# A conserved chromatin complex is sumoylated during acute ethanol stress in *Saccharomyces cerevisiae*

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

Doctor of Philosophy

University of Washington

2021

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Program Authorized to Offer Degree:

Molecular and Cellular Biology
University of Washington

University of Washington

**Abstract**

Acute ethanol stress induces sumoylation of conserved chromatin proteins in *Saccharomyces cerevisiae*

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Stress is a ubiquitous part of life which disrupts cellular function and, if unresolved, can irreparably damage essential biomolecules and organelles. All organisms are subject to stress in the form of unfavorable environmental conditions including extreme temperatures, hypoxia, reactive oxygen species, or shifts in osmolarity. To survive, organisms must sense these changes then react and adapt. One highly conserved adaptive response is sumoylation, which is a post-translational modification by the small ubiquitin-like modifier (SUMO) protein. SUMO is a broadly used signaling molecule capable of altering protein localization, interactions, solubility, regulating transcription and genomic integrity. In the context of environmental stresses, I find that *Saccharomyces cerevisiae* (budding yeast) exhibit unique and dynamic patterns of sumoylation as part of a concerted effort to return the cell to homeostatic conditions. These SUMO-based stress responses vary in kinetics and substrates in accordance with the type, severity, and duration of stress. I therefore conclude that, rather than employing a generalized SUMO stress response, yeast tailor their use of this highly versatile modification to suit the present environmental conditions.

**ACKNOWLEDGEMENTS**

I would first like to thank Rich Gardner for his unwavering guidance and support throughout my graduate experience. What initially began as a ‘fun’ rotation turned into an incredible lab home over the last six years. I do not think I would have learned or accomplished as much academically without him. During my time in the lab, I have also found my voice and the confidence to advocate for myself without fear and I thank Rich for providing me an environment to do that.

I would like to thank the members of my committee for their incredible support, specifically Sue Biggins, Dana Miller, and Alex Merz. Attempting to complete my PhD during the last year during a global pandemic and other significant events has been incredibly challenging. I thank you all for your support and holding me accountable for staying on track. Additionally thank you Sue for stepping in at the eleventh hour to take over as chair of my committee. To all of my committee members, thank you for your support and well wishes in the lead up to my defense.

Thank you to my amazing friends Lauren and Stef Loh and their small dog Gryffindor, Ari Gomez, Heather Borror, Chanelle Case-Borden, Trina Fernandes, Michael Mendoza, Elisa Morales-Merry, and everyone else who has provided much needed levity and support to me over the years. I truly do not know where I would be without you. I love you all.

Thank you to the MCB program for providing such a warm and supportive environment. I am specifically grateful for Maia Low and Denise Barnes; I have treasured every single chat we have had over the years and your support has meant more to me than I can ever express.

I would like to thank Gardner Lab members past and present for their camaraderie and support.

## DEDICATION

To my mother Alice Bradley for instilling in me the importance of education and for being my forever biggest supporter. You have given me the strength to push through on even the darkest of days and have shown me that it is never too late to chase your dreams. To my grandmother Irene Bradley I adore you and I dedicate this work to you and in the memory of my grandpa Don.

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**List of Abbreviations**

PTM Posttranslational modification

SUMO Small ubiquitin like modifier

UBL Ubiquitin like modifier

PrD Prion domain

GFP Green fluorescent protein

NLS Nuclear localization sequence

NPC Nuclear pore complex

SMC Structural maintenance of chromosomes

HU Hydroxyurea

ND Nocodazole

MMS Methyl methanesulfonate

ROS Reactive oxygen species

## Chapter 1: Introduction

Adaptation to changing environmental states is critical to cellular survival. Throughout the existence of a cell, it will be exposed to a myriad of exogenous stressors. Rapidly changing temperatures, hyper- or hypo- osmolarity, oxidative stress, genotoxic and others disturb cellular homeostasis leading to DNA, RNA, and protein damage. An effective cellular response activates stress pathways that alter protein interactions or activity and gene expression in an organized effort to reestablish homeostasis. The inability to quickly respond to stress cues can result in cell death. In response to changing environmental conditions, cells have the ability to utilize a number of distinct signaling pathways and posttranslational modifications to help promote adaptation and survival. This introduction will broadly explore three common stress responses and the stimuli required to activate them. I will then explore the role of key posttranslational modifications that have been shown to be integral in mounting an appropriate cellular response to stress.

### Environmental Stress

Exposure to environmental stress, is inevitable and the type of stressors living things are subjected to is vast. From air pollution and heavy metal exposure to rapidly changing temperatures, organisms have a great deal to contend with. Depending on the external environmental cue, cells can utilize several signaling pathways that aid in mounting an appropriate response. The three major cellular responses discussed here can be triggered, in some cases, by many different external environmental cues.

#### Heat Shock Response

The heat shock response was first characterized as the biochemical consequence of exposure to a slight increase in temperatures (~3-5°C above average) (Fulda *et al.*, 2010; Richter *et al.*, 2010). Subsequently it has been demonstrated that multiple exogenous stress stimuli can activate the heat shock response, such as: exposure to heavy metals, oxidative, and ethanol stress (Piper, 1995; Fulda *et al.*, 2010). Persistent exposure to these stresses results in protein damage which can lead to aggregation of misfolded proteins. In order to combat protein damage, the cell employs transcription factors known as heat shock factors (HSFs) (Fulda *et al.*, 2010; Richter *et al.*, 2010). HSF1 is required for the heat shock response and is conserved from

yeast to humans. Under non-stress conditions, inactive HSF1 monomers interact with Hsp90 in the cytoplasm (Masser *et al.*, 2020). After stress exposure, levels of misfolded proteins increase and directly compete with HSF1 for Hsp90 (Fulda *et al.*, 2010; Masser *et al.*, 2020). HSF1 release from Hsp90 triggers the transition from a monomer to a trimer that translocates from the cytoplasm to the nucleus where it binds promoters of target genes that inhibit apoptosis and promote cellular survival (Fulda *et al.*, 2010).

DNA Damage Response

There are several stress conditions that can trigger the DNA damage checkpoint pathway. Heavy metal exposure, UV damage, chemotherapy and other genotoxins all have been shown to damage DNA (Fulda *et al.*, 2010). Double strand breaks (DSBs) can result after exposure to any of the stressors mentioned above, these lesions are the catalyst for activation of the DNA damage response. Once DSBs are detected, the Mre-11-Rad50-Nbs1 (MRN) complex recruits ataxia telangiectasia mutated (ATM) to DNA lesions and phosphorylates checkpoint kinase 2 (Chk2, Rad53 in yeast) which then results in phosphorylation of downstream targets leading to cell cycle arrest where the DNA can be repaired, or activation of proapoptotic genes if damage is too severe (Fulda *et al.*, 2010; Warmerdam and Kanaar, 2010).

Osmotic Shock Response

Osmoregulation of fluids is essential for maintaining the balance of water in organisms. Disruption of this balance by either hyper- or hypo- osmolarity can lead to serious physiological consequences if left unchecked. Yeasts specifically possess a pathway that aids in cellular adaptation to hyperosmotic stress. In the presence of increased levels of osmolarity, the cell transduces signal through a MAPK pathway known as high osmolarity glycerol (HOG) (Hohmann, 2009). Through the HOG pathway, stress response genes are upregulated in response to phosphorylation of the MAP kinase *Hog1* (Hohmann, 2009). The mammalian ortholog of *Hog1* is *p38*, unlike *Hog1*, *p38* can also be stimulated by UV light (DNA damage), heat, growth factors and inflammatory cytokines (Zarubin and Han, 2005).

## Posttranslational Protein Modifications

Cells have developed systems to cope with a wide range internal and external stress conditions. In addition to promoting cellular survival through activation of stress response genes, a common feature in the cellular stress responses discussed above is the use of posttranslational protein modifications (PTMs) to activate downstream targets, integral in signaling for an effective response. PTMs come in many forms, ranging from chemical modifications like acetylation or phosphorylation, to ubiquitination and sumoylation where the PTM itself is a protein (ubiquitin, SUMO). The common characteristic all PTMs share is that they can alter protein function, activity, localization, stability and structure. Additionally, PTMs are critical to cellular survival because these reversible modifications allow the cell to rapidly respond to environmental stress (Conibear, 2020).

### Phosphorylation

Phosphorylation is the most common PTM in cellular signaling. Phosphate was first discovered in the protein Vitellin in 1906 (Pawson and Scott, 2005). By the late 1930s two forms of glycogen phosphorylase (b and a) an enzyme that catalyzes the rate-limiting step in glycogenolysis were discovered. At the time it was thought that phosphorylase a bound tightly to 5’ AMP and that it converted phosphorylase a to b and that action catalyzed the removal of 5’ AMP. While it was never shown that 5’AMP was removed from phosphorylase, this reaction was the first reported example of allosteric activation (Cohen, 2002).

In 1954, the first described enzymatic phosphorylation of proteins was reported (Burnett and Kennedy, 1954). Following this, Edwin Krebs and Edmond Fischer discovered that inactive phosphorylase b could be converted to the active form in the presence of Magnesium, ATP and an enzyme they called a phosphorylase kinase (Krebs and Fischer, 1956; Cohen, 2002; Pawson and Scott, 2005). Subsequently, they demonstrated that phosphorylase b phosphorylation was mediated by a phosphorylase b kinase. Phosphorylase b kinase is controlled by a cAMP-dependent protein kinase (PKA), lending support to the early concept of protein kinase cascades (Krebs and Fischer, 1956; Walsh *et al.*, 1968; Cohen, 2002; Pawson and Scott, 2005). By the 1980s the crystal structure of phosphorylase a was solved and for the first time it was demonstrated that activation by either cAMP or phosphorylation resulted in a conformational change of phosphorylase a (Barford and Johnson, 1989; Pawson and Scott,

2005). During this time, G proteins and G-protein coupled receptors were also discovered and this knowledge coupled with PKA phosphorylation, resulted in clearer understanding of canonical signal transduction we know today (Pawson and Scott, 2005).

A well-known phosphorylation event that is necessary for regulating protein functions via subsequent signal transduction is serine/threonine phosphorylation (Pawson and Scott, 2005). In addition to serine/threonine phosphorylation, retroviral cytoplasmic oncoproteins, v-Src, v-Abl, and v-Fps produce phosphotyrosine as the product of protein kinase activity. Tyrosine phosphorylation is necessary for malignant transformation (Cohen, 2002; Pawson and Scott, 2005). Dysfunctional tyrosine kinase activity has been linked to human oncogenes, including Bcr-Abl, indicative of chronic myelogenous leukemia (CML) (Eckhart *et al.*, 1979; Cohen, 2002; Pawson and Scott, 2005). Similar to serine/threonine phosphorylation; phosphorylation of regulatory tyrosine residues may induce conformational changes in a target substrate stimulating enzymatic activity (Barford and Johnson, 1989).

Twenty years after the first protein kinase cascade was described by Edwin Krebs additional kinase cascades were characterized. The mitogen-activated protein kinase (MAPK) cascade was identified when it was discovered that a protein kinase activated by insulin phosphorylated MAP (Ray and Sturgill, 1987). Organisms such as plants, yeasts, and humans utilize numerous MAPK pathways for transduction of an array of extracellular stimuli. From the first discovery of phosphate over 100 years ago, to early studies of protein phosphorylation, its importance in nearly every physiological cellular process is evident.

Ubiquitin and Ubiquitination

Discovered in the mid 1970s, ubiquitin is a posttranslational protein modification involved in nearly all eukaryotic processes (Swatek and Komander, 2016). Ubiquitin is a highly conserved 8.6-kDa (76 amino acids) protein that when attached to target substrates can perform a myriad of functions. Ubiquitin is a compact and highly stable protein that adopts the β-grasp fold conformation with a flexible C-terminal tail (Komander and Rape, 2012). The main core of ubiquitin is rigid but the loop containing Leu8 maintains some flexibility important for substrate binding (Komander and Rape, 2012). The 96% sequence homology between yeast and human ubiquitin reinforces its evolutionary importance in eukaryotes. Ubiquitin typically modifies substrate proteins by covalently attaching to lysine's through an isopeptide bond

(Clague *et al.*, 2015). In addition to lysine attachment ubiquitin can attach to threonine, serine, cysteine residues and the amino terminus of target substrates (McDowell and Philpott, 2013). A key feature of ubiquitin is its ability to form chains; 7 lysine's in the N-terminus are sites for chain assembly (Komander and Rape, 2012). Formation of distinct linkages of ubiquitin to substrate (mono, multimono, homogeneous chain, mixed chain, branched chain, and unanchored chain), can elicit distinct responses in the cell, thereby greatly increasing the signaling potential of this small but versatile PTM (Komander and Rape, 2012).

Ubiquitin is covalently attached to target substrates via a multi-step enzymatic cascade. First the C-terminal glycine of ubiquitin is activated in an ATP-dependent manner via an E1 activating enzyme. At this point ubiquitin is linked via a thioester bond to a cysteine residue of the E1. The activated ubiquitin is then transferred to a cysteine residue of a ubiquitin-carrier protein (E2). Finally, ubiquitin is C-terminally attached via an amide isopeptide linkage. This linkage is catalyzed by a ubiquitin-protein ligase (E3), to the ε-amino group of the substrate protein's lysine residue (Hershko and Ciechanover, 1998). E3 ligases aid in the regulation of an array of cellular processes. Due to this, there are over 1000 ubiquitin E3 ligases encoded in the human genome (De Bie and Ciechanover, 2011). In order to prevent ubiquitination from being constitutively 'active' it can be reversed by deubiquitinating enzymes (DUBs). Similar to the variety of E3 ligases, there are over 100 DUBs encoded in the human genome (Komander and Rape, 2012).

Ubiquitin, when linked to a specific lysine (Lys48) in the form of mono, poly, or branched chains signals for substrate degradation by the proteasome (Pohl and Dikic, 2019). The proteasome is a large protease complex that degrades substrates tagged with ubiquitin into small peptides (Lecker *et al.*, 2006). The interaction between ubiquitin and the proteasome is known as the ubiquitin proteasome system (UPS), an important cellular quality control system (Pohl and Dikic, 2019). As a quality control system, the UPS has an important role in protein quality control, in which protein quality control machinery target misfolded proteins for degradation (Pohl and Dikic, 2019). In addition to targeting aberrant proteins the UPS also serves in a regulatory capacity. For instance, the UPS plays an integral role in the cell cycle by degrading cyclins thereby allowing progression from G1 to S (Glotzer *et al.*, 1991; Dang *et al.*, 2020).

Ubiquitination can also function nonproteolytically; tagged substrates can be used to alter localization, activity, and protein-protein interactions. For example, p53, a transcription factor undergoes Mdm2-mediated multimonoubiquitination it is shuttled out of the nucleus into the cytoplasm (Li *et al.*, 2003). In yeast, plasma membrane proteins can also be internalized after being monoubiquinated (Komander and Rape, 2012). With respect to alteration of protein activity, this can be accomplished by activating target substrates via ubiquitination of inhibitors. Finally, ubiquitination can help regulate protein-protein interactions. A well-studied example of this is the role of PCNA in the DNA damage response. After DNA damage, PCNA a homotrimer that encircles DNA is monoubiquitinated (Moldovan *et al.*, 2007; Freudenthal *et al.*, 2010). Once ubiquitinated, PCNA then acts as a scaffold and recruits proteins involved in DNA repair (Moldovan *et al.*, 2007; Freudenthal *et al.*, 2010). From the molecular perspective, it is clear that ubiquitin is a major regulator of many cellular processes. Dysfunction in ubiquitination and the UPS can result in multiple disease states, from cancer to neurodegeneration. Thus, much of the current research in the field has been dedicated to elucidating potential druggable targets within the system (Fhu and Ali, 2021; Kiely-Collins *et al.*, 2021).

