and found that loss of the PrD resulted in reduced sumoylation of Cyc8 (Figure 5.9). This was particularly striking given that the $Cyc8^{\Delta PrD}$ mutant had dramatically increased steady state levels (Figure 5.9, input blot). This result supports the model in which Cyc8 is at risk of misfolding during osmotic stress and SUMO acts as a solubilizing factor.

One possibility is that signaling cascades of the hyperosmotic stress response direct Siz1 to sumoylate a portion of the pool of Cyc8 as a preventive measure against the chance of

misfolding. An alternative possibility is that actively misfolding or misfolded forms of Cyc8 are specifically targeted, either by factors in addition to sumoylation machinery, or by Siz1 itself. In the latter scenario, how would the SUMO ligase directly recognize a misfolding substrate? When we looked at the sequence of Siz1 for clues, we were surprised to find that Siz1 is highly enriched for asparagine residues, especially in one region spanning residues 493-554. Asparagine-rich regions have been shown to contribute to prion and amyloid formation (Halfmann et al., 2011). An intriguing possibility is that misfolding of the glutamine-rich PrD of Cyc8 enables a specfic interaction with Siz1's asparagine-rich region during hyperosmotic stress. This is easy to test: we can delete the asparagine-rich region and determine this mutation affects Cyc8 sumoylation. This experiment and other investigations into how Siz1 targets Cyc8 and Tup1 for sumoylation during hyperosmotic stress will be very important, because little is known about how SUMO ligases recognize their substrates.

Strains deficient in Cyc8 sumoylation show growth defects under extreme hyperosmotic stress

We wondered if the gene expression and cell biological effects we observed in sumoylation-deficient Cyc8 mutants related at all to cells' ability to handle hyperosmotic stress. I performed serial dilution growth assays in which strains expressing different combinations of wild type and mutant Cyc8 and Tup1 were grown on normal and hyperosmotic stress media (1.2M sorbitol). I did not observe any differences in growth across strains growing on hyperosmotic stress media (Figure 5.10). This could be because growth on agar media might not reflect the same physiological conditions as in liquid media, which I used for all other experiments. Alternatively, perhaps the gene expression and cell biological effects we observe do not result in differences in overall growth during hyperosmotic stress, at least at the intermediate level of severity of stress that we had been using in our experiments. We decided to challenge the strains on more extreme hyperosmotic stress media. I observed that at the highest levels of osmolyte (1.5M NaCl), most isolates of strains expressing sumoylation-deficient Cyc8 grew more poorly than strains expressing wild type Cyc8 (Figure 5.11). Several repeats of this experiment showed occasional isolates of sumoylation-deficient Cyc8 strains growing similar to wild type Cyc8 strains (an example is shown in Figure 5.11), suggesting that some isolates may have be suppressor mutations that rescue normal growth. There appeared to be no major growth differences between strains expressing wild type and sumoylation-

deficient Tup1 in a wild type Cyc8 background (Figure 5.11), consistent with the epistasis of Cyc8 sumoylation that we observed in our other experiments.

What is the function of Tup1 sumoylation?

We have observed that both Tup1 and Cyc8 need to complex together for their hyperosmotic stress-dependent sumoylation, but our gene expression and cell biological assays revealed that sumoylation of Tup1 does not drive the same outcomes as sumoylation of Cyc8. While we found that Cyc8 sumoylation is required to prevent the formation of Cyc8 and Tup1 inclusions during hyperosmotic stress, we still don't know the functional purpose for Tup1 sumoylation.

Unlike Cyc8, perhaps Tup1 sumoylation encodes information to direct specific gene expression changes. While our microarray data suggest that sumoylation of Cyc8 is the more important modfiication in terms of gene expression control during hyperosmotic stress, we noticed a cluster of genes in that seemed most affected in the Tup1 sumoylation deficient mutant (bottom portion of Figure 5.1; separated cluster in Figure 5.12, left). Many of these genes reside in within 20kb of a chromosome end (Figure 5.12, right), suggesting that Tup1 sumoylation may be important for regulating gene expression of loci in subtelomeric regions.

It is known that subtelomeric genes, while normally silent, can be activated during certain stress conditions. (Mak et al., 2009; Mazor and Kupiec, 2009; Smith et al., 2011). In this case, it appears that some are upregulated when Tup1 sumoylation is lost but not under normal conditions, suggesting that sumoylated Tup1 could be an important factor for actively maintaining gene expression in the context of silent chromatin during stress. This is interesting given that Tup1 has been implicated in telomere silencing (Chang et al., 2004), but little mechanistic detail is known.

Another interesting idea to consider about Tup1 sumoylation is that the sumoylation site that we and others have identified (lysine 270) lies in the middle of a region known to bind the tails of histones H3 and H4 (Edmondson et al., 1996). This region of Tup1 has also been shown to be important for repression by the Tup1-Cyc8 complex (Tzamarias and Struhl, 1994). Future experiments should assess if sumoylation of Tup1 affects histone binding, and if this relates to Tup1's function during hyperosmotic stress.

Adaptation to hyperosmotic stress and the transience of Cyc8 sumoylation/inclusion formation

The primary purpose of the yeast adaptive response to hyperosmotic stress is to that equalize hyperosmotic imbalance, mainly by facilitating the accumulation of intracellular osmolytes like glycerol (Klipp et al., 2005; Saito and Posas, 2012). The response to hyperosmotic stress contrasts with the responses to other stresses, like heat shock or high ethanol, which induce cellular changes that mitigate damage but cannot buffer the stress itself. We find it interesting that the sumoylation dynamics reflect these differences among stresses; heat shock and high ethanol induce chronic increases in sumoylation whereas hyperosmotic stress induces transient increases in sumoylation.

An important advantage of our studies is that we could observe the dynamics of a native, normally expressed prionogenic protein under physiologically relevant conditions. This enabled us to discover an endogenous *in vivo* misfolding transition state of the Cyc8 prionogenic protein. Cyc8 that cannot be sumoylated forms inclusions upon hyperosmotic stress, but the inclusions are transient. The dynamics of Cyc8 inclusion formation during hyperosmotic stress mirror the dynamics of sumoylation, supporting a model in which sumoylation protects Cyc8 during a specific time frame of misfolding susceptibility. The small abundant inclusions of sumoylation-deficient Cyc8 are reminiscent of inclusions called Stress Foci or Q-bodies that form in the minutes immediately after the overexpression of misfolded proteins (Escusa-Toret et al., 2013; Spokoini et al., 2012). Stress-Foci/Q-bodies coalesce from small abundant inclusions into a few large inclusions (Escusa-Toret et al., 2013; Spokoini et al., 2012). Sumoylation-deficient Cyc8 inclusions do not coalesce into large inclusions with time, but disappear between 30 and 60 minutes of hyperosmotic stress—the time in which yeast cells have adjusted intracellularly to the stress by increasing internal osmolytes. A key question then arises. Would sumoylation-deficient Cyc8 adopt an amyloid inclusion state if the cell cannot accumulate sufficient intracellular osmolytes to ameliorate the hyperosmotic stress? Answering this question will require a way to ablate the yeast cell's capacity to accumulate intracellular osmolytes, which is a complex response that has many redundant mechanisms (Saito and Posas, 2012). One way to test this is to ablate the osmotic stress response by deletion of the master osmotic stress response regulator *HOG1*. Interestingly, we and others have found that deletion of *HOG1* prolongs the sumoylation of Tup1 and Cyc8 (Figure 5.13, (Abu Irqeba et al., 2014)). It will be important to determine if we also see prolonged inclusion persistence in cells expressing sumoylation-deficient Cyc8.

Acknowledgements

Sarah Clowes first made the observation that sumoylation persists in *hog1*Δ cells during hyperosmotic stress.

Maitreya Dunham was incredibly helpful with our microarray work, from key discussions about microarray experimental design and analysis to assistance in submitting our array data to public repositories. Other members of the Dunham lab, including Emily Mitchell, Giang Ong, and Celia Payen helped me with the microarray experiments.

Aaron Gitler and Susan Lindquist shared positive control plasmids that we used for the SDD-AGE experiment.

Much of the data and text of this chapter are derived from the following manuscript:

Oeser M.L. and Gardner R.G. Sumoylation of a prionogenic transcription corepressor prevents its inclusion formation during hyperosmotic stress. *Manuscript submitted for review*.