Ubiquitin like proteins

In addition to ubiquitin, eukaryotes utilize a family of proteins that covalently attach to an array of substrates, and can alter protein-protein interactions, activity and localization. Proteins in this family are classified as ubiquitin-like proteins (UBLs) (Van Der Veen and Ploegh, 2012; Vierstra, 2012). Similar to their namesake, UBLs share sequence homology and three-dimensional structures analogous to ubiquitin. UBLs attach by their C-terminal tails to substrates via an enzymatic cascade (Van Der Veen and Ploegh, 2012; Vierstra, 2012). Unlike ubiquitin, UBLs have fewer known targets and significantly less E2 conjugating enzymes and E3 ligases, suggesting that they likely emerged later than ubiquitin.

Some well-studied eukaryotic UBLs include: NEDD8, ISG15, ATG8 and ATG12. NEDD8 (neural precursor cell-expressed, developmentally downregulated) initially was one of ten *Nedd* genes identified in embryonic mouse brain (Enchev *et al.*, 2015). NEDD8 expression is increased during the early stages of development. Additionally, the enzymes required for neddylation are overexpressed in cancers. Neddylation is an essential process in eukaryotes with the exception of budding yeast, where it is conserved but not required (Enchev *et al.*, 2015). Of all the known UBLs, NEDD8 shares the highest sequence homology with ubiquitin

(~60%). The major target of neddylation is the cullin-RING ligases, which comprise the largest ubiquitin E3 ligase family. Cullins serve as a scaffold for proteins in the assembly of the RING E3 ligases (Van Der Veen and Ploegh, 2012; Enchev *et al.*, 2015). A notable recently discovered target of NEDD8 is the tumor suppressor PTEN. Neddylation of PTEN results in import of PTEN into the nucleus leading to tumorigenesis via enhancement of PI3/Akt signaling and stabilization of fatty acid synthesis (Xie *et al.*, 2020).

ISG15 was the first UBL identified and is the product encoded by interferon-simulated gene 15. There are two domains of ISG15 that share a high sequence homology to ubiquitin. ISG15 expression is induced by type I interferons (INF$\alpha$ and INF$\beta$), secreted from virally infected cells (Van Der Veen and Ploegh, 2012). ISG15 targets viral and cellular proteins, while some targets identified by proteomic studies are induced by interferon and have a known role in antiviral immunity many other targets do not (Van Der Veen and Ploegh, 2012). While the function of ISGylation was initially unclear, recent work has uncovered its role in maintaining genome integrity, specifically by modification of target proteins involved in the DNA damage response (Sandy *et al.*, 2020). After translesion DNA synthesis (TLS) is triggered in response to DNA damage, it then needs to be 'deactivated' to prevent mutagenesis. After DNA lesion bypass, modification of activated PCNA by ISG15 is essential in terminating TLS (Park *et al.*, 2014). ISGylation also plays a role in protein degradation, autophagy, attenuation of hypoxia, activation of cytokines and a host of other cellular processes (Sandy *et al.*, 2020).

ATG8 and ATG12 are major components of autophagy machinery and are essential for autophagosomal membrane growth and share very little sequence homology with ubiquitin (Van Der Veen and Ploegh, 2012). Atg8 modifies lipids rather than proteins and is a major element in autophagosome biogenesis. In addition to autophagy, ATG8s can be integrated into phagosomes, macropinosomes, endosomes and entotic vacuoles where they fuse with lysosomes to degrade extracellular cargo (Nieto-Torres *et al.*, 2021). ATG8s can also interact with viral components and play an integral role in host defense through degradation by autophagy of viral proteins, and host cell machinery (Nieto-Torres *et al.*, 2021). ATG12 forms a multimeric complex through homo-oligomerization with Atg-5 and Atg16L. This complex is located on the outer membrane of the autophagosome where it functions as an E3 ligase for Atg8 (Van Der Veen and Ploegh, 2012).

One of the most recently discovered UBLs is the Ubiquitin-fold modifier 1 (UFM1) similar to its UBL family members, UFM1 conjugates to target substrates via a three-step enzymatic cascade (Van Der Veen and Ploegh, 2012). UFM1 is conserved across eukaryotes with the exception of yeast. Ufmylation is a reversible modification that is involved in several cellular processes (Van Der Veen and Ploegh, 2012). Modification by UFM1 has been linked to endoplasmic reticulum (ER) stress, hematopoiesis, GPCR biogenesis and fatty acid metabolism (Wei and Xu, 2016). Recently, it has been reported that the MRE11, an integral part of the DNA damage response machinery complex, is modified by UFM1. Ufmylation at key lysine's (Lys 281, Lys 282) on MRE11 promotes interaction with telomere binding factor, TERF2 and is essential for maintaining telomere length (Lee *et al.*, 2019; Banerjee *et al.*, 2020). While the newest of the UBLs, emerging evidence has demonstrated that UFM1 modification has been linked to an array of human diseases from schizophrenia to cancer (Banerjee *et al.*, 2020).

Currently there are 16 known ubiquitin like modifiers. Similar to ubiquitin, UBLs are integral to an array of physiological processes within a cell. While some well-studied UBLs have been discussed above, the most recognized UBL is the small ubiquitin like-modifier (SUMO). The following chapters will explore the history, function, and role this small but mighty posttranslational modification plays in cellular adaptation and stress responses.

## Chapter 2: Role of SUMO and sumoylation during stress

The small ubiquitin like-modifier (SUMO) is a posttranslational protein modification that is structurally similar to ubiquitin. In *Saccharomyces cerevisiae*, *SMT3*, an essential gene encoding SUMO was isolated as a high copy suppressor of mutation in *MIF2* (Meluh and Koshland, 1995). Although first discovered in yeast, SUMO is highly conserved across eukaryotes and plants (Mannen *et al.*, 1996; Lapenta *et al.*, 1997; Vierstra and Callis, 1999). Some organisms such as *Saccharomyces cerevisiae* possess a single SUMO gene, while plants and humans have several. SUMO is a 11kDa protein with a C-terminal β-grasp fold nearly indistinguishable from ubiquitin. Though structurally analogous, SUMO shares little sequence homology with ubiquitin (~20%). SUMOs have 20 additional amino acid residues located at the N-terminus in the form of an unstructured tail that aid in chain formation (Bayer *et al.*, 1998; Johnson, 2004).

SUMO genes encode small C-terminal precursor peptides that are cleaved by SUMO specific proteases resulting in a mature Gly-Gly carboxy terminus. Mature SUMO is activated by an E1 heterodimer (UBA2/AOS1- yeast and humans; SAE1a/SAE1b- plants) in an ATP-dependent manner resulting in a thioester bond between the C-terminal Gly residue and a Cys residue in the activating enzyme (Johnson *et al.*, 1997). SUMO is then transferred to the E2 (UBC9- yeast and humans; SCE- plants) conjugating enzyme where it forms a thioester bond between the catalytic Cys residue of the E2 and the C-terminal Gly of SUMO (Desterro *et al.*, 1997; Johnson and Blobel, 1997). Finally, SUMO is transferred from the conjugating enzyme to its target substrate via an E3 ligase that catalyzes transfer. SUMO is attached by an isopeptide bond between its C-terminal Gly residue and a Lys residue of the target substrate (Figure 2.1). Sumoylation can result in the addition of a single SUMO or a chain. Unlike ubiquitin, SUMO chains are unbranched (Cheng *et al.*, 2006). While a consensus site for SUMO attachment has been identified, not all target substrates contain the ΨKxE motif, where Ψ is an aliphatic hydrophobic residue and x represents any amino acid (Johnson and Blobel, 1999; Hoege *et al.*, 2002; Johnson, 2004). During stress, sumoylation dynamically changes by rapidly attaching and detaching from substrates and sumoylation is in part, regulated by stress (Morrell and Sadanandom, 2019; Filippopoulou *et al.*, 2020; He *et al.*, 2020).

**Transcriptional regulation**

SUMO and sumoylation have a prominent role in transcriptional regulation and in some aspects this regulation is linked to environmental stress. A notable example of this dynamic is yeast Cyc8-Tup1 transcriptional co-repressor complex, that is rapidly and transiently sumoylated during hyperosmotic stress (Oeser *et al.*, 2016). The Cyc8-Tup1 co-repressor complex is required for repression of genes from four distinct gene classes (Tzamarias and Struhl, 1995). Through gene expression analysis of genes previously reported to be repressed by the Cyc8-Tup1 co-repressor complex were upregulated in sumoylation deficient mutants when compared to wild type after exposure to hyperosmotic stress (Oeser *et al.*, 2016). Another major finding from this study was that the genes upregulated in non-sumoylatable Cyc8 and Tup1 mutants were distinct. During hyperosmotic stress, Tup1 regulates genes proximal to telomeres while hexose transporters and other genes regulated by Cyc8 are glucose repressed (Oeser *et al.*, 2016). These findings are of particular interest with respect to the Cyc8 mutant data because hexose sugars can serve as intracellular osmolytes under high salt conditions.

Additional studies in human cells have also demonstrated that sumoylation has an essential role in regulation of transcription during stress conditions. One study reported that Notch1, a component of the Notch signaling pathway is sumoylated during cellular stress. The Notch signaling pathway regulates stem cells during development and dysfunction in the pathway can lead to developmental defects and cancer (Carrieri and Dale, 2017; Giuli *et al.*, 2019; Pagliaro *et al.*, 2021). The Notch pathway is affected by a range of stress conditions such as irradiation, hypoxia, and oxidative (Gustafsson *et al.*, 2005; Oh *et al.*, 2013). In HeLa cells it has been reported that the intracellular domain of Notch1 is sumoylated in the nucleus after heat stress (Antila *et al.*, 2018). Subsequent to sumoylation of the intracellular domain induced by heat stress, expression of gene targets critical for determination of cell fate become repressed (Antila *et al.*, 2018). Repression of gene targets during heat stress is facilitated via sumoylation of Notch1 and subsequent recruitment of HDAC4 to sites of transcription (Rosonina *et al.*, 2017; Antila *et al.*, 2018).

Besides regulating transcription via interaction with specific transcription factors/complexes which in some cases result in the eventual recruitment of co-regulators that can either repress or upregulate transcription of target genes, SUMO modifications can also physically interfere with other posttranslational modifications (Rosonina *et al.*, 2017). This

interference has been shown to impede phosphorylation and acetylation of specific transcription factors. With respect to phosphorylation, transcription factor STAT5 which regulates transcription of genes essential in development and the immune system (Recio *et al.*, 2019) can be modified by several different PTMs including SUMO (Bosque *et al.*, 2017; Rosonina *et al.*, 2017). Since the lysine's necessary for sumoylation of STAT5 (Lys696 and Lys700) are in such close proximity to the tyrosine required for phosphorylation (Tyr694) sumoylation of STAT5 physically inhibits phosphorylation and transcriptional activation of STAT5 (Van Nguyen *et al.*, 2012; Rosonina *et al.*, 2017). A similar dynamic has been observed in the context of acetylation. HIC1 a product of a tumor suppressor gene when acetylated blocks its ability to interact with MTA1, an HDAC component that subsequently reduces its ability to repress gene targets (Van Rechem *et al.*, 2010; Rosonina *et al.*, 2017). Sumoylation of HIC1 however, restores gene repression by reestablishing its interaction with MTA1 (Van Rechem *et al.*, 2010; Rosonina *et al.*, 2017).

**Protein activity and localization**

Sumoylation can affect protein activity and localization during stress conditions in the cell. One of the first reported instances of SUMO directly impacting localization was illustrated with small GTPase RanGAP. RanGAP plays an integral role in nuclear import and the unmodified protein is located in the cytoplasm (Gill, 2004). Upon sumoylation however, RanGAP is shuttled to the nucleus where it interacts with Nup358 a component of the nuclear pore (Matunis *et al.*, 1996; Gill, 2004). Another well-known example of sumoylation's impact on subnuclear localization during cellular stress was shown in cardiomyocytes. An essential kinase complex IKK, involved in the regulation of NF-kappaB signaling undergoes sumoylation in certain contexts (Gill, 2004; Ramana *et al.*, 2004; Le *et al.*, 2017). The IKK kinase complex contains NEMO, a regulatory subunit that is generally found in the cytoplasm (Huang *et al.*, 2003; Le *et al.*, 2017). Under oxidative stress, however, NEMO is sumoylated resulting in increased NF-kappaB signaling and subsequent translocation of NEMO from the cytoplasm to the nucleus (Huang *et al.*, 2003; Le *et al.*, 2017). SUMO proteases have also been shown to shuttle between the cytoplasm and nucleus during stress conditions. In yeast, Ulp1 is typically localized to the nuclear pore complex (NPC) in the nucleus under normal unstressed conditions in *Saccharomyces cerevisiae* (Li and Hochstrasser, 2003). After exposure to high levels of ethanol however, Ulp1 translocates to the nucleolus where it co-localizes with Noc3 and Nop62 components of the NPC (Sydorskyy *et al.*, 2010). While this translocation is not a direct result of

sumoylation per se, it is important to note that upon ethanol stress, the NPC becomes more soluble, and this increased solubility releases Ulp1 (Sydorskyy *et al.*, 2010). This finding is notable because as a protease Ulp1 cleaves SUMO and during ethanol stress steady state levels of SUMO increase. It has been speculated that Ulp1 and the NPC act as a type of sensor for ethanol stress and that at above a threshold that has yet to be determined, Ulp1 is released and the sumoylation response is activated (Sydorskyy *et al.*, 2010).

**Chromatin and Genome Integrity**

In recent years there have been several studies illustrating the important role sumoylation plays in maintaining chromatin structure and overall genome integrity specifically in response to cellular stress conditions. Replication of DNA is a cornerstone for maintaining overall genome integrity but DNA replication like many other cellular processes can be negatively impacted by stress (Huen and Chen, 2010; Tian *et al.*, 2021). In order to mitigate potential DNA damage as a result of stress, the cell has developed mechanisms like replication fork reversal (Ciccia and Elledge, 2010; Huen and Chen, 2010; Tian *et al.*, 2021). A recent study has shown that even in this tightly regulated response to stress, sumoylation has an essential role. In mammalian cells arrested in S-phase, TOPIIA a DNA topoisomerase that relieves strain on newly replicated chromatids accumulates at stalled replication forks (Tian *et al.*, 2021). Subsequently fork stalling promotes sumoylation of TOPIIA and facilitates fork reversal through the recruitment of additional factors (Tian *et al.*, 2021). Whole genome ChIP-seq in *Arabidopsis thaliana* has also demonstrated that after heat shock, distribution of SUMO on chromatin changes (Han *et al.*, 2021). After heat stress, SUMO becomes enriched at genes necessary for mounting a stress response (Han *et al.*, 2021). This result bolsters the work we and others have done showing that SUMO dynamically interacts with chromatin during stress.

While I have only scratched the surface of the interplay between SUMO and the cellular stress response, it is evident even with the examples outlined above that SUMO and sumoylation change dynamically during cellular stress conditions and impact virtually all cellular processes. Since targets of SUMO are largely distinct between stress conditions, there are many open questions remaining in the field with respect to identification of target substrates and the functional reasons why they are sumoylated. Given the breadth of environmental stresses this thesis will briefly discuss work I completed examining hyperosmotic and heavy metal stress before delving more deeply into acute ethanol induced sumoylation and my discoveries.