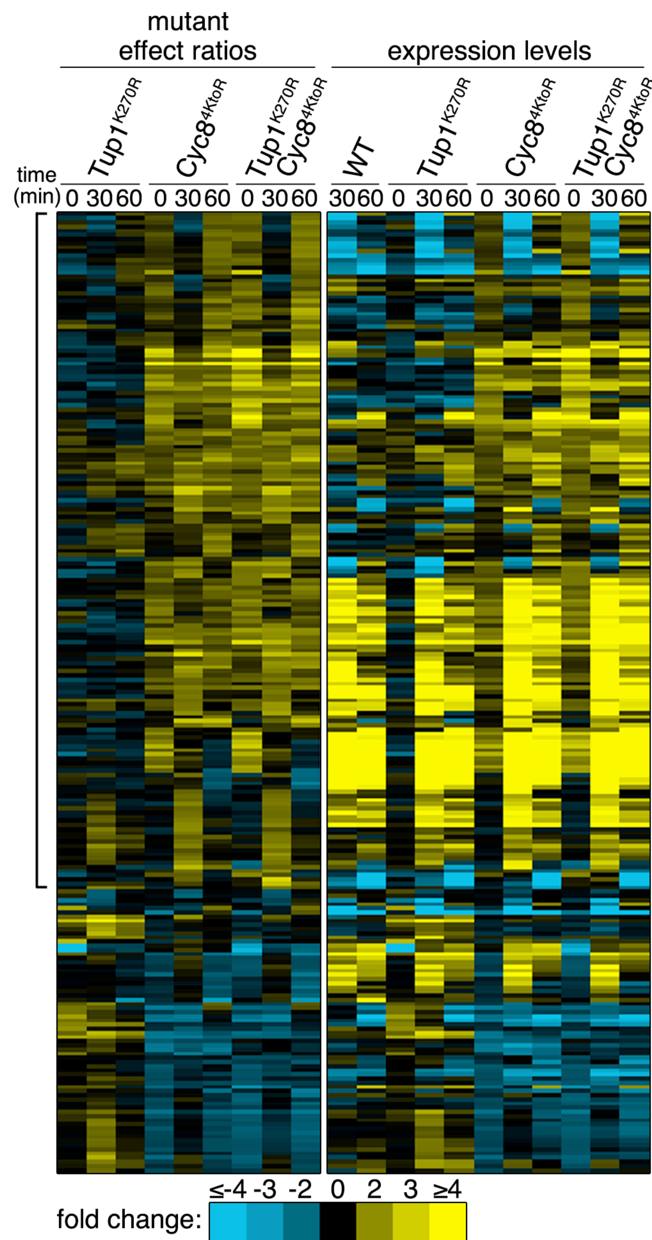


Figure 5.1. Distinct alterations in transcription occur with the sumoylation-deficient Tup1 and Cyc8 mutants. RNA was collected from biological duplicate samples of wild type and mutant (expressing sumoylation-deficient Tup1^{K270R} and/or Cyc8 ^{4KtoR}) strains that were exposed to 0, 30, or 60 minutes of hyperosmotic stress (1.2M sorbitol). Microarray experiments were performed in which the wild type, unstressed replicate sample served as the reference for comparison with all other samples with a set of replicates. Log_2-transformed gene expression ratios were averaged across replicate comparisons. Hierarchical clustering of mutant effects that are ≥1.57-fold during

hyperosmotic stress is shown here. Mutant effects were calculated as the $\log_2$ gene expression ratio for a given mutant sample minus the $\log_2$ ratio of the wild type sample for each time point across the time course. Genes appear in the clustering if they exhibited mutant effects of at least 1.57-fold in at least two instances across all comparisons. Vertical bracket highlights genes for which the Cyc8 mutant strains show increased expression levels relative to levels in wild type strains.

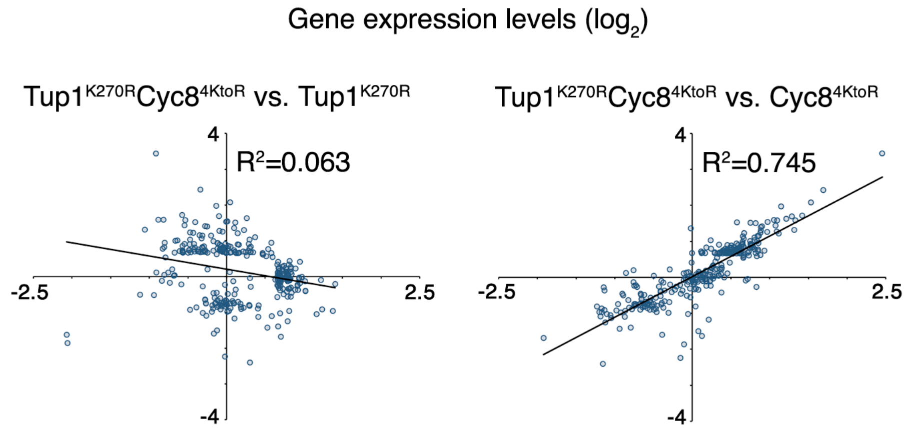


Figure 5.2. Gene expression of the double sumoylation-deficient mutant is similar to that of the Cyc8 sumoylation-deficient mutant. Scatter plots depicting the correlation of double sumoylation-deficient mutant gene expression (Tup1^{K270R}Cyc8^{4KtoR}) and single sumoylation-deficient mutant gene expression (Tup1^{K270R} or Cyc8^{4KtoR}). Gene expression data are from the unstressed time points of the data shown in Figure 5.1.

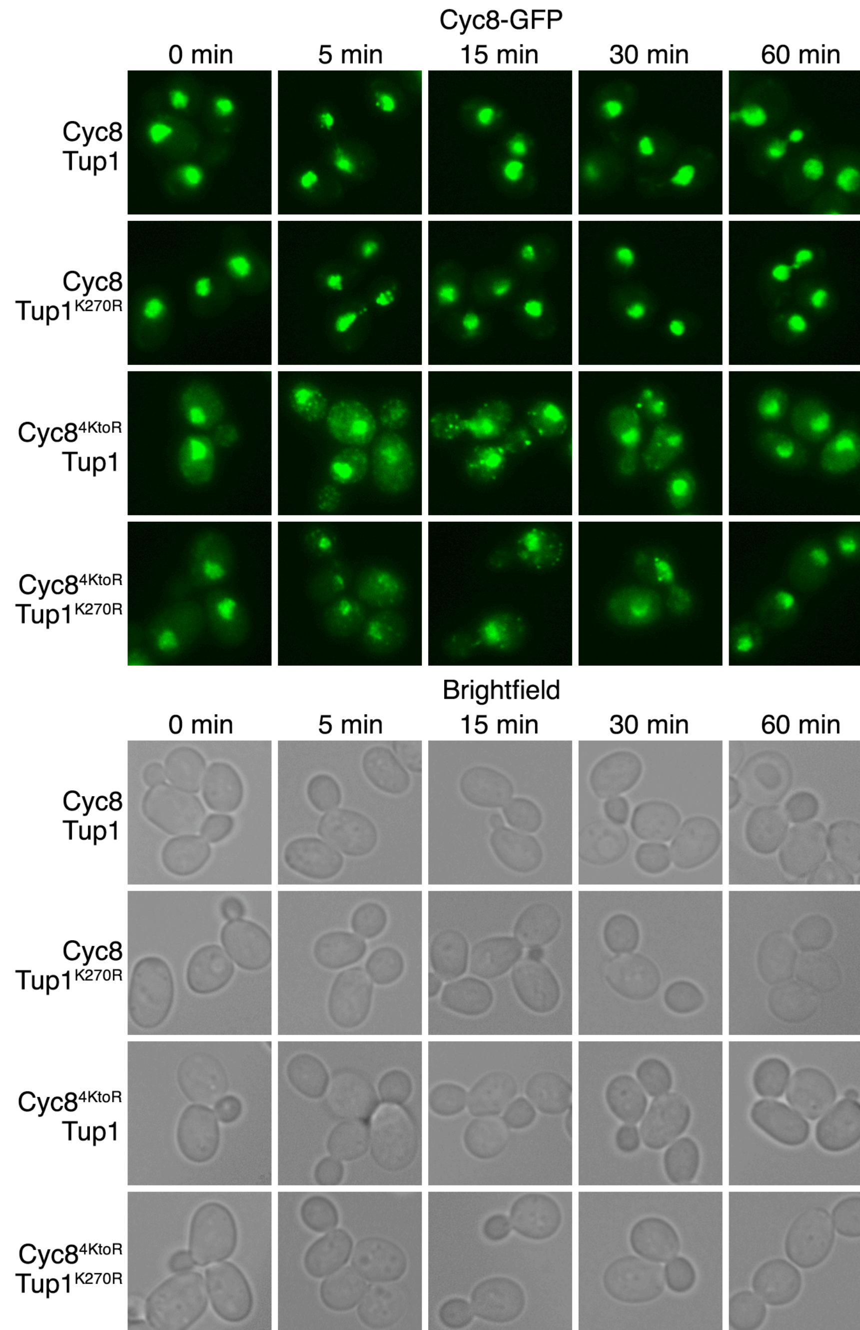


Figure 5.3. Loss of Cyc8 sumoylation leads to inclusion formation of Cyc8. Strains expressing combinations of wild type and/or sumoylation-deficient mutant versions of Cyc8 and Tup1, tagged with GFP or untagged, were exposed to a time course of hyperosmotic stress (1.2M sorbitol), fixed at the times indicated, and imaged by fluorescency microscopy. Top: GFP fluorescence images of cells in which wild type or mutant Cyc8 is GFP-tagged and wild type or mutant Tup1 is untagged. Bottom: Brightfield images of the same fields of cells.