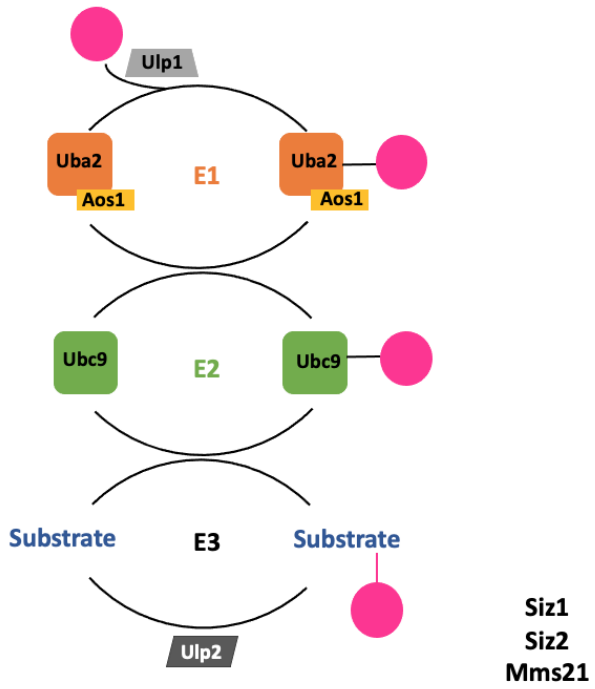


**Figure 2.1 Yeast SUMO enzymatic cascade**

## Chapter 3: Investigation of SUMO modifications and prionogenic protein switching during hyperosmotic stress and preliminary investigation of heavy metal stress induced sumoylation

When I first joined the Gardner lab, I intended to work on distinct projects that investigated different environmental stress conditions and the sumoylation of specific targets. These projects included following up a study on the sumoylation of prionogenic Cyc8 during hyperosmotic stress that was initially discovered by previous graduate student Michelle Oeser. Additionally, I was interested in identifying proteins that were sumoylated during heavy metal stress, ethanol stress and understanding their functions.

Prionogenic proteins have the ability to dynamically switch conformations (Alberti *et al.*, 2009). A well-known conformation results in the formation of insoluble protein aggregates known as amyloids, which in humans are historically thought to be pathogenic. As an example, amyloid formation is linked to several neurodegenerative disorders like Alzheimer's, Parkinson's, Huntington's and ALS (Shao and Diamond, 2007). Prions, however, can confer considerable cellular benefits. Studies in yeast, plants, and human neurons have revealed that prionogenic conformational switching to non-amyloid states leads to cellular adaptation that is advantageous during exogenous stress. The adaptive conformational switching in prionogenic proteins is thought to be beneficial because, within a population of cells some of the prionogenic proteins are already in an adaptive state (Alberti *et al.*, 2009; Halfmann *et al.*, 2010; Newby and Lindquist, 2013). This concept is known as cellular 'bet-hedging', and is often seen in microbial communities that elicit heritable responses to evolve new phenotypes during rapidly changing environmental conditions (Halfmann *et al.*, 2010; Newby and Lindquist, 2013). A major open question in the field is to determine how post-translational modifications control prionogenic conformational switching.

Michelle's work previously showed that, in *Saccharomyces cerevisiae*, hyperosmotic stress causes a rapid, but transient increase in the post translational modification of proteins by the SUMO (Oeser *et al.*, 2016). Hyperosmotic stress is a relevant stress to understand because in humans, osmoregulation of body fluids is essential for maintaining the balance of water. Disruption in this balance by hyperosmotic stress, can result in increased levels of salt in the blood (hypernatremia) as well as kidney damage. Mass spectrometry analysis revealed that Cyc8, a highly-conserved transcription co-repressor protein (Oeser *et al.*, 2016), is highly

sumoylated during hyperosmotic stress. Further analysis showed that Cyc8 forms inclusions during hyperosmotic stress and that this inclusion formation is regulated by sumoylation of Cyc8 (Oeser *et al.*, 2016). Cyc8 contains a conserved glutamine-rich (Q-rich) prion domain (PrD) which forms the [OCT+] prion when overexpressed (Patel *et al.*, 2009). Sumoylation of Cyc8 coincides with the cells ability to reverse inclusion formation. Given this dynamic we proposed that sumoylation may keep the PrD of Cyc8 in a soluble state which protects against prion formation (Oeser *et al.*, 2016). Based on these findings I attempted to characterize the PrD of Cyc8 by determining if Cyc8 inclusion formation alleviates transcriptional repression as well as determining if the PrD is required for upregulation of transcription.

**Is Cyc8's prion domain required for transcriptional de-repression?**

Early studies illustrated that the Cyc8 co-repressor complex was required for repression of thousands of genes and that the complex does not bind directly to the promoter (Tzamarias and Struhl, 1994, 1995). Through truncation analysis and yeast 2-hybrid screens, it was shown that the amino-terminal 175 residues of Cyc8 (Figure 3.1) are necessary and sufficient for formation of the Cyc8 transcription co-repressor complex *in vivo* (Tzamarias and Struhl, 1994, 1995). These findings were intriguing because the PrD and sumoylation sites of Cyc8 are not essential for establishment of transcriptional repression (Tzamarias and Struhl, 1994). We hypothesized that the PrD of Cyc8 is required for transcriptional de-repression rather than repression. To test this, I performed chromatin immunoprecipitation (ChIP) on two strains generated by Michelle: Cyc8, and Cyc8 with the PrD deleted. In Michelle's study she reported that 62% of upregulated genes are repressed by glucose and some were hexose transporters. She specifically found distinct differences in gene expression in the hexose transporters *HXT6* and *HXT7* (Oeser *et al.*, 2016). From her results we chose to examine binding of wild-type Cyc8 and the mutant to the promoters of HXT6 and HXT7. I found that compared to wild type Cyc8 lacking its PrD had decreased binding at HXT6 and HXT7 promoters at peak sumoylation (15 minutes) suggesting that the PrD is required for interaction with the genome during hyperosmotic stress (Figure 3.2).

**Determine if Cyc8 inclusion formation alleviates transcriptional repression**

We previously reported that sumoylation of Cyc8 coincides with the cells ability to reverse inclusion formation and hypothesized that sumoylation acts as a solubilizing factor that keeps the PrD of Cyc8 from forming amyloids during hyperosmotic stress. In our proposed

model, under basal conditions the Cyc8 co-repressor complex is associated with chromatin and genes are repressed (Figure 3.3A) but after exposure to environmental stress conditions Cyc8 complexes are released from chromatin and form inclusions. This inclusion formation leads to activation (de-repression) of RNAPII and transcription and gene expression of stress-related genes (Figure 3.3B). Once stress-related genes have been expressed, Cyc8 inclusions become sumoylated (Figure 3.3C), and sumoylation aids to rapidly reverse inclusions to re-establish repression of Cyc8-target genes (Figure 3.3D). Since Cyc8's sumoylated PrD is not required for establishment of transcriptional repression (Tzamarias and Struhl, 1994, 1995), we hypothesized that the conserved glutamine (Q) rich content of Cyc8's PrD is essential for transient inclusion formation during hyperosmotic stress. Next, I wanted to determine if the Cyc8 PrD mutants had any noticeable growth defects after exposure to hyperosmotic stress. To test, I performed spot dilution assays utilizing the following four strains: $Cyc8^{WT}$, $Cyc8^{Non\text{-}SUMO}$, $Cyc8^{PrD-}$, $Cyc8^{Non\text{-}SUMO/PrD-}$. Interestingly I found that when compared to controls Cyc8 with the prion domain deleted exhibited slightly slower growth, suggesting that the prion domain of Cyc8 has a role in maintaining normal growth in budding yeast (Figure 3.4).

A major question I tried to answer was to determine the effects altering the QA repeats and the polyQ tract of Cyc8's PrD had on inclusion formation and transcriptional de-repression. It has been shown that there is genetically diverse variation within the poly Q tract and QA repeats of Cyc8's PrD (Figure 3.5) in different wild *S. cerevisiae* strains (Gemayel *et al.*, 2015). Intriguingly gene expression profiles of shorter repeat lengths differed from the longer ones (Gemayel *et al.*, 2015). This finding was exciting because it is well known that expansion of polyQ tracts in humans increases the chances of amyloid formation, which can lead to neurodegeneration (Shao and Diamond, 2007). Therefore, it is likely that modification of the polyQ tract and QA repeats in Cyc8 will shift the transient inclusion state we reported during HOS (Oeser *et al.*, 2016) to a more stable amyloid state. To test the relationship between the polyQ tract and QA repeat length in Cyc8 during HOS, I initially made three different QA variants: short (22QA), normal (32QA), and long (40QA) with a wild-type (31Q) in both sumoylatable and non-sumoylatable versions. To elucidate the effects the variants had on Cyc8 sumoylation I exposed the strains to HOS over a 60-minute time course and analyzed inclusion formation by fluorescence microscopy (Figure 3.6). All three strains exhibited transient inclusion formation that began at 5 minutes post stress exposure, peaking at 15 minutes before resolving after the full hour. Given that I did not observe any noticeable changes between the strains, I next decided to examine whether my pilot strains had any discernible phenotypic and growth

defects by performing spot dilution tests on plates containing high salt (Figure 3.7). Compared to controls I did not observe any differences in growth in the Cyc8 PrD variants. To be certain I was not missing potential changes in the variant strains after exposure to hyperosmotic stress, I performed SUMO pulldowns with the 22QA/31Q and 32QA/31Q strains (Figure 3.8). While I had robust inputs, I did not detect any SUMO conjugates, this could have been due to the fact that my strains were tagged with GFP rather than HSV.

When I started this project, I planned on making additional Cyc8 QA/Q variants but cloning especially with the longer repeats was nearly impossible for me to achieve. After nearly a year of attempts and my preliminary microscopy data not showing distinct differences between variants, I chose to attempt a different approach. I was fortunate to have all the variants that were reported in the 2015 Molecular Cell paper titled "Variable Glutamine-Rich Repeats Modulate Transcription Factor activity," (Gemayel *et al.*, 2015) sent to me. After receiving all ten strains I successfully PCR amplified the following PrD's from Cyc8: 0QA, 14QA, 20QA, 37QA, 31QA, 33QA, 53QA, 55QA, 90QA, and 105QA (Figure 3.9). I then cloned the following PrDs: 20QA, 33QA, 55QA, 90QA, and 105QA into Cyc8-GFP and verified by sequencing. I subsequently transformed the 33QA variant into yeast and completed a preliminary experiment. Unfortunately, I could not detect GFP or inclusions by fluorescence microscopy. This is could be due to a frameshift mutation that may have occurred during cloning.

**Heavy metal stress induced sumoylation**

While our group had been studying the effects of sumoylation induced by hyperosmotic stress we were also incredibly interested in studying sumoylation induced by heavy metal stress. The study of heavy metals is relevant especially from an environmental and public health perspective. Specifically, with respect to the water supply pollution by heavy metals can be either man made or naturally occurring. Heavy metal exposure is toxic and a threat to humans and marine life. I was interested in investigating heavy metal stress in the context of sumoylation because we and others have shown that sumoylation aids in cellular adaptation and survival. Initially I wanted to examine global sumoylation in yeast after exposure to lead. We chose to first start with lead because at the time we as a nation were all looking on in horror to the Flint Michigan water crisis. Studying lead induced sumoylation responses proved to be a challenge however, because when I first attempted to make YPD media with lead (III) nitrate, the lead immediately precipitated out of solution. Due to this complication I searched the literature for how to expose yeast cells to lead and found that by switching the media from YPD

to MES (Sousa and Soares, 2014) a buffer that does not complex with lead. I then with the help of summer student Felicia Blandov, exposed our $His_6$-FLAG-*SMT3* strain to a 60 minutes of 100uM lead stress. I chose to start with a 100uM because it had been previously reported that yeast cells remain viable over a 48 hour period in MES (Sousa and Soares, 2014). By Western analysis we observed significant high molecular weight SUMO conjugates accumulate between 15 and 60 minutes (Figure 3.10).

In addition to piloting lead stress induced sumoylation I also investigated whether sodium arsenite could also elicit a sumoylation response. I first used two relatively high concentrations of sodium arsenite, 25 and 50mM. After a 60-minute time course I found that I was not detecting the expected high molecular weight SUMO conjugates (Figure 3.11). I concluded that I was likely killing my cells with such high concentrations and decided to take a different approach to determine an ideal concentration. To that end I chose to examine significantly lower arsenite concentrations and ask whether cultures exhibited normal growth. I examined the following arsenite concentrations in liquid culture: 0mM, 0.375mM, 0.750mM, and 1.0mM. I found that all arsenite exposed cultures grew slower than untreated with 1mM unsurprisingly the slowest (Figure 3.12). Given this data the 0.375mM arsenite concentration seemed like the best concentration because compared to the untreated doubling time of 70 minutes, 0.375mM cultures were approximately 20 minutes behind at 90 minutes (Figure 3.12). While 0.375mM of arsenite looked like the ideal concentration to pursue, I chose to examine global sumoylation after arsenite stress over 60 minutes for all concentrations for completeness (Figure 3.13). Altogether sumoylation induced by arsenite was observed at 15 minutes before returning to levels below the initial baseline untreated 0 time point.

While I unfortunately could not complete the intriguing environmental stress and sumoylation stories above, this work allowed me to gain a much broader understanding to the vast role SUMO has within the cell. With respect to the hyperosmotic stress and Cyc8 project it will be interesting to determine if the length of the PrD truly is important to effective gene repression. Conversely, the pilot projects on heavy metals have me considering what potential proteins could be sumoylated after exposure. Some initial thoughts make me think that there would be some overlap from the proteins I identified in my Mass Spectrometry analysis examining acute ethanol. In that screen the top candidates that appeared to be sumoylated were chromatin-associated transcription factors and proteins required for chromatin maintenance. The remainder of this work will examine my findings and conclusions on the acute ethanol response in yeast and the role that sumoylation might be playing.

**Acknowledgements**

Thank you to Michelle Oeser for her previous work that helped to inform much of the work in this chapter

Thank you to Matt Kaeberlein's lab and Mitchell Lee for help with the Bioscreen C and data analysis


**Figure 3.1 Schematic of Cyc8**

Region of Cyc8 required for transcriptional repression denoted by bracket. Prion domain (green box) SUMO sites denoted by vertical blue lines.

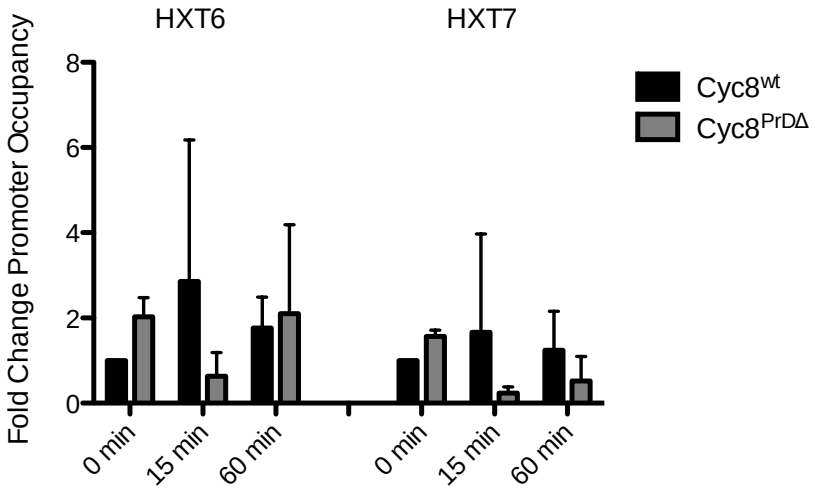


**Figure 3.2 PrD necessary for interaction with the genome**

Indicated cells were grown in triplicate in rich medium, treated with 1.2M sorbitol, and collected at denoted timepoints. Cyc8-DNA complexes were crosslinked with formaldehyde, immunoprecipitated via incubation with an anti-HSV antibody, and analyzed by qPCR with specific primers. Changes in Cyc8 promoter occupancy were corrected to ACT1 and represented as fold change over pre-stress condition. Error bars indicate SD.

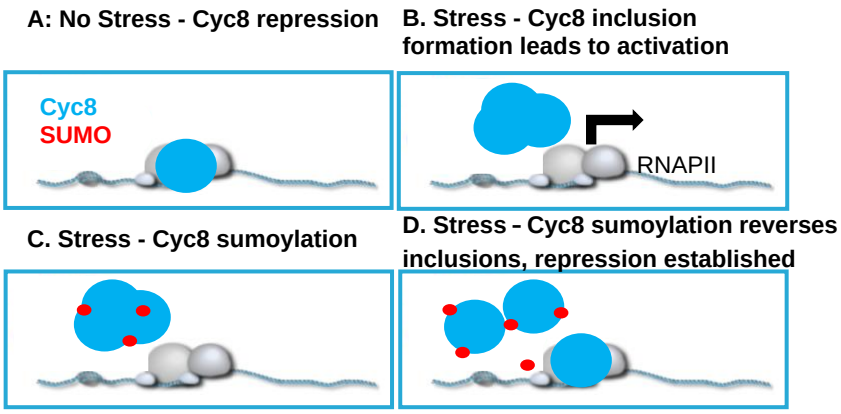


**Figure 3.3 Proposed model of Cyc8 inclusion formation**

Model of Cyc8 inclusion formation and transcriptional de-repression via sumoylation during HOS (Oeser *et al.*, 2016).

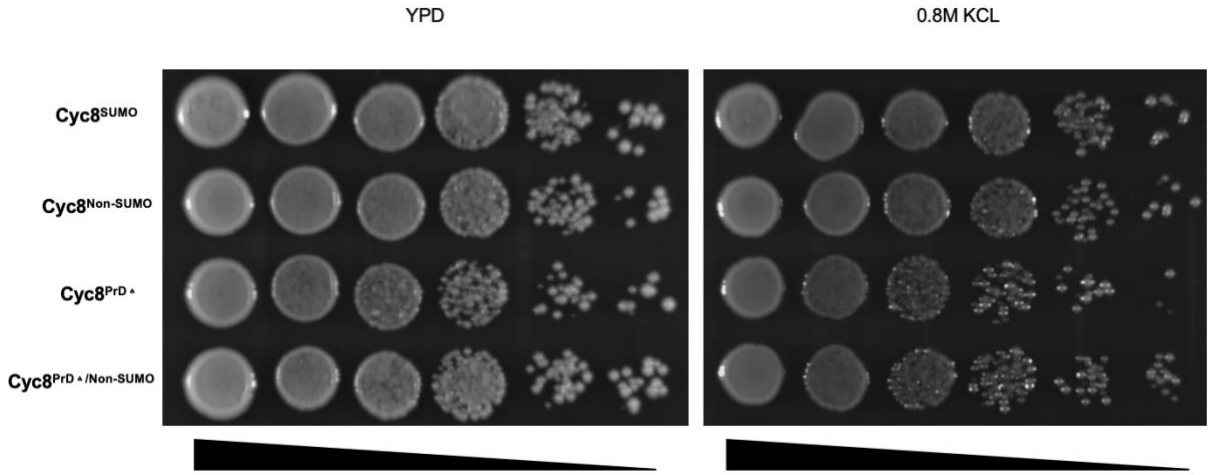


**Figure 3.4 Growth of Cyc8 PrD deficient mutants during hyperosmotic stress**

$His_6$-FLAG-*SMT3* cells ($Cyc8^{WT}$, $Cyc8^{Non\text{-}SUMO}$, $Cyc8^{PrD-}$, $Cyc8^{Non\text{-}SUMO/PrD-}$) were grown to mid-log phase in rich liquid media then 10-fold serially diluted onto rich media plates with and without salt and incubated at 30C for 3 days.

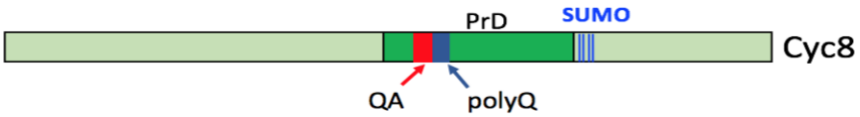


**Figure 3.5 Schematic of Cyc8 PrD**

PrD marked by dark green rectangle, QA region (red), PolyQ (dark blue), and SUMO sites vertical blue lines.

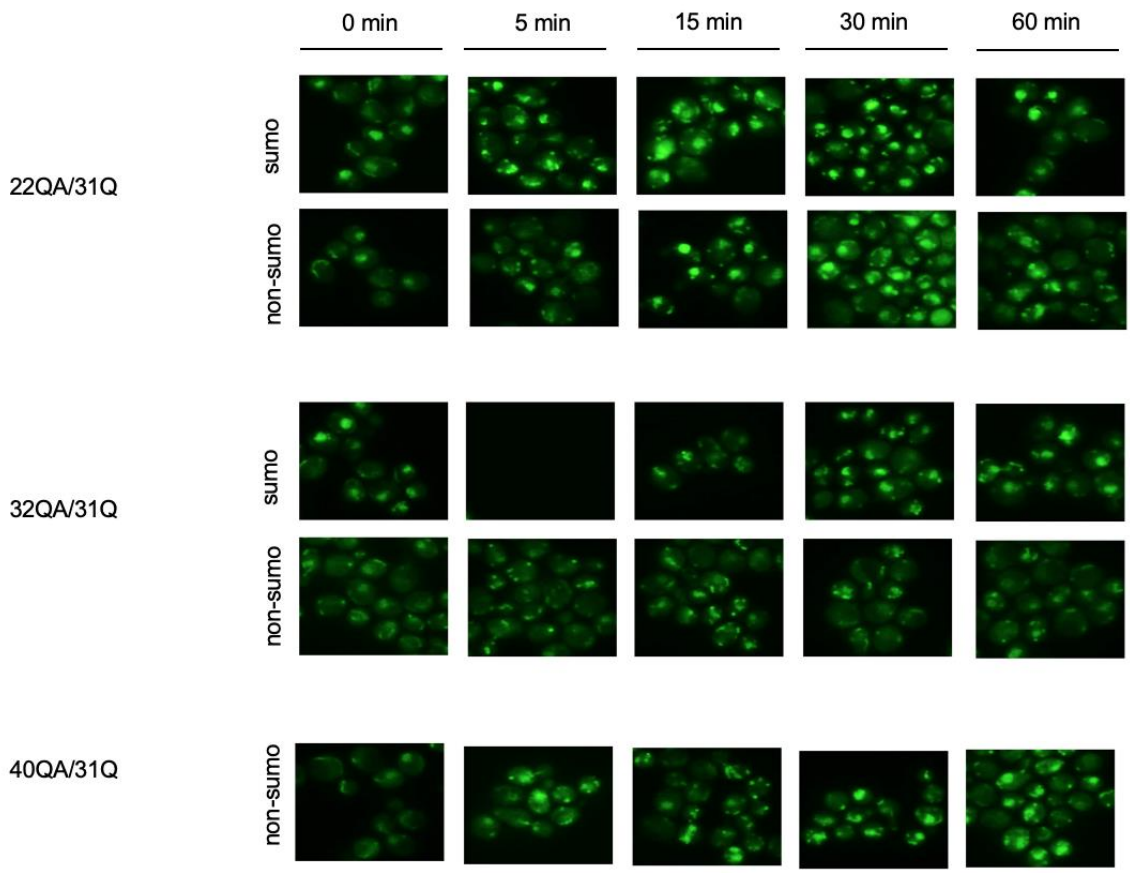


**Figure 3.6 Cyc8 QA variant hyperosmotic stress induced inclusions**

Cyc8 QA variant cells were exposed to 1.2M sorbitol over a 60-minute time course. Cells fixed at indicated timepoints and imaged by fluorescent microscopy.

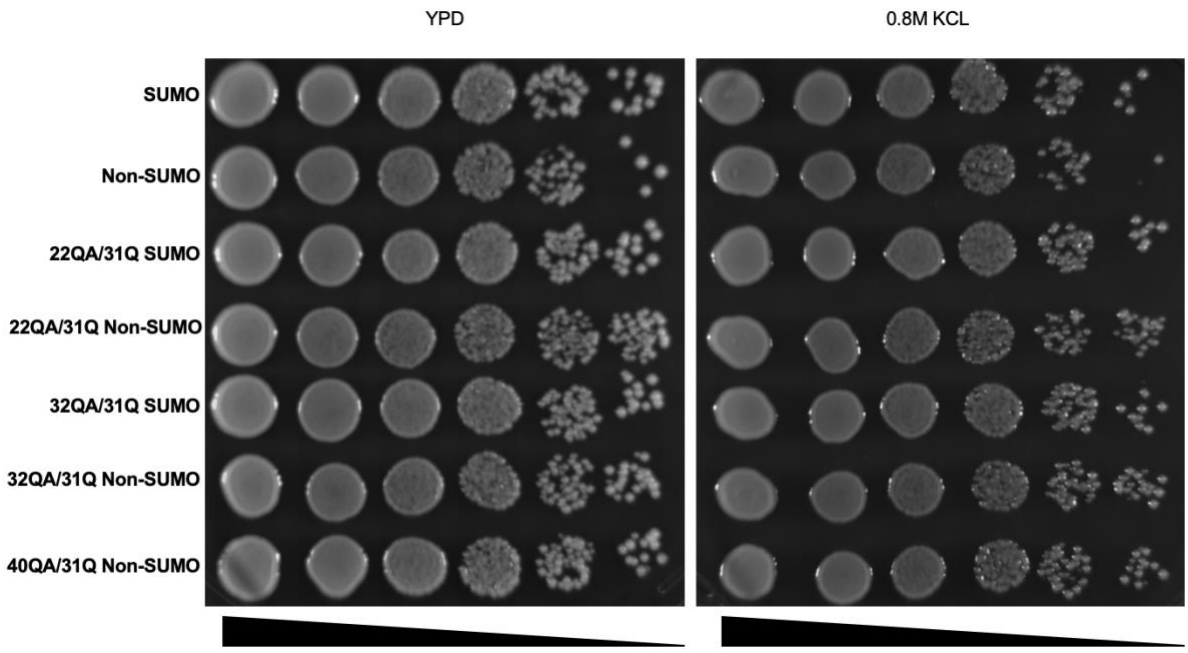


**Figure 3.7 Cyc8 QA variant growth during hyperosmotic stress**

Cyc8 QA variant strains with $His_6$-FLAG-SMT3 were grown to mid-log phase in rich liquid media then 10-fold serially diluted onto rich media plates with and without salt and incubated at 30C for 3 days.

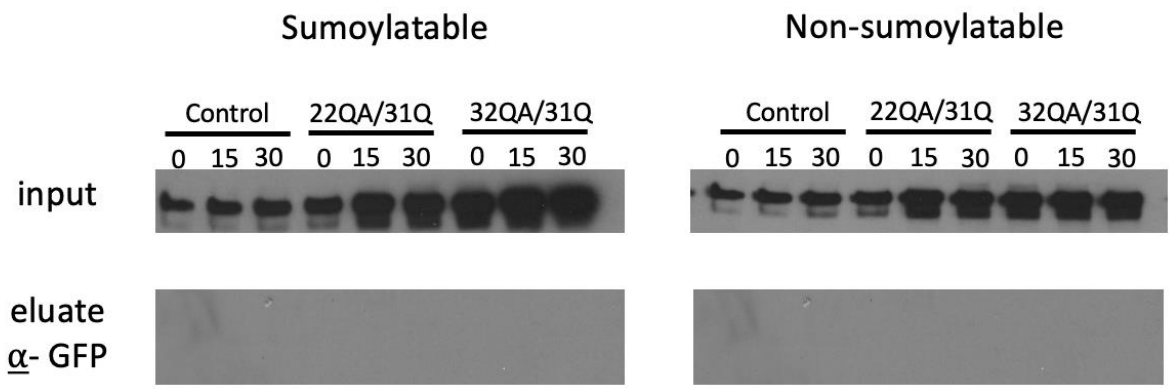


**Figure 3.8 Sumoylation of Cyc8 QA variants during hyperosmotic stress**

Cells expressing $His_6$-FLAG-*SMT3* and either C-terminally 3xHSV epitope tagged Cyc8 (wt, 22QA/31Q, or 32QA/31Q) from their endogenous promoters were subject to 1.2M sorbitol over a 30-minute time course. Cell lysates (input) and purified sumoylated proteins (eluate) were examined via Western analysis using an anti-GFP antibody to detect wild type Cyc8 and the Cyc8 PrD variants.

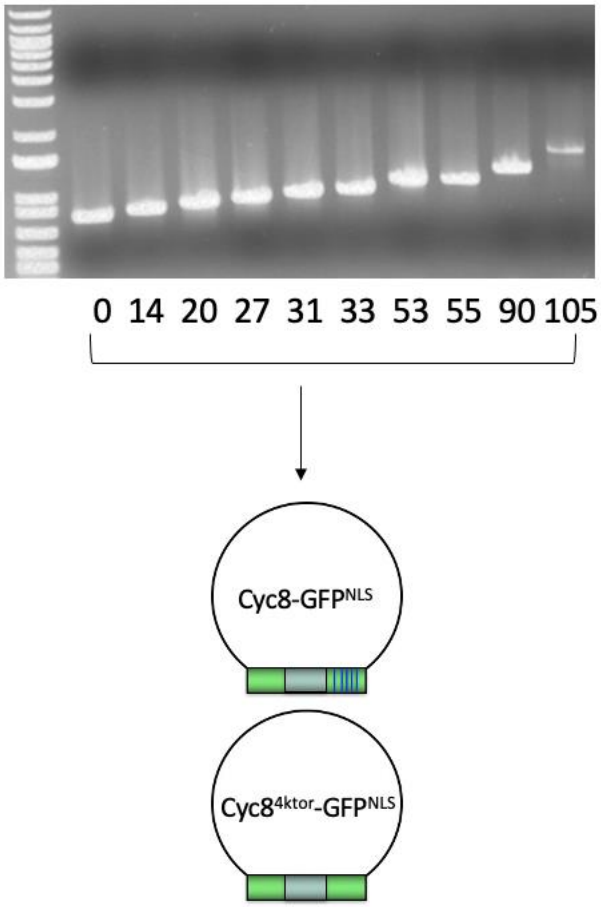


**Figure 3.9 PCR amplification of variant Cyc8 prion domains**

PCR amplification of variant Cyc8 prion domains ranging from 0-105. Fragments were amplified with restriction site sequences for digestion and cloning into Cyc8-GFP plasmids containing an NLS.

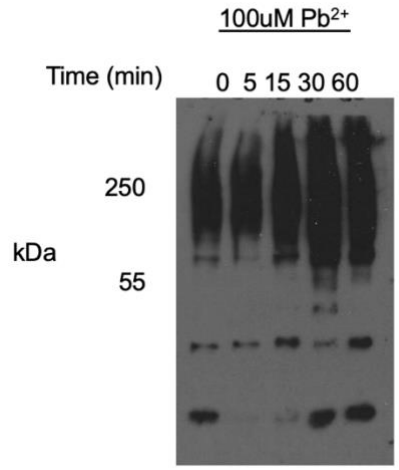


**Figure 3.10 Lead stress induces sumoylation**

$His_6$-FLAG-*SMT3* global sumoylation patterns after 100uM lead stress over 60 minutes. Changes in sumoylation patterns were examined by Western analysis using an anti-FLAG antibody.