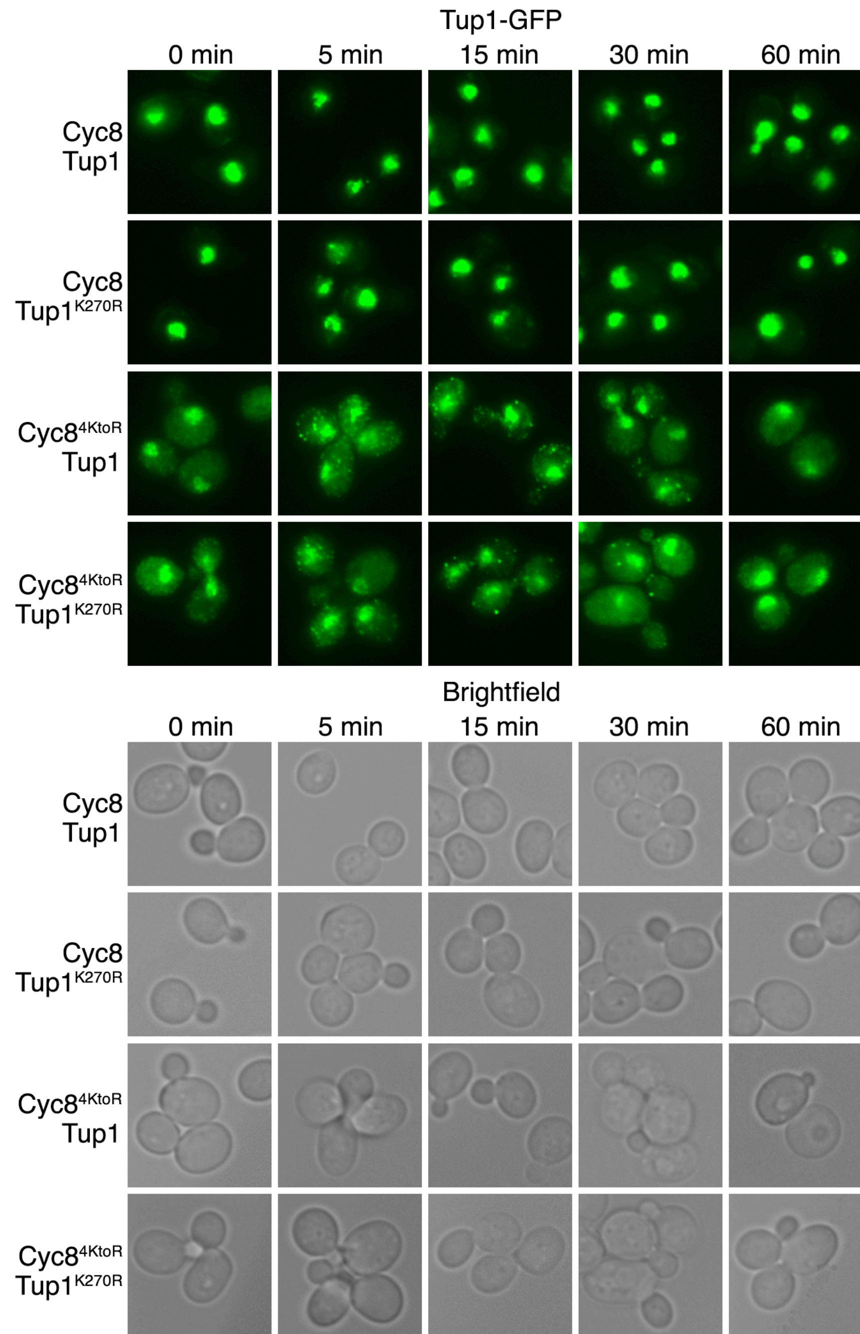


Figure 5.4. Loss of Cyc8 sumoylation leads to inclusion formation of Tup1. Strains expressing combinations of wild type and/or sumoylation-deficient mutant versions of Cyc8 and Tup1, tagged with GFP or untagged, were exposed to a time course of hyperosmotic stress (1.2M sorbitol), fixed at the times indicated, and imaged by fluorescency microscopy. Top: GFP fluorescence images of cells in which wild type or mutant Tup1 is GFP-tagged and wild type or mutant Cyc8 is untagged. Bottom: Brightfield images of the same fields of cells.

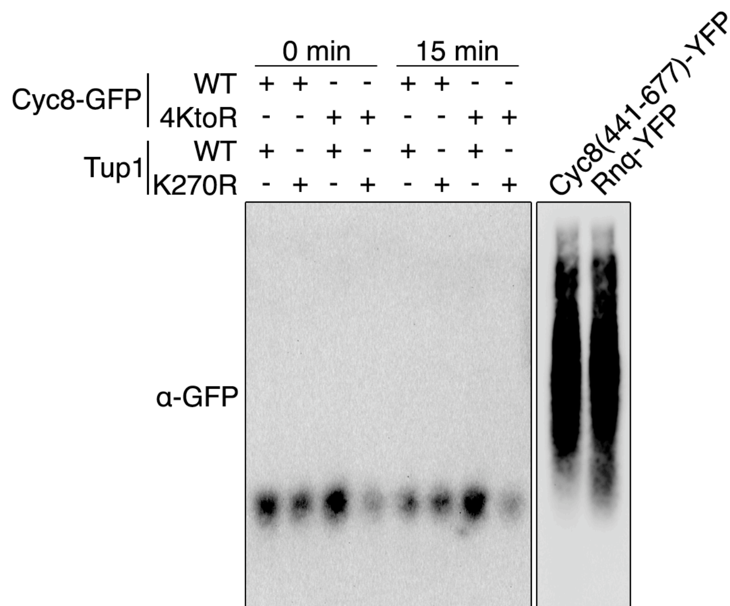


Figure 5.5. Sumoylation-deficient Cyc8 and Tup1 mutants do not form SDS-resistant amyloids. Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) of lysates from the strains used in Figure 5A. Cells were exposed to 0 or 15 minutes of hyperosmotic stress (1.2M sorbitol), and lysates were mixed with semi-denaturing buffer before loading into the agarose gel. Lysates from strains overexpressing either Rnq1-YFP or Cyc8(441-677)-YFP are also included as positive controls for SDS-resistant amyloid formation. Western analysis was performed with anti-GFP antibody. The two images are different exposures of the same blot.

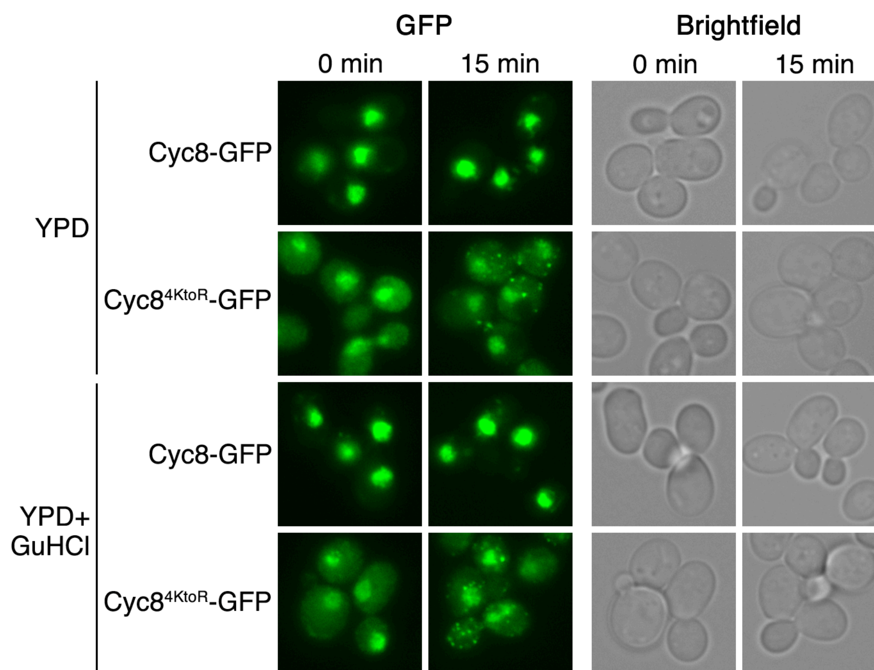


Figure 5.6. Passage on guanidine hydrochloride does not affect formation of Cyc8 inclusions. Left: GFP fluorescence microscopy images of cells expressing GFP-tagged wild type or sumoylation-deficient Cyc8 after three passages on either YPD or YPD+4mM guanidine hydrochloride (GuHCl). Cells were exposed to 0 or 15 minutes of hyperosmotic stress (1.2M sorbitol), fixed, and imaged by fluorescence microscopy. Right: Brightfield images of the same fileds of cells.

Tetratricopeptide repeats (TPRs)