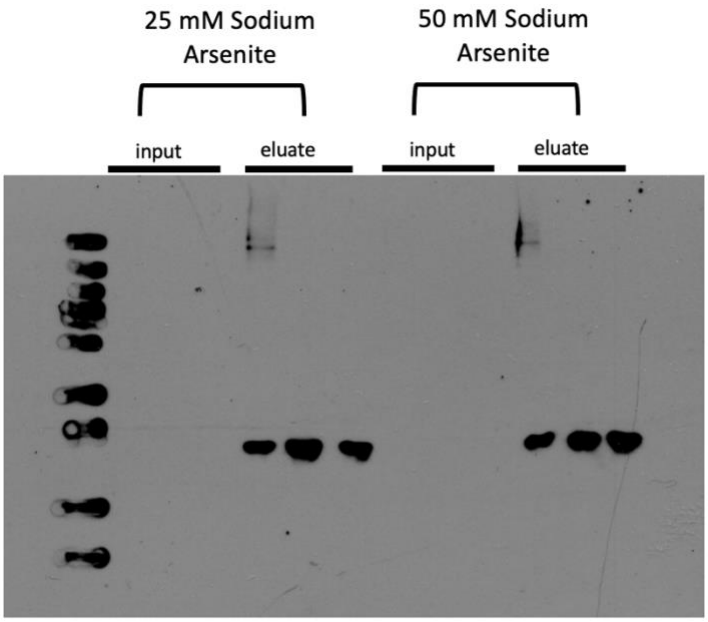


**Figure 3.11 High arsenite does not cause sumoylation**

$His_6$-FLAG-*SMT3* sumoylation patterns after 25mM or 50mM arsenite stress over 60 minutes. Changes in sumoylation patterns were examined by Western analysis using an anti-FLAG antibody.

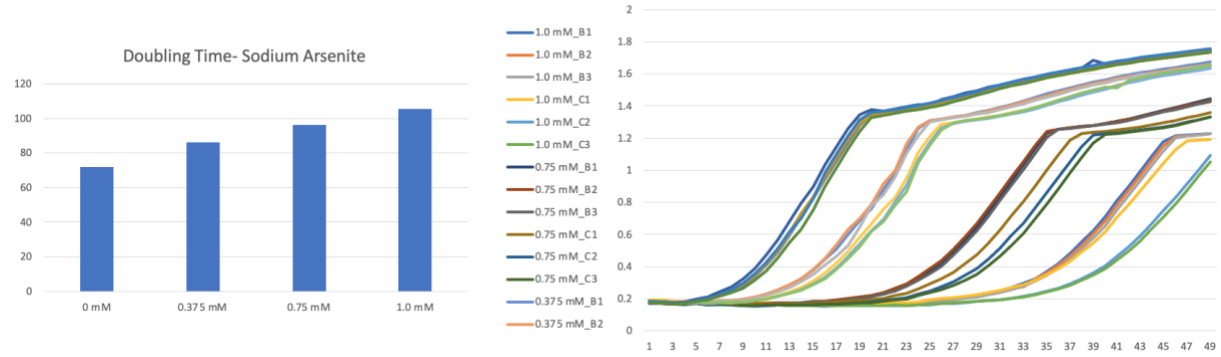


**Figure 3.12 Narrowing down arsenite concentrations**

Quantitative measure of growth rates in liquid culture generated by Bioscreen C automated growth curve analysis. Cells were grown in triplicate at 30C in rich media with 0mM, 0.375mM, 0.750mM, and 1.0mM sodium arsenite for 48 hours with continuous shaking. Absorbance at 600nm was measured every 30 minutes and average absorbance (at 600nm) was plotted versus time. Doubling time was calculated using YODA software (Olsen *et al.*, 2010).

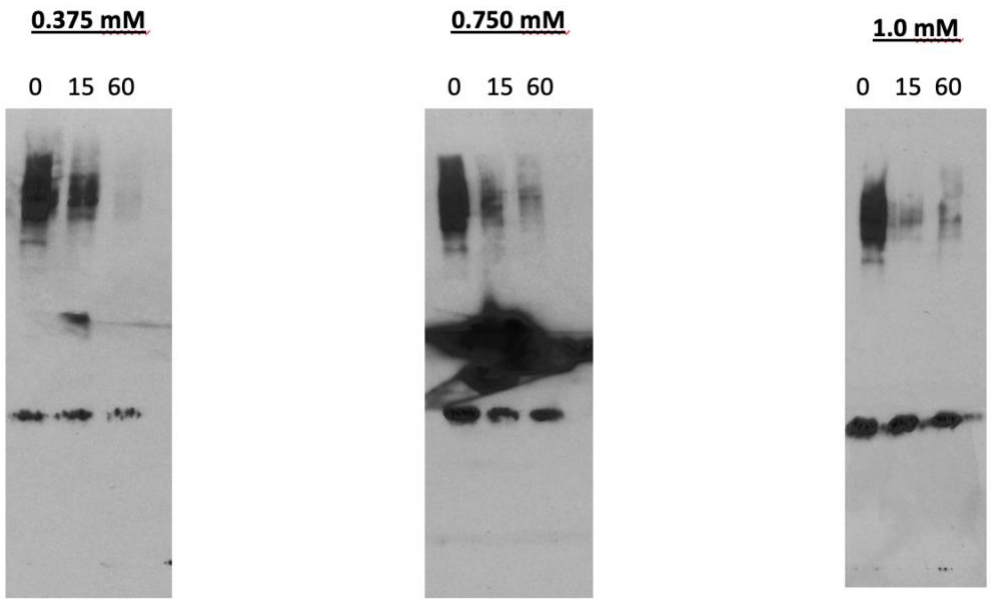


**Figure 3.13 Lower arsenite concentrations elicit a sumoylation response**

$His_6$-FLAG-*SMT3* sumoylation patterns after 0.375mM, 0.750mM, or 1.0mM arsenite stress over 60 minutes. Changes in sumoylation patterns were examined by Western analysis using an anti-FLAG antibody.

## Chapter 4: Acute ethanol exposure induces sumoylation of Smc5-Smc6 chromatin complex

As discussed in the previous chapters there has been much work done to elucidate the sumoylation's role in the cellular response to exogenous stress conditions. While significant work has examined sumoylation dynamics during heat stress, hyperosmotic stress, heavy metal stress, and chronic ethanol stress much less attention has been focused on the acute ethanol stress response. Utilization of different yeast strains for the ethanol production purposes have been exploited for centuries (Mohd Azhar *et al.*, 2017; Parapouli *et al.*, 2020). Given the industrial importance of ethanol production, many studies have reported distinct differences in ethanol tolerance among laboratory and industrial yeast strains (Lewis *et al.*, 2010; Steensels and Verstrepen, 2014). While it has been shown that yeast cells can tolerate relatively high concentrations of ethanol, this does not prevent them from experiencing cellular stress during acute and chronic ethanol exposure. Chronic exposure to high concentrations of ethanol can alter membrane fluidity, lipid composition, increase reactive oxygen species (ROS) production through oxidative phosphorylation decoupling in the mitochondria and cause protein misfolding (Auesukaree, 2017). Despite these studies, there is little known about the molecular determinants and cellular processes that contribute to ethanol tolerance. The goal of this project was to examine the effects of acute ethanol stress on protein sumoylation in *Saccharomyces cerevisiae*.

### Global sumoylation kinetics in yeast depend on ethanol concentration

Our group previously examined global sumoylation response patterns over time in *S. cerevisiae* to various stressors that included ethanol (10% v/v) (Oeser *et al.*, 2016). In that study, acute exposure to a high concentration of ethanol resulted in the steady accumulation of SUMO conjugates over a 60-minute time course. While I was initially interested in sumoylation induced by 10% (v/v) ethanol, I also wanted to determine if the sumoylation patterns observed remained unchanged at ethanol concentrations lower than 10% (v/v). Utilizing a yeast strain where the endogenous SUMO gene, *SMT3*, was tagged with a $His_6$-FLAG sequence at its 5' end (Figure 4.1A), ethanol induced sumoylation was examined at the following concentrations (v/v): 1%, 2.5%, 5%, 7.5%, and 10%. At concentrations lower than 10% the ethanol sumoylation response was transient, with a pronounced increase in SUMO conjugates at 15 minutes that returned to basal levels by 60 minutes (Figure 4.1B), indicating that at lower ethanol

concentrations yeast is mounting an adaptive response that mitigates the need for chronic sumoylation.

To determine what levels of ethanol affect yeast cell growth, the effect of ethanol concentration on cell growth under chronic exposure to different ethanol concentrations (1%, 2.5%, 5%, 7.5%, and 10%) was examined. To do this, liquid culture growth assays were performed over a period of 24 hours. Cells in 1% ethanol exhibited growth identical to cells with no ethanol treatment, while cells in 2.5% and 5% ethanol were delayed before entering into exponential growth. Cells in 7.5% and 10% ethanol, however, did not achieve exponential growth during the 24-hour time period (Figure 4.2). To be certain that acute exposure of ethanol did not impact overall cell growth, I performed spot dilution tests on media lacking ethanol after cells were exposed to the same concentrations of ethanol as listed above for 1 hour in liquid culture. No growth deficiency was observed between untreated and ethanol-treated cells (Figure 4.3), indicating that acute ethanol exposure does not impact cell growth as observed with chronic exposure.

**A chromatin structure maintenance complex is sumoylated during acute ethanol exposure**

To identify proteins sumoylated during acute ethanol stress, I utilized a label-free mass spectrometry (MS) approach. With this approach changes in total peptide counts for a protein were used as a proxy for protein abundance (Oeser *et al.*, 2016). Although the transient nature of sumoylation at lower concentrations of ethanol was of interest, sumoylation at 10% ethanol was chosen for this experiment to maximize the potential proteins that could be identified at early and late timepoints. Similar to previous studies from our group, metal affinity chromatography was utilized to enrich for sumoylated species from $His_6$-FLAG-*SMT3* cell lysates derived from cultures prior to ethanol treatment and after ethanol treatment, in this case 5- and 60-minutes post-ethanol exposure (Figure 4.4). Sumoylated proteins were subsequently isolated by gel purification and subject to trypsin digestion prior to MS analysis. Total peptide counts were examined to determine which proteins showed increases at 5 minutes or 60 minutes of 10% ethanol exposure.

Proteins were classified as being sumoylated in response to acute ethanol stress if the summed peptide counts from the 5-minute and 60-minute replicates exceeded the 0-minute peptide counts by 3-fold or greater and were statistically significant ($p \leq 0.1$) in their changes

(Oeser *et al.*, 2016) (Table 4.1). From my MS analysis, 18 proteins appeared to be sumoylated during acute ethanol exposure (Table 4.1), with 15 out of the 18 identified as chromatin binding and 7 of the 18 identified as chromatin-binding transcription factors by a Gene Ontology (GO) analysis (Table 4.2: Gcr1, Tec1, Hap1, Ste12, Cst6, Met4, and Upc2). Of the 18 proteins identified, the top three proteins were chromatin structural proteins Top2, Smc5, and Smc6 (Figure 4.5).

The Smc5-Smc6 chromatin complex

Acute ethanol induced sumoylation of Smc5 and Smc6 was intriguing because they form a highly conserved complex. The structural maintenance of chromosomes complex (SMC) 5/6 is one of four eukaryotic SMC complexes: cohesion, condensin, dosage compensation complex and Smc5/6 (Aragón, 2018). Smc5 and Smc6 like their family members are ring shaped complexes that contain a trimeric core with a pair of SMC ATPases and a conserved kleisin domain. Characterized by their V-like structure, where a pair of SMC's fold onto themselves through a hinge domain to bring the N- and C-terminal domains together to form an ATPase that has the ability to bind DNA (Aragón, 2018) (Figure 4.6). The complex was first identified in fission yeast (*S. pombe*) by a screen identifying mutants that caused radiation sensitivity (Lehmann *et al.*, 1995). In addition to the core SMC ATPases the Smc5 and Smc6 complex consists of six additional subunits known as non-SMC elements (NSEs; Nse 1-6) (Sergeant *et al.*, 2005; Zhao and Blobel, 2005; Aragón, 2018). One of the NSEs in the Smc5-Smc6 complex is a SUMO E3 ligase (Nse2/Mms21).

Given their essentiality, early studies examining isolated hypermorphic alleles of Smc5 and Smc6 demonstrated that DNA damaging agents lead to defects in DNA repair (Lehmann *et al.*, 1995; Torres-Rosell *et al.*, 2005; Aragón, 2018). Subsequent studies of the complex reported that inactivation of Smc5 and Smc6 in humans, chickens and plants lead to defects in homologous recombination (Aragón, 2018). Additionally, Smc5 and Smc6 are enriched around DNA double strand breaks after DNA replication and have roles in stabilizing replication forks and removal of homologous recombination intermediates (Bermúdez-López *et al.*, 2010; Aragón, 2018). While most well-known for aiding in genome integrity, it has been reported that Smc5 and Smc6 has a role in inhibiting transcription of the Hepatitis B viral genome in human cells (Decorsière *et al.*, 2016; Murphy *et al.*, 2016) and regulating developmental processes in *Arabidopsis thaliana* (Liu *et al.*, 2014; Kwak *et al.*, 2016). It has been previously reported that

Smc5 and Smc6 are sumoylated, however it has not yet been shown that the complex is sumoylated after exposure to ethanol stress.

While subsequent experiments focused solely on gaining a better understanding of Smc5 and Smc6 sumoylation induced by acute ethanol exposure, I wanted to verify the top three candidates of the MS screen. Smc5, Smc6, and Top2 were confirmed to be sumoylated during acute ethanol stress by enriching for sumoylated proteins from $His_6$-FLAG-*SMT3* cell lysates. To do this, I C-terminally 3xHSV tagged versions of Smc5, Smc6, and Top2 that were expressed from their endogenous promoters. By Western analysis I found that each protein exhibited multiple higher molecular weight SUMO conjugates after 10% (v/v) ethanol stress over a 2-hour period (Figure 4.7). Smc5, Smc6, and Top2 sumoylation was verified over a longer time period to confirm that sumoylation remained stable in 10% ethanol. The ethanol-induced sumoylation of Smc5, Smc6, and Top2 was quantified by measuring the intensity of the entire sumoylation ladder for each protein at each time point of ethanol exposure. While this does not provide absolute values as is the case with single bands, it was good for comparison of ethanol treatment with no treatment. Using this method there was a clear increase in ethanol induced sumoylation that was statistically significant at all time points relative to no treatment (Figure 4.7). Even though the ethanol induced sumoylation of Top2 was confirmed, I specifically chose to pursue Smc5 and Smc6 because their sumoylation during acute ethanol stress has not yet been reported. To that end, I also examined Smc5 and Smc6 ethanol induced sumoylation after exposure to 5% (v/v) ethanol (Figure 4.8). While transient sumoylation was observed globally at lower concentrations of ethanol, including 5%, Smc5 and Smc6 were sumoylated over a two-hour exposure to ethanol. The higher weight SUMO conjugates observed at 10% ethanol however were not present at the lower concentration.

Given the significant laddering of higher order SUMO conjugates after acute ethanol exposure observed in Smc5 and Smc6, I wanted to determine if this was due to multiple mono-sumoylation events or a chain of poly-sumoylation. As later experiments will demonstrate, Smc5 and Smc6 data confirm each other, therefore only Smc5 was used for this experiment. To decipher the type of sumoylation observed, 3xHSV-Smc5 was expressed in a yeast strain where all lysine residues of the SMT3 gene itself have been mutated to arginine residues (Esteras *et al.*, 2017). After exposure to acute ethanol, a predominant single sumoylation band was observed (Figure 4.9). However, a slightly higher molecular weight sumoylated species was observed which indicates that there may be additional mono-sumoylation sites if SUMO chain

formation is eliminated. While identification of all Smc5 and Smc6 sumoylation sites is of interest for future studies, for this work I chose to examine the broader features of acute ethanol induced Smc5 and Smc6 sumoylation.

**The E3 SUMO ligase Mms21 is necessary for the sumoylation of Smc5 and Smc6 during acute ethanol stress**

After identifying that acute ethanol exposure resulted in the sumoylation of Smc5 and Smc6, it was important to identify the enzymes required for their ethanol induced sumoylation. Unlike the ubiquitination system, which has over 100 E3 ubiquitin ligases in yeast (De Bie and Ciechanover, 2011), the sumoylation system has only four known E3 SUMO ligases: Siz1, Siz2, Cst9, and Mms21 (Hay, 2001; Gill, 2004). It has been previously reported that the E3 for Smc5 and Smc6 is Mms21 during DNA damage (Duan *et al.*, 2009; Bermúdez-López *et al.*, 2015; Liang *et al.*, 2018), but it was unclear if this was the case during acute ethanol exposure. *SIZ1*, *SIZ2*, and *CST9* are not essential genes and can be deleted. *MMS21* is essential and I therefore used an auxin-inducible degron (AID) depletion strategy (Nishimura *et al.*, 2009; Havens *et al.*, 2012), where Mms21 was fused to an auxin degron and its depletion was induced by addition of auxin to the media. After depletion of Mms21 by addition of auxin for 90 minutes, Smc5 and Smc6 sumoylation induced by acute ethanol exposure decreased approximately 75% during the 60-minute time course (Figure 4.10), consistent with the similar reduction in Mms21 levels. Next, I investigated if the non-essential E3s were involved in sumoylation induced by acute ethanol exposure. Complete loss of Siz1, Siz2, or Cst9 did not significantly reduce Smc5 and Smc6 sumoylation during acute ethanol stress (Figure 4.11). While Mms21 is the likely E3 SUMO ligase for ethanol induced sumoylation of Smc5 and Smc6 because it is known to interact with the complex (Zhao and Blobel, 2005; Stephan *et al.*, 2011; Xaver *et al.*, 2013), there remains the possibility that the non-essential E3 SUMO ligases (Siz1, Siz2, and Cst9) share some redundancy and individual deletions may not have a pronounced effect on Smc5 and Smc6 ethanol induced sumoylation.

**Smc5 and Smc6 sumoylation patterns during other stress conditions**

We previously reported that global sumoylation patterns and kinetics differ between distinct stress conditions (Oeser *et al.*, 2016). To gain better insight into Smc5 and Smc6 sumoylation, their sumoylation patterns during heat stress (42°C) and hyperosmotic stress

(1.2M sorbitol) were examined. After exposing cells to 42°C heat shock over a time course of 60 minutes, Smc5 and Smc6 high weight SUMO conjugates had a similar banding pattern to that of acute ethanol but accumulated at a much slower rate (Figure 4.12). While Smc5 and Smc6 were not identified to be sumoylated in our prior analysis of hyperosmotic stress (Oeser *et al.*, 2016), strains were also subjected to hyperosmotic stress (1.2M sorbitol) over 60 minutes to be thorough. The results were much more complicated and not as intuitive as ethanol and heat stress, however. After exposure to hyperosmotic stress there was a rapid decrease in Smc5 and Smc6 sumoylation after 5 minutes before a rapid increase at 15 minutes that then remained stable for the duration of the time course (Figure 4.13). The decrease in Smc5 and Smc6 sumoylation is consistent with what was previously observed during hyperosmotic stress; proteins are desumoylated to provide a free pool of SUMO that is readily available for Tup1 and Cyc8 sumoylation (Oeser *et al.*, 2016). There was an increase in Smc5 and Smc6 sumoylation following the initial decrease during hyperosmotic stress; this might be a return to basal sumoylation seen prior to hyperosmotic stress as the cells adapt to the stress and Tup1-Cyc8 are desumoylated thus increasing the free SUMO pool (Oeser *et al.*, 2016). Conversely, it is possible that Smc5 and Smc6 are increasingly sumoylated during the later time points. It is difficult to assess the indirect rebound effects on global sumoylation as SUMO pools are restored during adaptation to hyperosmotic stress versus the direct regulated events of protein sumoylation at later stages of adaptation.

**Acknowledgements**

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

Bradley AI, Marsh NM, Borror HR, Mostoller KM, Gama AI, Gardner RG (2021). Acute ethanol stress induces sumoylation of conserved chromatin structural proteins in Saccharomyces cerevisiae. Mol Biol Cell. 32, 1121-1133.

Thank you, to Luis Aragon's group, for the gift of the non-sumoylatable SUMO strain

Thank you to Matt Kaeberlein's group for use of the Bioscreen C and help with data analysis

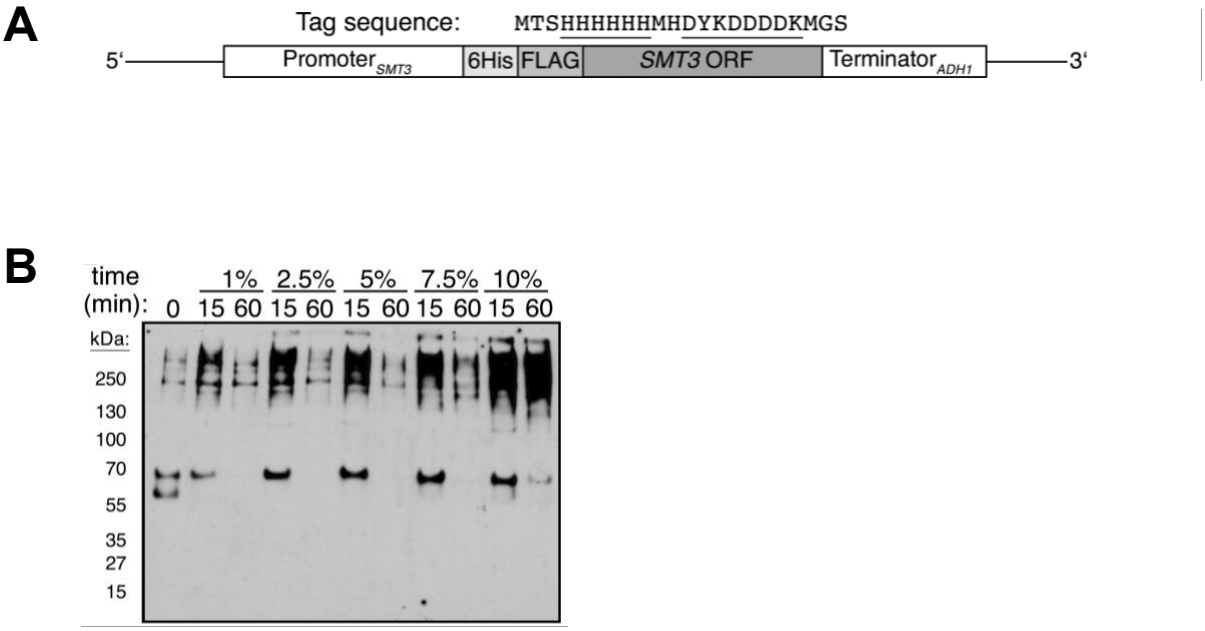


**Figure 4.1 Acute ethanol induced sumoylation is transient at lower concentrations**
(A) Schematic for $His_6$-FLAG-SMT3 located at the *SMT3* locus and expressed from the endogenous promoter. $His_6$-FLAG sequence shown, sequences underlined. (B) Comparison of global sumoylation changes that occur during acute cellular exposure of ethanol (1%, 2.5%, 5%, 7.5%, and 10% v/v) over 60 minutes. Changes in sumoylation patterns were examined by Western analysis using an anti-FLAG antibody.

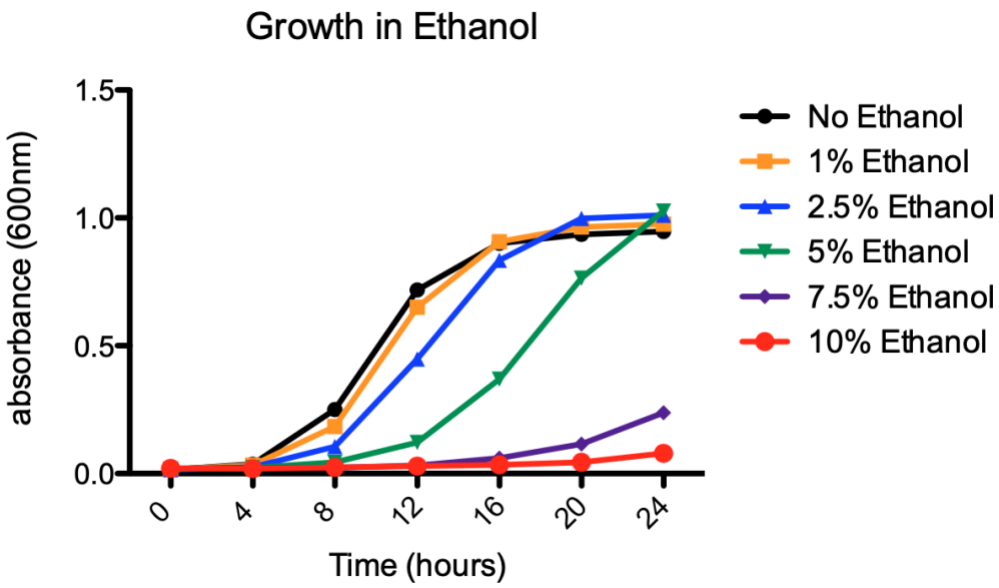


**Figure 4.2 Chronic ethanol exposure slows growth at high concentrations**

Quantitative measure of growth rates in liquid culture generated by Bioscreen C automated growth curve analysis. Cells were grown in triplicate at 30°C in rich media with 0%, 1%, 2.5%, 5%, 7.5%, or 10% ethanol for 24 hours with continuous shaking. Absorbance at 600nm was measured every 30 minutes and average absorbance (at 600nm) was plotted versus time. Error bars show SD for triplicate samples.

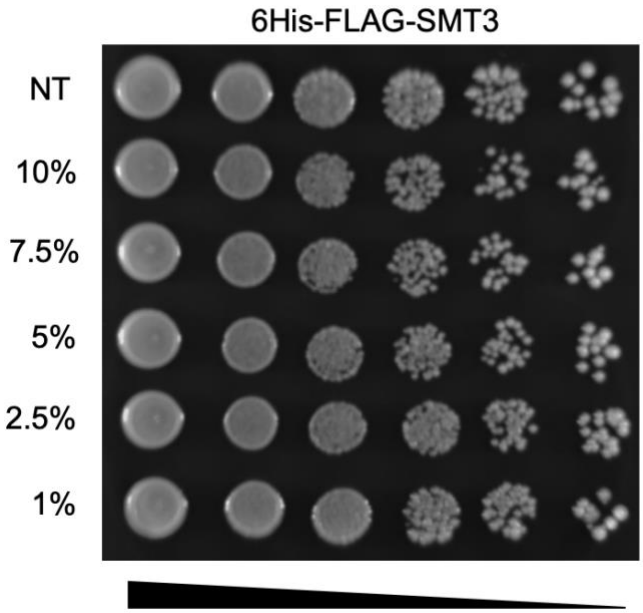


**Figure 4.3 Acute ethanol exposure does not affect cell growth**

$His_6$-FLAG-*SMT3* cells were grown to mid-log phase in rich liquid media then treated for 60 minutes in rich media with 0%, 1%, 2.5%, 5%, 7.5%, or 10% ethanol before being 10-fold serially diluted onto rich media plates lacking ethanol and incubated at 30°C for 3 days.

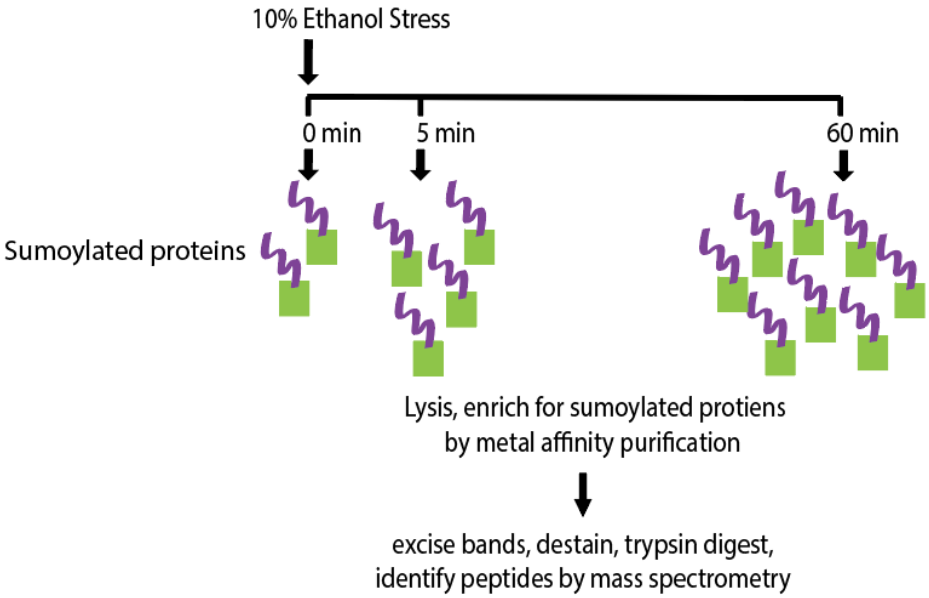


**Figure 4.4 Mass Spectrometry strategy**

Strategy to identify proteins sumoylated during acute ethanol exposure.

| Accession | Gene | Total Peptides | | | Description |
|---|---|---|---|---|---|
| | | 0 min | 5 min | 60 min | |
| YNL088W | TOP2 | 24 | 109 | 104 | Topoisomerase II |
| YOL034W | SMC5 | 6 | 36 | 40 | Role in DNA replication and repair; in complex with Smc6 |
| YLR383W | SMC6 | 1 | 12 | 25 | Role in DNA replication and repair; in complex with Smc5 |
| YPL075W | GCR1 | 0 | 9 | 16 | Transcription activator |
| YOR038C | HIR2 | 1 | 3 | 13 | Regulation of histone gene transcription |
| YBR083W | TEC1 | 0 | 3 | 10 | Transcription factor, filamentation genes |
| YGR071C | ENV11 | 1 | 2 | 9 | Vacuolar function |
| YBL097W | BRN1 | 2 | 3 | 9 | Subunit of condensin complex |
| YLR256W | HAP1 | 1 | 21 | 9 | Zinc finger transcription factor |
| YDR485C | VPS72 | 1 | 6 | 8 | Htz1p-binding component of the SWR1 complex |
| YIL126W | STH1 | 1 | 4 | 8 | ATPase component of RSC chromatin remodeling complex |
| YHR084W | STE12 | 0 | 3 | 6 | Transcription factor activated by MAPK |
| YBR081C | SPT7 | 2 | 2 | 6 | Subunit of SAGA complex |
| YIL036W | CST6 | 0 | 17 | 4 | Basic leucine zipper (bZIP) transcription factor |
| YJL092W | SRS2 | 0 | 4 | 1 | DNA helicase and DNA-dependent ATPase |
| YKL054C | DEF1 | 0 | 4 | 1 | RNAPII degradation factor |
| YNL103W | MET4 | 0 | 10 | 0 | Leucine-zipper transcriptional activator |
| YDR213W | UPC2 | 0 | 7 | 0 | Sterol regulatory element binding protein |

**Table 4.1** Proteins sumoylated during acute ethanol exposure

| GO Terms from the molecular function Ontology | | | |
|---|---|---|---|
| GO Term (GO ID) | Genes Annotated | GO Term Usage | Genome Frequency |
| DNA binding (GO:0003677) | TEC1, UPC2, ENV11, STE12, CST6, STH1, SRS2, DEF1, HAP1, SMC6, MET4, TOP2, SMC5, HIR2, GCR1 | 15 of 18 genes, 83.33% | 602 of 6411 annotated genes, 9.39% |
| DNA-binding transcription factor activity (GO:0003700) | TEC1, UPC2, STE12, CST6, HAP1, MET4, GCR1 | 7 of 18 genes, 38.89% | 180 of 6411 annotated genes, 2.81% |

**Table 4.2** Gene Ontology analysis of MS hits

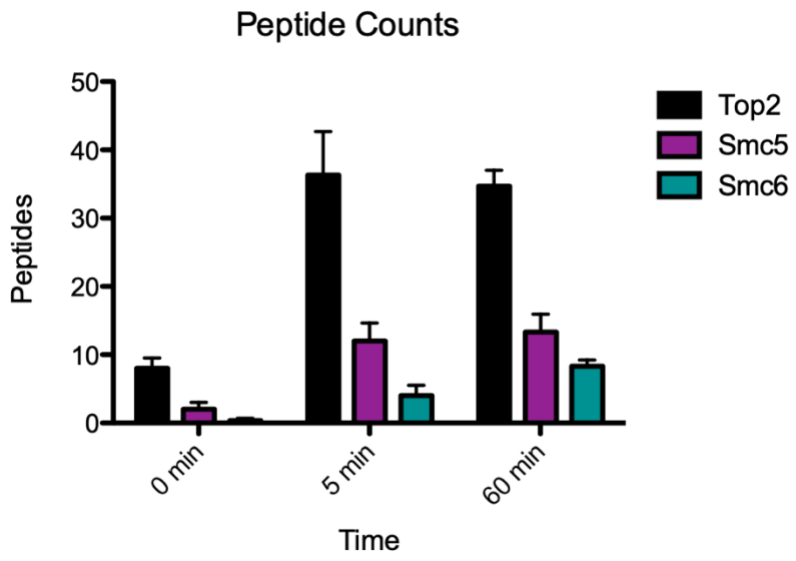


**Figure 4.5 Ethanol induced sumoylation MS peptide counts**

Total peptide counts identified for the top 3 proteins (Top2, Smc5, and Smc6) at 0, 5 and 50 minutes of 10% acute ethanol stress.