SUMO sites

1 2 3 4 5 6 7 8 9 10 PrD

```
1   MNPGGEQTIM EQPAQQQQQQ QQQQQQQQQQ AAVPQQPLDP LTQSTAETWL
51  SIASLAETLG DGDRAAMAYD ATLQFNPSSA KALTSLAHLY RSRDMFQRAA
101 ELYERALLVN PELSDVWATL GHCYLMLDDL QRAYNAYQQA LYHLSNPNVP
151 KLWHGIGILY DRYGSLDYAE EAFAKVLELD PHFEKANEIY FRLGIIYKHQ
201 GKWSQALECF RYILPQPPAP LQEWDIWFQL GSVLESMGEW QGAKEAYEHV
251 LAQNQHHAKV LQQLGCLYGM SNVQFYDPQK ALDYLLKSLE ADPSDATTWY
301 HLGRVHMIRT DYTAAYDAFQ QAVNRDSRNP IFWCSIGVLY YQISQYRDAL
351 DAYTRAIRLN PYISEVWYDL GTLYETCNNQ LSDALDAYKQ AARLDVNNVH
401 IRERLEALTK QLENPGNINK SNGAPTNASP APPPVILQPT LQPNDQGNPL
451 NTRISAQSAN ATASMVQQQH PAQQTPINSS ATMYSNGASP QLQAQAQAQA
501 QAQAQAQAQA QAQAQAQAQA QAQAQAQAQA QAQAQAHAQA QAQAQAQAQA
551 QAQAQAQQQQ QQQQQQQQQQ QQQQQQQQQQ QQQQQQQLQP LPRQQLQQKG
601 VSVQMLNPQQ GQPYITQPTV IQAHQLQPFS TQAMEHPQSS QLPPQQQQLQ
651 SVQHPQQLQG QPQAQAPQPL IQHNVEQNVL PQKRYMEGAI HTLVDAAVSS
701 STHTENNTKS PRQPTHAIPT QAPATGITNA EPQVKKQKLN SPNSNINKLV
751 NTATSIEENA KSEVSNQSPA VVESNTNNTS QEEKPVKANS IPSVIGAQEP
801 PQEASPAEEA TKAASVSPST KPLNTEPESS SVQPTVSSES STTKANDQST
851 AETIELSTAT VPAEASPVED EVRQHSKEEN GTTEASAPST EEAEPAASRD
901 AEKQQDETAA TTITVIKPTL ETMETVKEEA KMREEEQTSQ EKSPQENTLP
951 RENVVRQVEE DENYDD*
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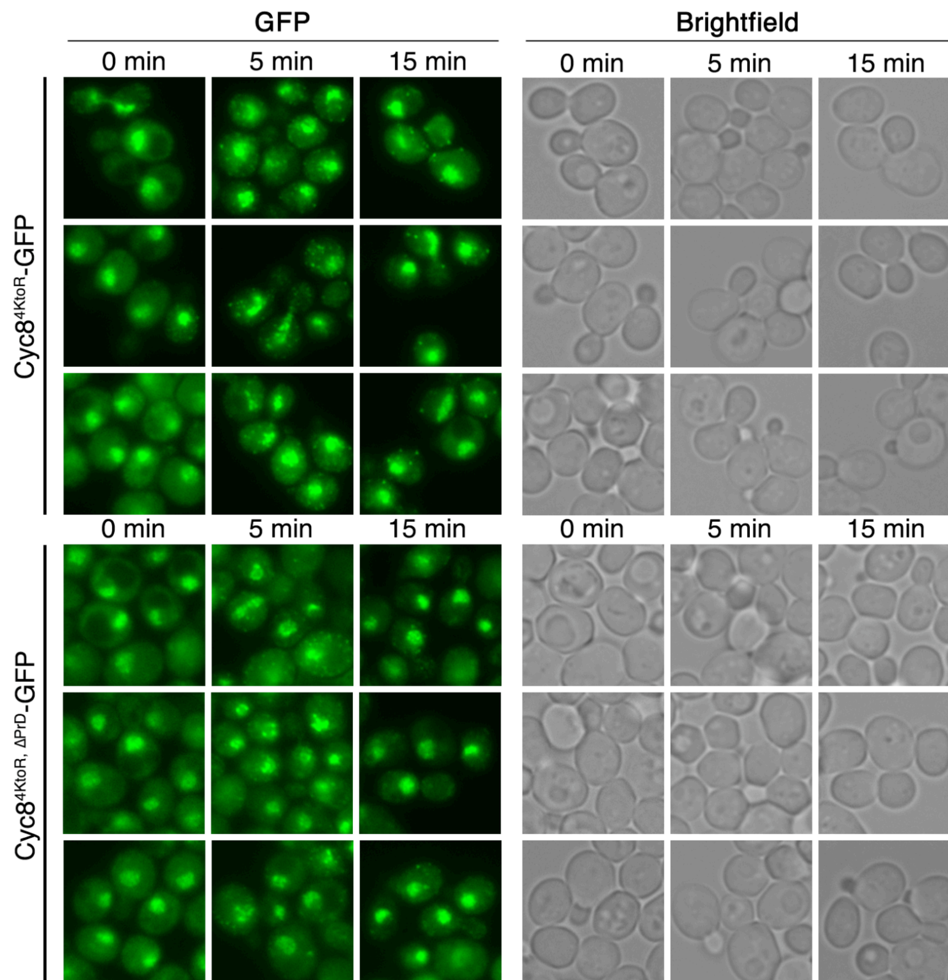
Figure 5.7. Schematic representation of the topology and amino acid sequence of Cyc8.



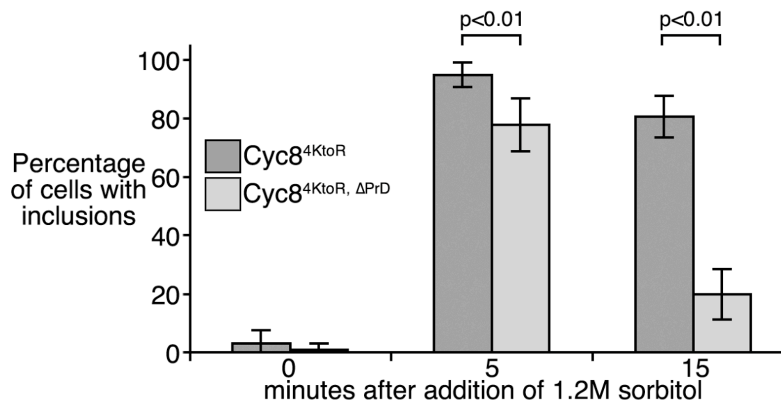


Figure 5.8. The prion domain of Cyc8 contributes to the persistence of inclusions of sumoylation-deficient Cyc8. Top left: Fluorescence microscopy images of cells expressing GFP-tagged, sumoylation-deficient Cyc8 with or without the PrD (residues 441-677). Cells were exposed to 0, 5, or 15 minutes of hyperosmotic stress (1.2M sorbitol), fixed, and imaged by

fluorescence microscopy. Top right: Brightfield images of the same fields of cells. Bottom: Analysis of the data. Five fields of cells for each condition, with ≥10 cells/field, were counted for the presence of inclusions. Data represent the average percentage of cells that contained inclusions within the five fields. Error bars are the standard deviation. A two-tailed, heteroscedastic student’s t-test was used to determine significance.

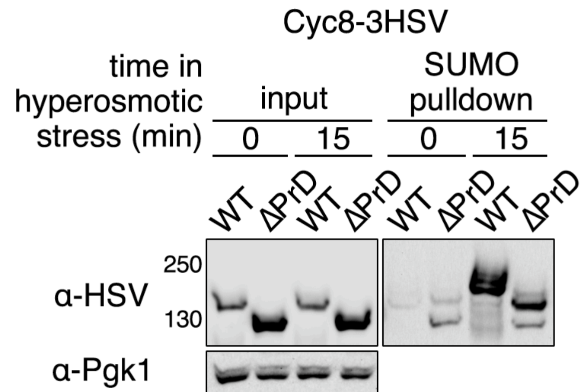


Figure 5.9. Cyc8 lacking the prion domain undergoes reduced sumoylation during hyperosmotic stress. *6His-FLAG-SMT3* cells expressing either wild type Cyc8-3HSV or Cyc8$^{\Delta PrD}$-3HSV (Cyc8 lacking residues 441-677) were examined for Cyc8 sumoylation: cell lysates (input) and purified sumoylated proteins (SUMO pulldown) were subject to western analyses using anti-HSV or anti-Pgk1 antibodies to detect Cyc8 or Pgk1, respectively.

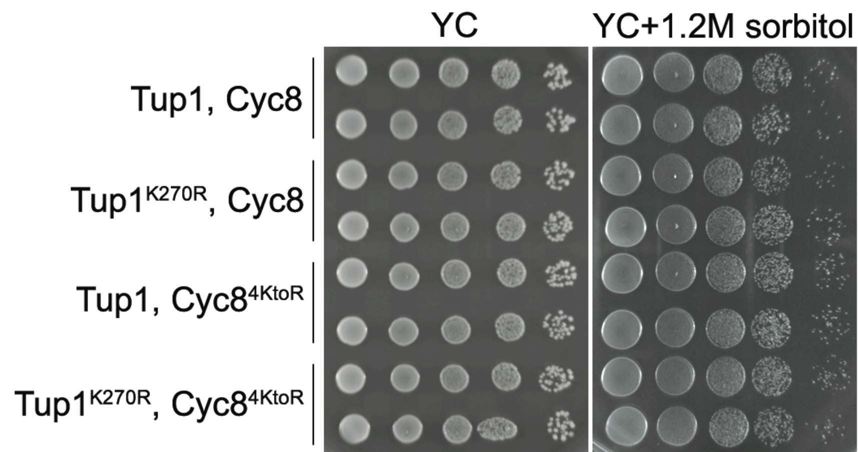


Figure 5.10. Strains expressing wild type and sumoylation-deficient mutants of Tup1 and Cyc8 do not exhibit differences in growth under intermediate hyperosmotic conditions. Ten-fold serial dilutions of cells were spotted to assess normal growth (yeast complete media) or growth during hyperosmotic stress (yeast complete media+1.2M sorbitol). The strains are expressing various versions of Tup1 and Cyc8. Shown are two isolates per strain. Plates were incubated at 30˚C for 3 days.

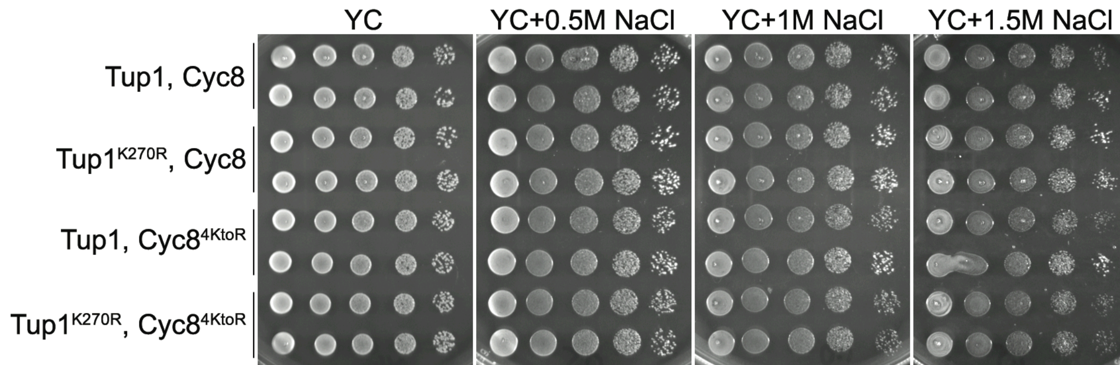


Figure 5.11. Some isolates of strains expressing sumoylation-deficient Cyc8 show a slight growth defect under extreme hyperosmotic stress. Ten-fold serial dilutions of cells were spotted to assess normal growth (yeast complete media) or growth during increasing severity of hyperosmotic stress (yeast complete media+0.5M NaCl, 1M NaCl, or 1.5M NaCl). The strains are expressing various versions of Tup1 and Cyc8. Shown are two isolates per strain. Plates were incubated at 30˚C for at least 3 days (cells growing on extreme hyperosmotic stress media required longer periods of growth).

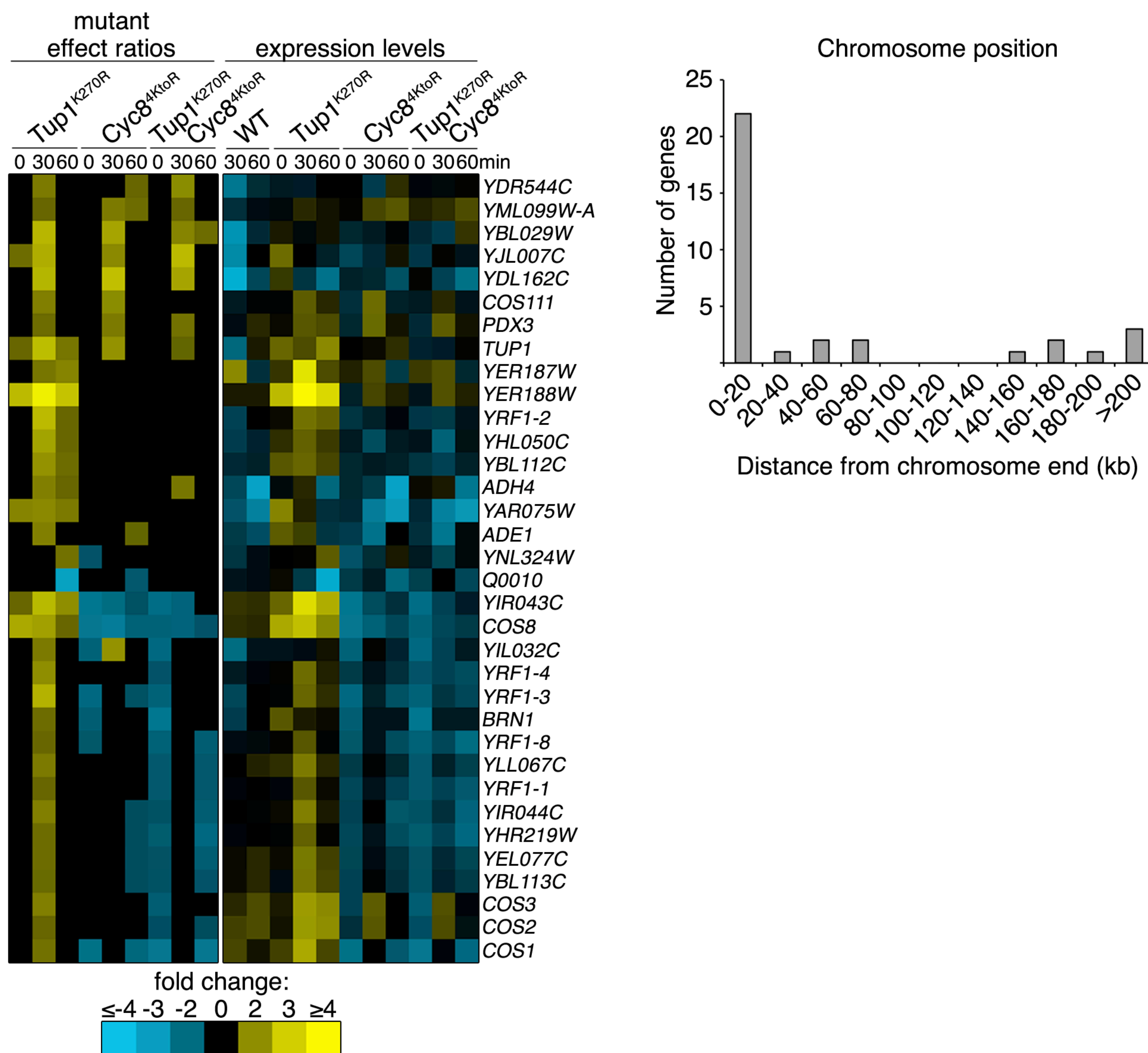


Figure 5.12. Loss of Tup1 sumoylation results in transcriptional changes at subtelomeric genes. Left: A reclustered version of the data from the bottom section of the clustering in Figure 5.1. Right: Chromosome position (distance in kb from nearest chromosome end), binned based on 20kb distance increments.

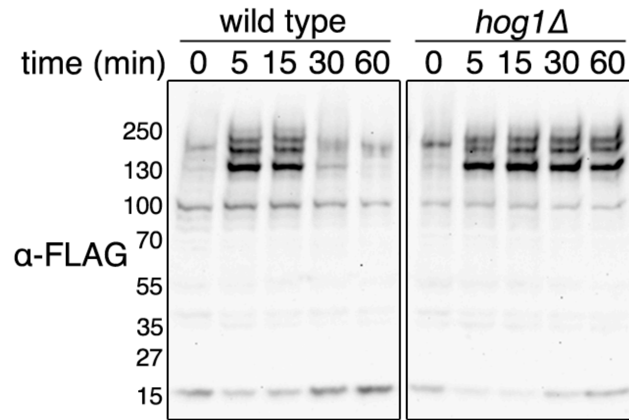


Figure 5.13. Loss of *HOG1* prolongs sumoylation during hyperosmotic stress. *6His-FLAG-SMT3* wild type and *hog1*Δ cells were subjected to hyperosmotic stress (1.2M sorbitol). Changes in sumoylation patterns were examined by western analysis using an anti-FLAG antibody. The left and right panels were cropped from the same exposure of the same blot.

CHAPTER SIX:

CONCLUSIONS

In a recent review, Grace Gill observed, "Something About SUMO Inhibits Transcription" (Gill, 2005). That statement seems to resonate with the studies that I have presented here. I have presented evidence that SUMO seems to contribute to the inhibition of transcription through two distinct pathways, highlighting the broad functionality of this Ubl.

I have found that the rapid and transient sumoylation of Cyc8 and Tup1 during in hyperosmotic stress protects against inclusion formation and loss of repression by Tup1-Cyc8. SUMO, through its apparent action as a solubilizing factor, therefore functions to maintain gene repression during stress. In separate studies, I have discovered transcriptional activation function that is normally inhibited by SUMO interaction in a predicted SUMO-targeted ubiquitin ligase. This protein's activation activity appears to be another example of SUMO inhibition of transcription. Transcriptional activation activity of this protein is interesting, considering that Uls1 is involved in transcriptional silencing at the telomeres, regions known to depend on SUMO for their maintenance but also known to switch transcriptional states during certain environmental contexts, including some stress conditions.

Transcriptional switching through SUMO

Why would a protein that functions in silencing also activate transcription? There are examples of proteins with both silencing and activating functions (e.g. the repressor-activator protein Rap1), so it appears that these dual functionalities are not necessarily mutually exclusive. In fact, the silencing protein Sir3 has been found to transiently concentrate at the *GAL1* locus during activation (Radman-Livaja et al., 2011). Furthermore, both silent chromatin and actively expressed loci share an interesting feature in yeast: many genes, both highly and minimally expressed, have been found to associate with components of the nuclear periphery, including nuclear pore components (Brown and Silver, 2007; Casolari et al., 2004).

Interestingly, regions of silent chromatin in yeast (and heterochromatin regions in other eukaryotes) form clusters at the nuclear periphery. Both Sir4 and the Ku proteins can interact with protein components of the nuclear periphery (Andrulis et al., 2002; Hediger et al., 2002), and the inner nuclear periphery is highly enriched for Sir proteins, Rap1, and Ku proteins (Gotta

et al., 1996; Maillet et al., 1996; Taddei et al., 2009). Furthermore, artificial tethering of a weakened silent mating type locus to the nuclear periphery has been shown to restore silencing (Andrulis et al., 1998; Andrulis et al., 2004), highlighting the importance of nuclear architecture in chromatin silencing. Notably, Uls1 was found to be capable of this restoration-of-silencing function (Andrulis et al., 2004).

The activation of many genes in yeast also involves their localization to the nuclear periphery. Several studies have revealed that many inducible genes, which normally localize to the nucleoplasm, are targeted to nuclear pore complexes for full transcriptional induction (Ahmed et al., 2010; Brickner et al., 2007; Brickner and Walter, 2004; Cabal et al., 2006; Casolari et al., 2004; Taddei et al., 2006). This relocalization to the nuclear periphery can contribute to the "transcriptional memory" of these loci, altering the kinetics of future transcriptional activation events (Brickner et al., 2007; Brickner, 2009; Brickner and Walter, 2004; Light et al., 2013).

Could there be a need for factors that can interact with the machinery of both silent and active transcriptional contexts? The presence of strongly silenced and strongly active genes in close physical proximity to each other suggests the potential for such a need. Perhaps silencing/activating proteins could aid in the establishment of boundaries at the nuclear periphery, or mediate switching from silent to active and back again. There is growing evidence that normally silent subtelomeric genes can be quite dynamic in their expression. In a variety of stress conditions, transcriptional regulators are redistributed over normally silenced subtelomeric regions, inducing the expression of many different genes (Mak et al., 2009; Mazor and Kupiec, 2009; Smith et al., 2011).

SUMO has been shown to play a role in both positioning telomeres, in spatial coordination of gene activation, and in gene activation control in general. The SUMO ligase Siz2 promotes the sumoylation of Ku proteins and Sir4, and loss of Siz2 results in delocalization of telomeres from the nuclear periphery (Ferreira et al., 2011). Localization of the SUMO protease Ulp1, which regulates levels of sumoylated Cyc8 during carbon switching, appears to be important in driving derepression of the *GAL1* gene, a locus known to be positioned at the nuclear pore during activation (Texari et al., 2013). Finally, an interesting study found that while recruitment of Ubc9 and accumulation of SUMO at promoter regions occurs during the activation of several inducible genes, decreases in sumoylation during induction actually results in increased transcription of inducible genes (Rosonina et al.). This could indicate that SUMO is important for later downregulation of induced genes. Together, these findings illustrate that that

SUMO's involvement in transcriptional silencing, activation, and regulation is complex. It will be interesting to explore if Uls1 is involved in such transcriptional transitions as well.

SUMO, protein misfolding, and the environment

That environmental stress can induce protein misfolding is well established (Gidalevitz et al., 2011; Lindquist and Kelly, 2011). Our studies provide new evidence that sumoylation may act as a solubilizing factor during hyperosmotic stress in order to maintain proper transcriptional control. A major question we raise is whether sumoylation also acts as a solubilizing agent in response to other stresses, particularly those that cause protein misfolding. In our experiments, it appears that mainly Tup1-Cyc8 sumoylation occurs during osmotic stress, but in other stresses, many more proteins appear to be sumoylated. We wonder what portion of this pool of sumoylated proteins is modified with SUMO to protect against misfolding, given the exceptional solubility of SUMO.

Prions are a special type of misfolded protein and are defined by their ability to propagate through non-Mendelian inheritance (Prusiner, 2013). In yeast, many prionogenic proteins normally function in transcription or translation, which means their prion induction could potentially elicit new selective-pressure outcomes by increasing phenotypic variation (Liebman and Chernoff, 2012; Wickner et al., 2013). On the other hand, prion formation in humans has been largely linked to disease (Prusiner, 2013), though recent studies suggest prion formation might contribute to normal processes (Prusiner, 2013). The intriguing relationship between the environment and prions is a subject of intense study in yeast and other organisms. Various stresses and stimuli have been shown to alter prion induction, propagation, or loss (Chernova et al., 2011; Singh et al., 1979; Tuite et al., 1981; Tyedmers et al., 2008; Westergard and True, 2014), but little mechanistic detail of these processes is known.

We have uncovered new molecular details into modulation of a prionogenic protein by environmental stress. The formation of non-sumoylated Cyc8 inclusions correlates with derepression of many genes, which could increase phenotypic variation and be potentially beneficial or detrimental in specific environmental situations. In addition, non-sumoylated Cyc8 inclusions could represent an intermediate with increased potential to shift to a potentially harmful amyloid prion state. In the case of Cyc8, sumoylation appears to act as an important environmental buffer and modulator of these potential outcomes. We observe that under more extreme hyperosmotic stress, loss of sumoylation of Cyc8 appears to be detrimental to growth

in that particular stress. It would be interesting to also determine if hyperosmotic stress (at intermediate or extreme levels) can alter cells’ ability to grow in other environments—perhaps we would uncover new beneficial phenotypes. It is an intriguing idea that sumoylation of prionogenic or other misfolding-prone proteins in a cell population might act as a modulator of the adaptive potential of cells or organisms.

Final remarks

In this thesis, I have explored how the ubiquitin-like modifier SUMO is used to modulate cellular outcomes via transcription, with a particular focus on cellular contexts that require rapid transcriptional change, such as stress. I have presented evidence SUMO can act as a signal to inhibit transcription, and I have also shown that SUMO can promote transcriptional repression in a non-signaling manner by solubilizing transcriptional regulators that are at risk of misfolding during stress. Other studies have shown that SUMO regulates some cellular processes by modifying large groups of proteins that work together (e.g. for DNA repair) (Jentsch and Psakhye, 2013), and the current thinking is that this promotes interactions and signaling exchanges between sumoylated proteins. I am curious if, in addition to direct signaling effects, SUMO is also promoting the formation of specialized microenvironments that facilitate efficient completion of the process at hand.

The emerging picture of SUMO’s function in the cell is complex and fascinating. I am eager for future discoveries to illuminate a more complete view of SUMO’s role—and the role of less understood Ubls—in promoting cellular health in the face of ever-changing environments.

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APPENDIX I:

PLASMIDS AND STRAINS

Plasmids used in these studies

Plasmid name	Alias	Relevant gene(s)	Parent vector	Reference
pRG1194	pACYCDuet-1			
pRG1780	pETDuet-1			
pRG1781	pRSFDuet-1			
pRG1782		*UBA1*	pETDuet-1	(Rosenbaum et al., 2011)
pRG1783		Ubiquitin	pRSFDuet-1	(Rosenbaum et al., 2011)
pRG1784		Ubiquitin, *CDC34*	pRSFDuet-1	(Rosenbaum et al., 2011)
pRG2289		*SAN1-3HSV-8His, 2HA-GAL4AD-cdc68-1(NTD)*	pACYCDuet-1	(Rosenbaum et al., 2011)
pRG2310		*2HA-GAL4AD-cdc68-1(NTD)*	pACYCDuet-1	(Rosenbaum et al., 2011)
pRG2288		*SAN1-3HSV-8His, 2HA-GAL4AD-cdc13-1(NTD)*	pACYCDuet-1	(Rosenbaum et al., 2011)
pRG2309		*2HA-GAL4AD-cdc13-1(NTD)*	pACYCDuet-1	(Rosenbaum et al., 2011)
pRG2069		*SAN1-3HSV-8His, 2HA-GAL4AD-BGL2(20-313)*	pACYCDuet-1	(Rosenbaum et al., 2011)
pRG2065		*2HA-GAL4AD-BGL2(20-313)*	pACYCDuet-1	(Rosenbaum et al., 2011)
pRG2067		*SAN1-3HSV-8His, 2HA-GAL4AD-TEF2(190-458)*	pACYCDuet-1	(Rosenbaum et al., 2011)
pRG2063		*2HA-GAL4AD-TEF2(190-458)*	pACYCDuet-1	(Rosenbaum et al., 2011)
pRG2312		*8His-UBI1/RPL40A* (Ub sequence tagged with 8His)	pRS406	
pRG2313		*8His-UBI2/RPL40B* (Ub sequence tagged with 8His)	pRS406	
pRG2389		*8His-UBI2/RPS31* (Ub sequence tagged with 8His)	pRS406	
pRG2471		*8His-UBI4* (all Ub repeats tagged with 8His)	pBSII SK(+)	
pRG3990		P_{GPD}-*AFB2*	pGP5G-ccdB	(Havens et al., 2012)