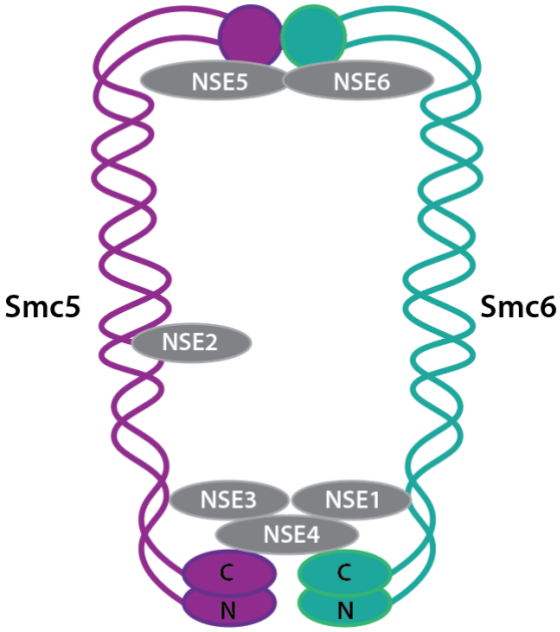


**Figure 4.6 Structural maintenance of chromosomes 5 and 6**

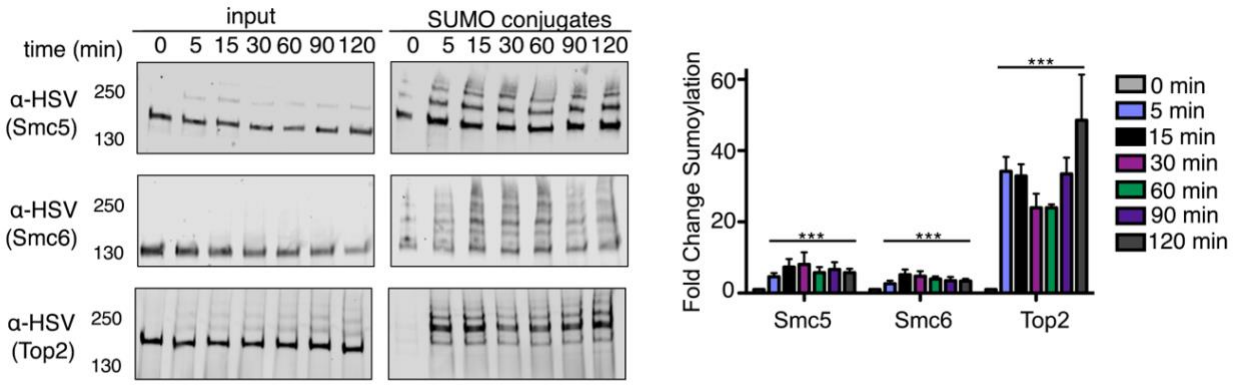


**Figure 4.7 Chromatin associated proteins are sumoylated during acute ethanol exposure**
Cells expressing $His_6$-FLAG-*SMT3* and either C-terminally 3xHSV epitope tagged Smc5, Smc6, or Top2 from their endogenous promoters were subject to acute ethanol (10% v/v) over 120-minute time course. Cell lysates (input) and purified sumoylated proteins (SUMO conjugates) were examined via Western analysis using an anti-HSV antibody to detect Smc5, Smc6, and Top2. Fold change sumoylation of Smc5, Smc6 and Top2 in 10% ethanol. Each value represents the mean and SEM values are indicated as bars (n=3), two-way ANOVA was used to compare untreated zero timepoint vs. subsequent timepoints in the presence of ethanol, significant differences ($P<0.0001$) are indicated (***).

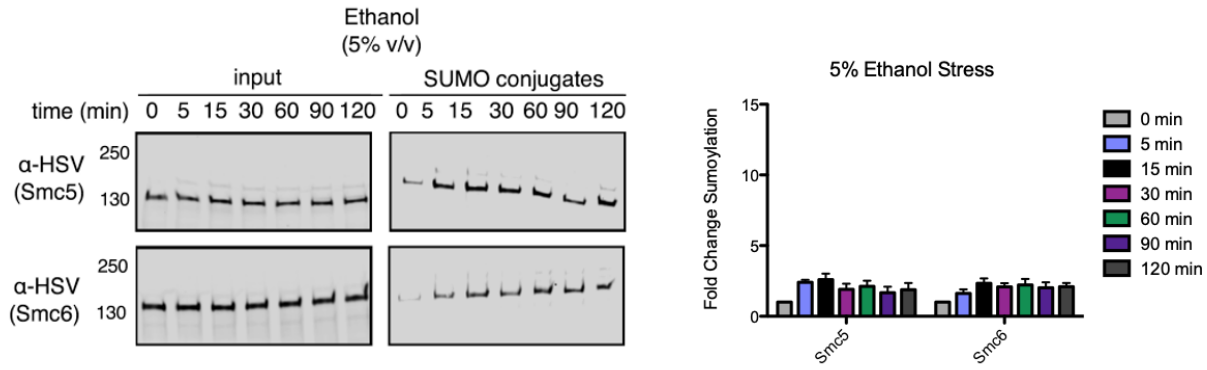


**Figure 4.8 Smc5 and Smc6 are not transiently sumoylated at lower acute ethanol concentrations**

Cells expressing $His_6$-FLAG-*SMT3* and either C-terminally 3xHSV epitope tagged Smc5 and Smc6 from their endogenous promoters were subject to acute ethanol (5% v/v) over 120-minute time course. Cell lysates (input) and purified sumoylated proteins (SUMO conjugates) were examined via Western analysis using an anti-HSV antibody to detect Smc5 and Smc6. Fold change sumoylation of Smc5 and Smc6 in 5% ethanol. Each value represents the mean and SEM values are indicated as bars (n=3).

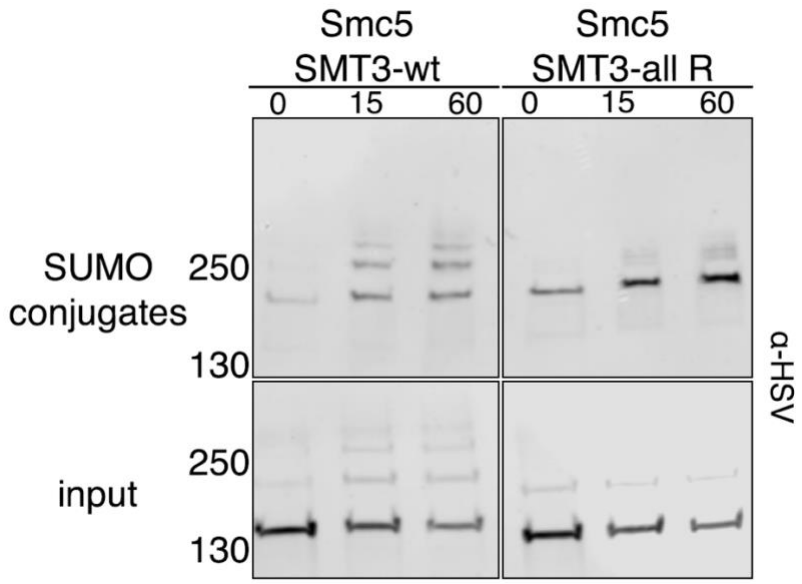


**Figure 4.9 Ethanol-induced Smc5 appears to be primarily a poly-sumoylation chain**

Cells with 3xHSV-Smc5 expressed from its endogenous promoter and expressing either $His_6$-*SMT3* that contains all lysine residues (SMT3-wt), or all lysine residues converted to arginine residues (SMT3-all R) were exposed to 10% ethanol over a 60-minute time course. Cell lysates (bottom panels) and metal affinity purified sumoylated proteins (top panels) were examined by Western analysis using an anti-HSV antibody.

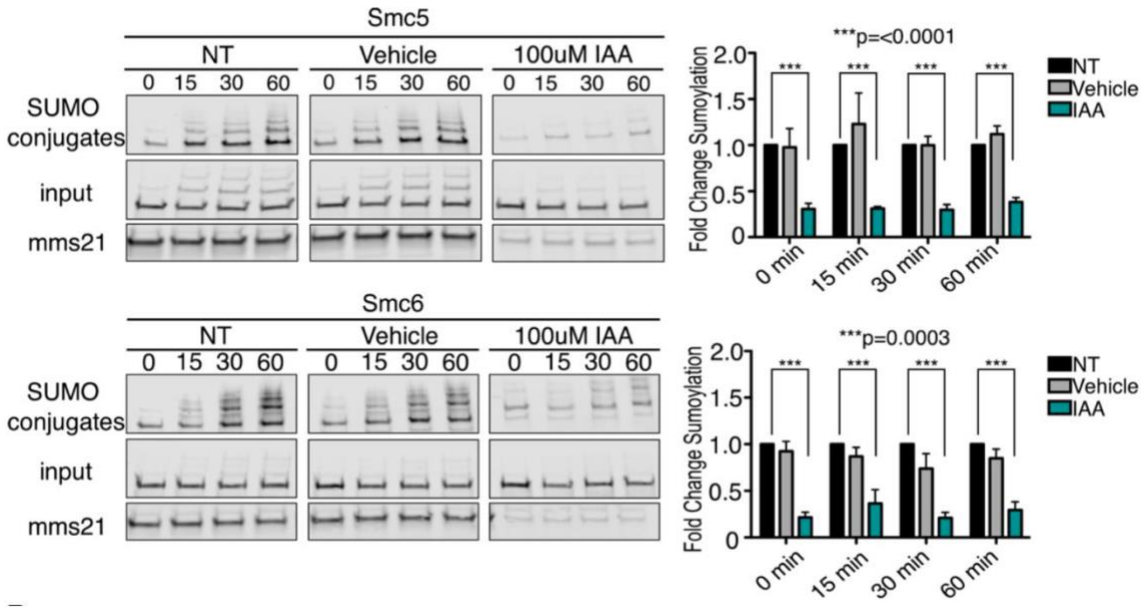


**Figure 4.10 The E3 SUMO ligase Mms21 promotes sumoylation of Smc5 and Smc6 during ethanol stress**

Cells expressing His6-FLAG-*SMT3*, *MMS21*-3HSV-AID and C-terminally 3xHSV tagged Smc5 or Smc6 from their endogenous promoters were grown to early log-phase then treated with NT, Vehicle, or auxin (IAA) for 90 minutes before exposure to acute ethanol for 60 minutes. Cell lysates (input) and purified sumoylated proteins (SUMO conjugates) were examined via Western analysis with an anti-HSV antibody to detect Mms21, Smc5 and Smc6. Inputs and Mms21 samples were run on the same gel and blot while SUMO conjugates were run separately on the same gel and blot. Blot images were cropped for optimal visualization. Fold changes in sumoylation were calculated utilizing values from Image Studio Lite software. Values were normalized to inputs and NT samples set to a value of 1.0. Error bars show SEM for triplicate samples. NT samples compared to IAA were significantly different in Smc5 and Smc6 with ***p=<0.0001 and ***p=<0.0003 respectively.

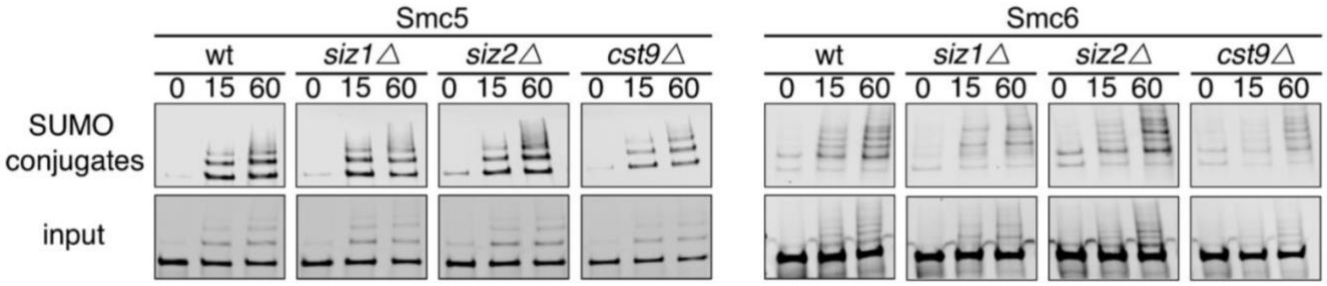


**Figure 4.11 Non-essential SUMO E3 ligases are not required for sumoylation of Smc5 and Smc6 during acute ethanol stress**

Wild-type (WT), *siz1*Δ, *siz2*Δ, or *cst9*Δ cells expressing $His_6$-FLAG-SMT3 and either 3xHSV Smc5 or Smc6 were subjected to 60-minutes of acute ethanol (10% v/v) exposure. Cell lysates (input) and metal affinity purified sumoylated proteins (SUMO conjugates) were examined by Western analysis with an anti-HSV antibody. Inputs and SUMO conjugates were run separately, but each on the same gel and blot. Blot images were only cropped for optimal visualization.

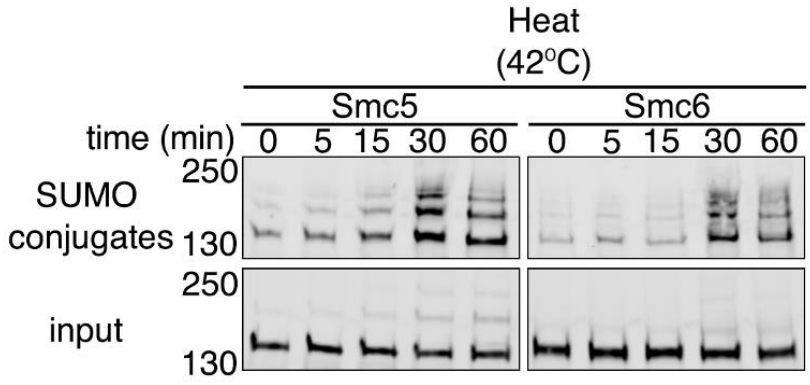


**Figure 4.12 Smc5 and Smc6 are sumoylated during heat stress**

Cells expressing $His_6$-FLAG-*SMT3* and C-terminally 3xHSV epitope tagged Smc5 or Smc6 from their endogenous promoters were exposed to heat (42°C) stress over a 60-minute time course. Cell lysates (input) and metal affinity purified sumoylated proteins (SUMO conjugates) were examined by Western analysis using an anti-HSV antibody.