pRG1673	pRS416 Gal-RNQ1-YFP	P_{GAL1}-*RNQ1-YFP*	pRS416	(Douglas et al., 2008)
pRG4190		*pAG424GAL-CYC8 PrD-EYFP*	pAG424GAL-ccdB-EYFP	(Alberti et al., 2009)
pRG4043		*TUP1*	pRS406	
pRG4129		*TUP1(K270R)*	pRS406	
pRG4059		*TUP1-3HA*	pRS406	

pRG4060		*TUP1(K270R)-3HA*	pRS406	
pRG4111		*TUP1(73-713)-3HA*	pRS406	
pRG4086		*TUP1-GFP*	pRS406	
pRG4087		*TUP1(K270R)-GFP*	pRS406	
pRG4020		*CYC8*	pRS405	
pRG4130		*CYC8(K735R,K736R,K738R,K748R)*	pRS405	
pRG4052		*CYC8-3HA*	pRS405	
pRG4079		*CYC8(1-900)-3HA*	pRS405	
pRG4080		*CYC8(1-830)-3HA*	pRS405	
pRG4081		*CYC8(1-745)-3HA*	pRS405	
pRG4082		*CYC8(1-715)-3HA*	pRS405	
pRG4084		*CYC8-3HSV*	pRS405	
pRG4113		*CYC8(K735R,K736R,K738R,K748R)-3HSV*	pRS405	
pRG4110		*CYC8(176-966)-3HSV*	pRS405	
pRG4085		*CYC8-GFP*	pRS405	
pRG4128		*CYC8(K735R,K736R,K738R,K748R)-GFP*	pRS405	
pRG4193		*CYC8(Δ441-677,K735R,K736R,K738R,K748R)-GFP*	pRS405	
pRG2665		*PGAL1-ULS1*	pRS404	
pRG2666		*PGAL1-ULS1(1-895)*	pRS404	
pRG2796		*PGAL1-ULS1(1-895,Δ7-10,Δ371-378,Δ471-474,Δ543-550)*	pRS404	
pRG117	pGAD-C3	*GAD*		(James et al., 1996)
pRG118	pGBD-C1	*GBD*		(James et al., 1996)
pRG2723		*GAD-SMT3(1-96)*	pGAD-C3	
pRG2652		*GBD-ULS1*	pGBD-C1	
pRG2728		*GBD-ULS1(Δ7-10)*	pGBD-C1	
pRG2729		*GBD-ULS1(Δ371-378)*	pGBD-C1	
pRG2730		*GBD-ULS1(Δ471-474)*	pGBD-C1	
pRG2731		*GBD-ULS1(Δ543-550)*	pGBD-C1	
pRG2753		*GBD-ULS1(Δ7-10,Δ371-378)*	pGBD-C1	
pRG2754		*GBD-ULS1(Δ7-10,Δ371-378,Δ471-474)*	pGBD-C1	
pRG2755		*GBD-ULS1(Δ7-10,Δ371-378,Δ543-550)*	pGBD-C1	
pRG2756		*GBD-ULS1(Δ471-474,Δ543-550)*	pGBD-C1	
pRG2757		*GBD-ULS1(Δ7-10,Δ471-474,Δ543-550)*	pGBD-C1	
pRG2758		*GBD-ULS1(Δ371-378,Δ471-474,Δ543-550)*	pGBD-C1	
pRG2759		*GBD-ULS1(Δ7-10,Δ471-474)*	pGBD-C1	
pRG2760		*GBD-ULS1(Δ7-10,Δ543-550)*	pGBD-C1	
pRG2761		*GBD-ULS1(Δ371-378,Δ471-474)*	pGBD-C1	
pRG2762		*GBD-ULS1(Δ371-378,Δ543-550)*	pGBD-C1	
pRG2767		*GBD-ULS1(Δ7-10,Δ371-378,Δ471-474,Δ543-550)*	pGBD-C1	

pRG3803		*GBD-ULS1(1-889)*	pGBD-C1	
pRG3804		*GBD-ULS1(1-889,Δ7-10,Δ371-378,Δ471-474,Δ543-550)*	pGBD-C1	
pRG2458		*ULS1-3HSV*	pRS406	
pRG3659		*ULS1(Δ7-10,Δ371-378,Δ471-474,Δ543-550)-3HSV*	pRS406	
pRG2555		*ULS1-3GFP*	pRS406	

Yeast strains used in these studies

Strain name	Alias	Genotype	Reference
RGY386	BY4741	*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0*	(Brachmann et al., 1998)
RGY4546		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 8His-UBI1/RPL40A, 8His-UB12/RPL40B, 8His-UBI3/RPS31, 8His-UBI4* (all Ub repeats in *UBI4* with 5' 8His tag)	(Richardson et al., 2012)
RGY5347		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 8His-UBI1/RPL40A, 8His-UBI3/RPS31, 8His-UBI4* (all Ub repeats in *UBI4* with 5' 8His tag)	
RGY4136		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, P_{GAL1}-RNQ1 URA3 2μ*	(Konopka et al., 2011)
RGY5266		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6*	
RGY5643		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, TUP1-3HA::NatMX*	
RGY5642		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, TUP1-3HA::NatMX*	
RGY5750		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, CYC8-3HSV::URA3*	
RGY5722		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, CYC8-3HSV::URA3*	
RGY5641		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ*	
RGY5645		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ*	
RGY5702		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ, cyc8Δ*	
RGY5758		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1-3HA::URA3, cyc8Δ::CYC8::LEU2*	
RGY5759		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1(K270R)-3HA::URA3, cyc8Δ::CYC8::LEU2*	
RGY5754		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1::URA3, cyc8Δ::CYC8-3HSV::LEU2*	
RGY5756		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1::URA3, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)-3HSV::LEU2*	
RGY5704		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, siz1Δ*	
RGY5744		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, siz1Δ, CYC8-3HSV::URA3*	
RGY5746		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, siz1Δ, TUP1-3HA::NatMX*	
RGY5705		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, siz2Δ*	

RGY5707		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cst9Δ	
RGY5749		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, MMS21-3HSV-IAA1.T10::KanMX, CYC1::AFB2::LEU2	
RGY5711		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, ULP1-3HSV-IAA1.10::KanMX, CYC1::AFB2::LEU2	
RGY5745		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, ULP1-3HSV-IAA1.10::KanMX, CYC1::AFB2::LEU2, CYC8-3HSV::URA3	
RGY5747		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, ULP1-3HSV-IAA1.10::KanMX, CYC1::AFB2::LEU2, TUP1-3HA::NatMX	
RGY5712		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, ULP2-3HSV-IAA1.10::KanMX, CYC1::AFB2::LEU2	
RGY5753		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, TUP1::TUP1(73-713)-3HA::URA3	
RGY5751		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, CYC8::CYC8(176-966)-3HSV::LEU2	
RGY5762		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1::URA3, cyc8Δ::CYC8::LEU2, isolate 1	
RGY5763		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1::URA3, cyc8Δ::CYC8::LEU2, isolate 2	
RGY5765		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1(K270R)::URA3, cyc8Δ::CYC8::LEU2, isolate 1	
RGY5766		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1(K270R)::URA3, cyc8Δ::CYC8::LEU2, isolate 2	
RGY5768		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1::URA3, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)::LEU2, isolate 1	
RGY5769		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1::URA3, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)::LEU2, isolate 2	
RGY5771		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1(K270R)::URA3, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)::LEU2, isolate 1	
RGY5772		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1(K270R)::URA3, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)::LEU2, isolate 2	
RGY5812		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1::URA3, cyc8Δ::CYC8-GFP::LEU2	
RGY5813		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1(K270R)::URA3, cyc8Δ::CYC8-GFP::LEU2	
RGY5814		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1::URA3, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)-GFP::LEU2	
RGY5815		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1(K270R)::URA3, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)-GFP::LEU2	
RGY5816		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1-GFP::URA3, cyc8Δ::CYC8::LEU2	
RGY5817		MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1(K270R)-GFP::URA3, cyc8Δ::CYC8::LEU2	

RGY5818		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1-GFP::URA3, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)::LEU2*	
RGY5819		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1(K270R)-GFP::URA3, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)::LEU2*	
RGY5822		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)-GFP::LEU2*	
RGY5823		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8(Δ441-677,K735R,K736R,K738R,K748R)-GFP::LEU2*	
RGY5824		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-3HSV::LEU2*	
RGY5826		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8(Δ441-677)-3HSV::LEU2*	
RGY1109	PJ69-4A	*MATa, his3Δ200, ura3-52, trp1-901, leu2-3,112, PGAL2-ADE2, PGAL7-lacZ, LYS2::PGAL1-HIS3, gal4Δ, gal80Δ*	(James et al., 1996)
RGY4151		*MATa, ade2Δ::hisG, met15Δ0, his3Δ200, ura3Δ0, trp1Δ63, lys2Δ0, leu2Δ0,TEL-VIIL::adh4::URA3, TEL-VR::ADE2, ppr1Δ::LYS2*	
RGY4188		*MATa, ade2Δ::hisG, met15Δ0, his3Δ200, ura3Δ0, trp1Δ63, lys2Δ0, leu2Δ0,TEL-VIIL::adh4::URA3, TEL-VR::ADE2, ppr1Δ::LYS2, uls1Δ::KanMX*	
RGY4180		*MATa, ade2Δ::hisG, met15Δ0, his3Δ200, ura3Δ0, trp1Δ63, lys2Δ0, leu2Δ0,TEL-VIIL::adh4::URA3, TEL-VR::ADE2, ppr1Δ::LYS2, sir4Δ::KanMX*	
RGY69	UCC3515	*MATalpha, ade2-101, his3Δ200, ura3-52, trp1Δ63, lys2-801, leu2Δ1, hmlalpha::URA3*	
RGY4191		*MATalpha, ade2-101, his3Δ200, ura3-52, trp1Δ63, lys2-801, leu2Δ1, hmlalpha::URA3, uls1Δ::KanMX*	
RGY4183		*MATalpha, ade2-101, his3Δ200, ura3-52, trp1Δ63, lys2-801, leu2Δ1, hmlalpha::URA3, sir4Δ::KanMX*	
RGY5651		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, uls1Δ::ULS1-3GFP::URA3, TUP1-mCherry::HIS3MX6*	
RGY5652		*MATa, met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, uls1Δ::ULS1-3GFP::URA3, CYC8-mCherry::HIS3MX6*	

APPENDIX II:

MATERIALS AND METHODS

Plasmids and strains

Plasmids and yeast plasmids used in these studies are listed in Appendix I. Standard cloning methods were used to generate genetic constructs. Standard yeast genetic methods were used (Guthrie and Fink, 1991). All gene deletions were verified by colony PCR, and genomic tagging was verified by colony PCR and western blotting. Auxin degradation strains were made by integration of PCR-generated, C-terminal genomic tagging constructs (which encode an in-frame fusion of a 3HSV-IAA1.NdC degron tag; IAA1.NdC is a variant of the IAA1 degron that was a gift from Jennifer Nemhauser). Details of the auxin-responsive tag will be described in a subsequent publication (Britney Moss and Jennifer Nemhauser).

In coli ubiquitination

Components of the San1 ubiquitination cascade were cloned behind T7*lac* promoters in Duet expression vectors (Novagen) that were maintained in *T7* Express competent *E. coli* (New England Biolabs) with appropriate antibiotic selection. Cells were grown at 37˚C to OD_{600} ~1.0 and induced with 800µM IPTG for 6 hours at 30˚C. Cells were lysed in Bugbuster (Novagen) with 10mM PMSF. To immunoprecipitate the 2xHA-tagged substrates, soluble extract was incubated for 16 hours at 4˚C with 1:2000 mouse anti-HA antibodies (Sigma) conjugated to 0.75mg/ml Protein A Dynabeads (Invitrogen) in IP buffer. Beads were washed 3x in IP buffer then incubated at 65˚C for 10 minutes in SUMEB (8M urea, 10mM MOPS, 10mM EDTA, 1% SDS, 0.01% bromophenol blue, pH 6.8).

Yeast growth and stress conditions

Cells were grown to a density of ~$1.5x10^7$ cells/ml at 30˚C in yeast complete (YC) media prior to stress induction. All 0 time point samples were collected before stress induction. For hyperosmotic stress, equal volumes of culture and YC+2.4M sorbitol were combined for a final concentration of 1.2M sorbitol. For heat stress, culture flasks were submerged in a 42˚C water bath to rapidly warm the culture and were then moved to shaking platform 42˚C incubator. For ethanol stress, equal volumes of culture and YC+20% ethanol were combined for a final

concentration of 10% v/v ethanol. Induction of Cyc8 PrD and Rnq1 was performed by growing cells in YC with 3% raffinose to mid-log phase at 30˚C to a density of ~$2x10^6$ cells/ml, then inducing expression with 3% galactose at 30˚C for 24 hours.

Auxin-dependent protein degradation

Cells were grown to a density of ~$1x10^7$ cells/ml in YC media at 30˚C. Indole-3-acetic acid (IAA, Fisher) was diluted 1:1000 from a 100mM stock solution in 95% ethanol for a final concentration of 100μM, or 95% ethanol was diluted 1:1000 for 0.095% ethanol for a control treatment. Cultures were incubated with 100μM IAA or 0.095% ethanol with shaking at 30˚C for 60 minutes prior to stress induction.

Sumoylated protein purification

15ml aliquots of cells were collected at each time point after stress and flash frozen in liquid nitrogen. Harvested cells were lysed by vortexing with glass beads at 4˚C in lysis buffer (8M urea, 50mM Tris pH 8.0, 0.05% SDS with 2mM PMSF and 10mM NEM). An aliquot representing 5% of the input was set aside. Cell lysates were incubated with TALON resin (Novagen) overnight at 4˚C. The resin was washed 3x with wash buffer (8M urea, 50mM Tris pH 8.0, 200mM NaCl, 0.05% SDS, 5mM imidazole). Sumoylated proteins were eluted from the column by addition of loading buffer (8M urea, 10mM MOPS, 10mM EDTA, 1% SDS, 0.01% bromophenol blue, pH 6.8) and incubation at 65˚C for 10 minutes. Sumoylated proteins were resolved by SDS-PAGE using 4-20% gradient gels. Western analyses were performed with rabbit anti-Smt3 (1:5000, a gift from Xiaolan Zhao), mouse anti-FLAG (1:2500, Sigma), mouse anti-HSV (1:2500, Novagen), mouse anti-HA (1:2500, Sigma), mouse anti-Pgk1 (1:5000, Abcam), or rabbit anti-GFP (1:2500, Sigma).

Semi-denaturing detergent agarose gel electrophoresis

Cells were grown in YC to a density of ~$1.5x10^7$ cells/ml at 30˚C. A volume of 15ml was collected for Cyc8-GFP strains and 5ml was collected for galactose-induced strains. All cells were flash frozen on dry ice and ethanol. Cells were lysed by vortexing with glass beads at 4˚C in a nondenaturing buffer (100mM Tris pH 7.5, 200mM NaCl, 1mM EDTA, 5% glycerol, 1mM DTT, 1% NP-40, 250U/ml Benzonase nuclease, 1mM $MgCl_2$, 2mM PMSF, and 10mM NEM).

Lysates were centrifuged for 2 minutes at 3,000 rpm to pellet unlysed cells, and the supernatant was mixed with 4X SDD-AGE loading buffer (2X TAE, 20% glycerol, 8% SDS, bromophenol blue) and incubated at room temperature for 5 minutes before loading. Samples were run on a 1.5% agarose gel containing 0.1% SDS for 6 hours at 4˚C and transferred to nitrocellulose by capillary transfer as previously described (Halfmann and Lindquist, 2008). Western blotting was performed with anti-GFP to visualize GFP- and YFP-tagged proteins.

Mass spectrometry analyses

Sumoylated proteins from cells exposed to 0 or 15 minutes of hyperosmotic stress were enriched by metal affinity chromatography as described above. Samples were run 1 cm into a 4-20% SDS-PAGE gel and slices were excised. Proteins in the gel slices were digested with trypsin and the digestion products were desalted and dried by vacuum centrifugation. Dried peptide mixtures were resuspended in 7μL of 0.1% formic acid. 5μL was analyzed using an LTQ OrbiTrap mass spectrometer (Thermo Scientific) at the Fred Hutchinson Cancer Research Center proteomics shared resource (Seattle, WA). Complete MS/MS methods were performed as previously described in (Richardson et al., 2012). The protein database search algorithm X!Tandem (Craig and Beavis, 2004) was used to identify peptides from the Saccharomyces Genome Database (http://www.yeastgenome.org). Peptide false discovery rates were measured using Peptide Prophet (Keller et al., 2002). Identified peptides were filtered using Peptide Prophet scores of ≥0.55 (~5% error rate).

Microarray analyses

Duplicate biological cultures were grown from isolates of independent transformations. Cultures were grown at 30˚C in YC to a density of ~$1.75x10^7$ cells/ml and 0, 30, and 60 minute hyperosmotic stress samples were harvested by fast filtration and flash frozen in liquid nitrogen. Total RNA was prepared from the cells by hot acid phenol extraction as described on the Dunham lab website (http://dunham.gs.washington.edu/protocols.shtml). Total RNA was labeled using the Quick Amp RNA labeling kit (Agilent) and Cy3- and Cy5-CTP (GE Healthcare). Labeled cRNA was purified with the RNeasy mini kit (Qiagen). Yield and dye incorporation were measured with a Nanodrop spectrophotometer (Thermo Scientific). Cy3- and Cy5-labeled samples were combined to obtain ≥1pmol dye and cRNA in each channel. Labeled cRNA combinations were mixed with blocking agent and fragmentation buffer (Agilent), then incubated

at 60˚C for 30 minutes in the dark. Hi-RPM hybridization buffer (Agilent) was used to stop the reaction. Samples were hybridized to yeast gene expression 8x15K microarray slides (Agilent) at 65˚C overnight. Gasket slides were removed in 6X SSPE/0.005% N-lauroylsarcosine. Array slides were washed 1 minute in 6X SSPE/0.005% N-lauroylsarcosine, 1 minute in 0.6X SSPE/0.005% N-lauroylsarcosine, and 30 seconds in acetonitrile.

Data were acquired using an Agilent scanner and feature-extracted and normalized with the Agilent software using default settings. All raw microarray data are available from the Princeton Microarray Database (http://puma.princeton.edu) and the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE57476. Average linkage hierarchical clustering was performed with Cluster 3.0 ((Eisen et al., 1998), http://rana.lbl.gov/EisenSoftware.htm), using the similarity metric "Correlation (uncentered)", and the cluster data were visualized with Java TreeView (http://jtreeview.sourceforge.net/). To evaluate experimental variation in sample handling and microarray procedures, we analyzed the distribution of the ratios at each feature of a "wt x wt" array in which wild type, unstressed replicate samples were cohybridized. The distribution of $\log_2$-transformed gene expression ratios was centered at 0 with a standard deviation of 0.22. We used this data to define a threshold for significant gene expression changes of three standard deviations from the mean, which corresponded to ±0.65 $\log_2$ units, or a 1.57-fold change in gene expression. Mutant effect ratios were determined by subtracting the values of wild type time points from the corresponding mutant time points (in $\log_2$ space).

RNA sequencing

Triplicate biological cultures were grown to a density of ~1.75×10^7 cells/ml in rich media (yeast extract, peptone, dextrose media) and harvested by centrifugations. Total RNA was extracted with the Nucleospin RNA kit according to the manufacturer's instructions. RNA concentration was measured using a Nanodrop spectrophotometer, and 2ug of total RNA was used for library construction. Library prep, sequencing, and data analysis were performed by the Fred Hutchinson Cancer Research Center genomics shared resource (Seattle, WA). Sequencing was performed with an Illumina HiSeq 2500, image analysis and base calling were performed with RTA v1.12 software (Illumina), and FASTQ files were generated with CASAVA v1.8 software (Illumina). Data were filtered for quality and a threshold of for 1 count per million in at least 3 samples was applied for all genes.

Microscopy

Aliquots of cells at each time point after hyperosmotic stress were removed, fixed in 4% paraformaldehyde solution for 15 minutes at room temperature and then washed with PBS. Cells were imaged on a Nikon Eclipse 90i with a 100X objective, filters for GFP (HC HiSN 0 Shift filter set with excitation wavelength (450–490 nm), dichroic mirror (495 nm), and emission filter (500–550 nm)), and a Photometrics Cool Snap HQ2 cooled CCD camera with NIS-Elements acquisition software.

Image processing

All blots were scanned using an Epson Perfection V350 Photo scanner at 300 dpi. All images were processed with a Mac iMac or Pro computer (Apple) using Photoshop CS (Adobe).

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EDUCATION

University of Washington Seattle, WA
PhD in Molecular and Cellular Biology June 2014
National Science Foundation Graduate Research Fellowship 2010-2013

Northwestern University Evanston, IL
BA in Biological Sciences 2006
Departmental honors June 2006

RESEARCH EXPERIENCE

University of Washington Seattle, WA
Predoctoral research associate with Dr. Richard Gardner 2008-2014

- Used genetics, biochemistry, and cell biology techniques to examine how yeast cells protect themselves from stress by modulating protein modification mechanisms
- Analyzed protein modification-mediated gene expression changes during cellular stress with real-time quantitative PCR and microarray analyses
- Designed and generated over 400 genetic constructs by cloning, subcloning, and PCR mutagenesis to build yeast and bacteria strains expressing proteins and pathways of interest
- Optimized a method for inducible, rapid depletion of essential yeast proteins
- Reconstituted a functional eukaryotic enzymatic cascade in a prokaryote to enable experiments on the minimal components of the system

Northwestern University Feinberg School of Medicine Chicago, IL
Research technologist with Dr. Franck Mauvais-Jarvis 2006-2008

- Managed laboratory activities: reagent ordering, chemical inventory, and safety compliance
- Researched the role of hormones in diabetes and obesity by querying gene and protein expression profiles and performing microscopy experiments with mammalian tissues and cells

Northwestern University Evanston, IL
Undergraduate research assistant with Drs. Teresa Horton and Jon Levine 2005-2006

- Used immunohistochemistry and microscopy to visualize signaling proteins in the mouse brain

University of New Hampshire Durham, NH
Summer research assistant with Dr. Vaughn Cooper 2004-2005

- Performed bacterial evolution experiments to evaluate rapid adaptation in virulent strains

SKILLS AND TECHNIQUES

- Design and assembly of genetic constructs via cloning and subcloning methods
- Yeast and bacterial strain development
- Design and implementation of genetic screens, selections, and enzymatic assays
- RNA extraction and gene expression analysis
- Protein expression and purification using a variety of affinity tags
- Fluorescence microscopy

COMMUNICATION AND LEADERSHIP EXPERIENCE

University of Washington Seattle, WA

Teaching assistant: introductory and advanced molecular biology September 2009-March 2010
Research mentor to a Bioengineering undergraduate student January 2011-June 2013
Research mentor to a visiting undergraduate student through the University of Washington Pharmacological Sciences Summer Diversity Program Summer 2012
Job shadow mentor to two local high school students Summer 2010

Seattle Expanding Your Horizons Seattle, WA
Organizing committee member and workshop presenter 2010-2013

- Helped coordinate and execute the Seattle Expanding Your Horizons conference, a day of science, math, and engineering workshops that serves middle school girls from the region
- Developed and carried out molecular biology laboratory workshops for students

University of Washington Molecular and Cellular Biology Student Symposium Seattle, WA
Organizing committee member 2009-2010

- Coordinated a student-run symposium featuring faculty speakers from across the U.S.

PRESENTATIONS

Oeser M.L., Gardner R.G. (2013) Rapid and transient sumoylation of a transcriptional corepressor complex in response to osmotic stress. Poster presentation at the Cold Spring Harbor Ubiquitin Family Meeting, Cold Spring Harbor, NY, May 2013.

Oeser M.L., Gardner R.G. (2011) Exploring signal integration of ubiquitin-like modifiers through the yeast SUMO-targeted ubiquitin ligase Uls1. Poster presentation at the University of Washington-Kobe University Joint Symposium on Integrative Membrane Biology and Signal Transduction Medicine, Kobe, Japan, December 2011.

Oeser M.L., Gardner R.G. (2010) Reconstituting the ubiquitination system in *E. coli*. Poster presentation at Proteomics of Protein Degradation and Ubiquitin Pathways, Vancouver, Canada, June 2010.

MANUSCRIPTS AND PUBLICATIONS

Oeser M.L., Gardner R.G. Sumoylation of a prionogenic transcription corepressor prevents its inclusion formation during hyperosmotic stress. *Manuscript submitted for review*.

Oeser M.L., Gopalan J., Engelhart E., Gardner R.G. Mutational analysis reveals a transcription activation domain in a SUMO-targeted ubiquitin ligase with silencing function. *In preparation*.

Gallagher P.S., **Oeser M.L.**, Abraham A.C., Kaganovich D., Gardner R.G. (2014) Cellular maintenance of nuclear protein homeostasis. *Cell Mol Life Sci* 71:1865-1879.

Rosenbaum J.C., Fredrickson E.K., **Oeser M.L.**, Garrett-Engele C.M., Locke M.N., Richardson L.A., Nelson Z.W., Hetrick E.D., Milac T.I., Gottschling D.E., Gardner R.G. (2011) Disorder Targets Misorder in Nuclear Quality Control Degradation: A Disordered Ubiquitin Ligase Directly Recognizes Its Misfolded Substrates. *Molecular Cell* 41:93-106.