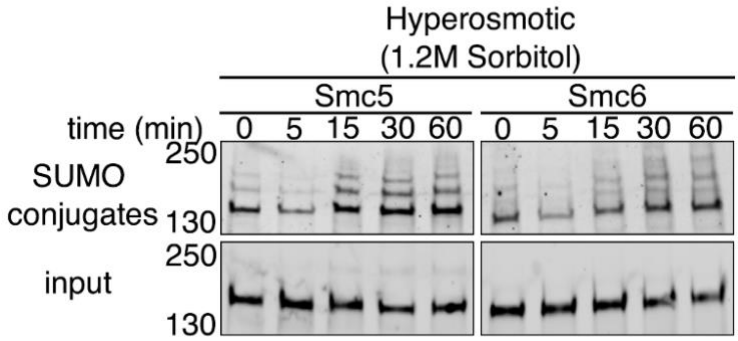


**Figure 4.13 Smc5 and Smc6 sumoylation patterns during hyperosmotic stress**

Cells expressing $His_6$-FLAG-*SMT3* and C-terminally 3xHSV epitope tagged Smc5 or Smc6 from their endogenous promoters were exposed to hyperosmotic (1.2M sorbitol) stress over a 60-minute time course. Cell lysates (input) and metal affinity purified sumoylated proteins (SUMO conjugates) were examined by Western analysis using an anti-HSV antibody.

## Chapter 5: Acute ethanol exposure causes discrete chromatin changes

In the previous chapter I explored the dynamics by which Smc5 and Smc6 are sumoylated during acute ethanol exposure. While Smc5 and Smc6 have known roles in maintaining genome integrity, I hypothesized that acute ethanol exposure may cause DNA damage and that sumoylation of Smc5 and Smc6 may aid in mitigating that damage in some way. Sumoylation of proteins is essential for progression through the cell cycle and SUMO conjugation to its targets dynamically changes during different stages of the cell cycle under normal conditions (Talamillo *et al.*, 2020). A common feature of yeast cells is that exposure to stress often affect progression through the cell cycle (Jorgensen and Tyers, 2004). As an example, yeast cells have been shown to halt in G1 after exposure to stress (Qu *et al.*, 2019). This pause is generally thought to allow cells the time to resolve or adapt to the stress before proceeding through the cell cycle (Qu *et al.*, 2019). Due to this, a major question we asked was when in the cell cycle is the Smc5 and Smc6 complex sumoylated during acute ethanol stress.

### Ethanol-induced Smc5 and Smc6 sumoylation occurs in G1 and G2/M phases but is reduced in S phase

Chromatin undergoes regulated changes during the cell cycle (Antonin and Neumann, 2016). Since the Smc5-Smc6 complex is associated with chromatin and its localization is altered specifically during S phase (Jeppsson *et al.*, 2014), I was interested in determining the stages of the cell cycle where Smc5 and Smc6 undergo ethanol-dependent sumoylation. I arrested cells in G1 using alpha-factor, early S phase using hydroxyurea (HU), and G2/M using nocodazole (ND). Cells arrested in G1 were generally observed to be either large and unbudded or with shmoo morphology. Cells arrested in S phase were generally observed to be large with small buds. G2/M arrested cells were generally observed as dumbbells (Figure 5.1). I next examined the DNA content of cells in asynchronous culture after arrest at each stage in the cell cycle by staining cells with SYTOX green followed by examination by flow cytometry (Haase and Reed, 2002). Untreated cells exhibited 1C and 2C DNA content profiles consistent with asynchronous growth (Figure 5.2). Cells arrested in G1 with alpha-factor had 1C DNA content with a slight shoulder indicating a small subset of cells in early S phase (Figure 5.2). Strains used in this study contain an intact *BAR1* gene, which encodes a protease that cleaves and inactivates alpha-factor (Ciejek and Thorner, 1979), therefore minor escape from G1 arrest was expected. Cells arrested in early S phase with HU had a 1C DNA content peak (Figure 5.2)

similar to what has been previously described (Paillé *et al.*, 2019). This peak is indicative of early S-phase arrest in asynchronous culture because hydroxyurea depletes dNTPs required for DNA synthesis in early S phase. Cells arrested in G2/M with nocodazole (ND) had a 2C DNA content peak as anticipated (Figure 5.2).

After confirming arrest at each cell-cycle stage by microscopy (Figure 5.1) and flow cytometry, I examined if Smc5 and Smc6 were sumoylated during each stage. I found that arrest in G1 allowed the same ethanol-induced sumoylation as asynchronous cells (Figure 5.3). I also found that G2/M arrest allowed Smc5 and Smc6 ethanol induced sumoylation similar to asynchronous cells (Figure 5.4). Interestingly, cells arrested in early S phase showed considerably reduced ethanol induced sumoylation of Smc5 and Smc6 (Figure 5.5). Next I wanted to determine if HU addition inhibited sumoylation globally within the cell, therefore we examined sumoylation of the transcriptional co-repressor Cyc8 during HU treatment and hyperosmotic stress (1.2M sorbitol), which is a condition we have previously found Cyc8 to be sumoylated (Oeser *et al.*, 2016). Hyperosmotic stress induced Cyc8 sumoylation still occurred after early S-phase arrest (Figure 5.6). These results ruled out an inhibitory effect of HU addition on global sumoylation. While we have previously shown that Cyc8 is not sumoylated during acute ethanol stress (Oeser *et al.*, 2016), I wanted to be certain that this was also the case after early S-phase arrest. I found that Cyc8 is not sumoylated after acute ethanol exposure during early S-phase arrest (Figure 5.7). These results demonstrate that Smc5 and Smc6 undergo ethanol-induced sumoylation during alpha-factor and ND arrests in G1 and G2/M phases respectively, but do not during HU arrest in early S phase.

**Are the acute ethanol induced sumoylation patterns of Smc5 and Smc6 unique from that of DNA damage?**

It has been reported that Smc5 and Smc6 are sumoylated after exposure to levels of methyl methanesulfonate (MMS) that induce DNA damage (Cremona *et al.*, 2012; Chung and Zhao, 2015; Sarangi *et al.*, 2015; Zapatka *et al.*, 2019). I wanted to verify that Smc5 and Smc6 were sumoylated during an acute time course of MMS exposure, and if the addition of ethanol with MMS treatment had any increased effect. I predicted that if both had the same effect, the addition of ethanol would not change Smc5 or Smc6 sumoylation beyond MMS treatment. I exposed cells to 0.033% MMS and found that Smc5 and Smc6 were sumoylated after addition of the DNA-damaging agent (Figure 5.8). However, addition of ethanol with MMS increased

Smc5 and Smc6 sumoylation when compared to MMS treatment alone but was generally equivalent to that of ethanol treatment alone (Figure 5.8). I conclude that ethanol treatment may have a stronger dominant effect on Smc5 and Smc6 sumoylation in an asynchronous culture when compared to acute MMS treatment due to the stages of cells in the cell cycle. The more pronounced effect of ethanol in an asynchronous culture may be due to the likelihood that maximal MMS induced sumoylation of Smc5 and Smc6 occurs during S phase. This would be analogous to the MMS induced sumoylation of the RecQ helicase Sgs1, which is limited to S phase (Bermúdez-López *et al.*, 2016), and I have shown that ethanol induced sumoylation of Smc5 and Smc6 occurs during G1 and G2/M. G1 and G2/M are the predominant cell-cycle phases in an asynchronous culture (Figure 5.2), therefore the results of this experiment are consistent with a dominant effect of ethanol on Smc5 and Smc6 sumoylation during asynchronous growth when compared to MMS treatment alone.

**Acute ethanol stress induces Rad52 foci formation but not Rad53 phosphorylation**

Chronic, long term treatment of yeast cells with ethanol has been reported to increase DNA mutation rates through error-prone DNA polymerases being recruited to replication forks delayed in their progression (Voordeckers *et al.*, 2020). Due to this I wanted to see if acute ethanol exposure led to any visible measure of chromatin structural alterations. It was not clear how to measure *in vivo* global chromatin structural changes during an acute response of 60 minutes or less. We searched for examples in the literature but could not find any publications to our knowledge that examined *in vivo* chromatin changes in yeast under acute conditions of stress. Therefore, I decided to first examine *in vivo* global chromatin structure using chromatin staining and histone H2B localization by fluorescence microscopy (Figure 5.9). Unfortunately, no obvious differences were observed over the 60-minute time course of acute ethanol treatment.

I next examined more specific measures of chromatin changes. *In vivo* ways to examine chromatin changes resulting from damaging agents like MMS, which is an alkylating agent that methylates guanine and adenine moieties (Beranek, 1990), have been developed through observing foci formed by the homologous recombination protein Rad52 fused to tdTomato (Estrem and Moore, 2019), and phosphorylation of the intra-S phase checkpoint kinase Rad53 (Sanchez *et al.*, 1996). Though several studies examining Rad52 foci are generally conducted after extensive DNA damage caused by irradiation (Smith *et al.*, 2019), we chose to remain with

the acute ethanol exposure conditions in an asynchronous culture that were used in all previous experiments. This was especially important since asynchronous cultures contain predominantly cells in G1 and G2/M phases where ethanol induced sumoylation is the most prominent.

For these studies, I examined cells that were either untreated, treated with 0.033% MMS or 10% ethanol alone, or 0.033% MMS plus 10% ethanol together. Compared to no treatment, 15 minute treatment of cells with either 10% ethanol or 0.033% MMS caused the formation of Rad52 foci in about 1% of cells in an asynchronous culture (Figure 5.10A), and this is consistent with that observed in prior studies examining MMS treatment in asynchronous cultures (Barlow and Rothstein, 2009; Smith *et al.*, 2019). There was no increase in Rad52 foci frequency in cells treated with both 10% ethanol and 0.033% MMS relative to cells treated with either stress individually. By this measure, ethanol and MMS have a similar effect on Rad52 foci formation (Figure 5.10B). Surprisingly, acute ethanol treatment did not induce phosphorylation of Rad53 whereas MMS did cause Rad53 phosphorylation (Figure 5.11), indicating that acute ethanol exposure does not trigger the intra-S phase checkpoint like MMS exposure (Barlow and Rothstein, 2010). Importantly, addition of ethanol and MMS together did not trigger Rad53 phosphorylation, suggesting that ethanol exposure effects are epistatic to MMS exposure effects. Altogether, our collective data suggests that acute ethanol stress impacts chromatin, but the effects have some differences compared with the DNA-damaging agent MMS.

**Does Acute ethanol stress increase reactive oxygen species (ROS) production resulting in Smc5-Smc6 sumoylation?**

Given the above data I wanted to attempt to identify the mechanism of acute ethanol induced sumoylation. I suspected that acute ethanol treatment led to DNA damage given my Rad52 foci results. Ethanol is known to have primary effects in the cell that include altering membrane fluidity and denaturing proteins (Tóth *et al.*, 2014). Chronic ethanol exposure has been shown to increase ROS production (Yang *et al.*, 2012), but it is not clear that acute ethanol exposure would have the similar effect. Therefore, I first wanted to determine if acute ethanol exposure resulted in ROS production. To test I exposed our $His_6$-FLAG-*SMT3* strain to 10% ethanol after a one-hour pretreatment of L-ascorbic acid (Vitamin C) for 60 minutes. I chose to pretreat with ascorbic acid specifically because it reacts with a variety of ROS at a faster rate than glutathione (Zhitkovich, 2020). Additionally, Vitamin C has been shown to detoxify singlet oxygen in the nucleus (Rubis *et al.*, 2019). Compared to the control, I found that global ethanol

induced sumoylation is reduced suggesting that acute ethanol stress leads to ROS production in some proteins (Figure 5.12). Next, I wanted to determine if addition of Vitamin C prior to acute ethanol stress specifically altered Smc5 sumoylation. There was no noticeable difference in sumoylation induced by ethanol in untreated cells and cells treated with Vitamin C (Figure 5.13). The mechanism of acute ethanol induced sumoylation remains unclear and future studies are needed to answer this critical question.

**Discussion**

Adaptation to stress is essential for cellular survival, and the cell utilizes distinct multifaceted approaches to re-establish homeostasis during particular stress conditions. In the absence of stress adaptation, prolonged cellular stress can lead to the irreversible damage of cellular components that can ultimately impact cell viability (Figure 5.14). In the case of ethanol, chronic exposure to high concentrations of ethanol leads to alterations in membrane fluidity and lipid composition, increased production of reactive oxygen species (ROS) through altering oxidative phosphorylation in the mitochondria, and cause protein denaturing and misfolding (Kato *et al.*, 2011, 2019; Auesukaree, 2017). For this study, I chose to examine proteins that become sumoylated during acute ethanol exposure. Acute ethanol exposure was of interest because while there has been much work done on the role of chronic ethanol exposure, very little research has been done on the cellular effects of acute ethanol. My mass spectrometric analysis identified 18 proteins that become increasingly sumoylated during acute ethanol exposure, and 15 of the 18 proteins are chromatin-associated (Table 4.1). This is not unexpected as protein sumoylation is known to regulate multiple chromatin-associated processes including the DNA damage checkpoint (Wu *et al.*, 2014; Munk *et al.*, 2017), regulation of chromosome structure (Tanaka *et al.*, 1999; Nacerddine *et al.*, 2005), and chromosome movement (Seeber and Gasser, 2017; Zhao, 2018).

Although not the focus of my study, it is important to comment on the scope of proteins identified in my MS analysis. Of the 18 proteins discovered, 7 are known to function as transcription factors (Table 4.1 and 2: Gcr1, Tec1, Hap1, Ste12, Cst6, Met4, and Upc2). The particular functions of these transcription factors reflect the ways in which ethanol alters cellular physiology. The cellular effects of ethanol exposure include alterations in membrane fluidity and lipid composition (Tóth *et al.*, 2014), changes in glucose and amino acid uptake (Yang *et al.*, 2012), a reduction in the activities of glycolytic enzymes (Tóth *et al.*, 2014), and disruption of

membrane integrity (Stanley *et al.*, 2010). In terms of membrane fluidity, Upc2 is a transcription factor that undergoes regulated cleavage from the ER membrane to activate sterol biosynthesis genes (Joshua and Höfken, 2017), and it is known that ethanol exposure leads to the increased synthesis and presence of unsaturated fatty acids and ergosterol in the membrane (Henderson and Block, 2014). For changes in glucose and amino acid uptake, Gcr1 regulates genes involved in glycolysis (Hossain *et al.*, 2016), and Met4 regulates genes in the sulfur amino acid biosynthesis pathway (McIsaac *et al.*, 2012). Tec1 and Ste12 are involved in regulating filamentous and invasive growth pathways (Mayhew and Mitra, 2014). Cst6 is also known to regulate stress and carbon utilization pathways (Pohlers *et al.*, 2017), and deletions are sensitive to ethanol stress (Liu *et al.*, 2016). Altogether, it is notable that the cellular processes affected by ethanol exposure are regulated by transcription factors that are sumoylated during acute ethanol stress. Alteration of transcription factor activity in these pathways is consistent with yeast cells mounting an adaptive response to manage the cellular dysfunction that occurs with exposure to ethanol. Further studies are needed to verify the ethanol-induced sumoylation of these transcription factors and to understand the transcriptional responses that might occur through their ethanol-induced sumoylation and subsequent alteration of their function.

In addition to transcription factors, major proteins that showed increased sumoylation upon ethanol exposure were Smc5, Smc6, and Top2, which are known to form a highly conserved chromatin structure complex (Aragón, 2018). The Smc5-Smc6 complex is one of four highly conserved structural maintenance of chromosomes (SMC) complexes found in eukaryotes and is best known for its role in DNA repair and overall genome stability (Aragón, 2018). It has been described that Smc5 and Smc6 sumoylation occurs as a regulatory consequence of stalled replication forks and has a functional role in modulating replication associated repair and error free DNA bypass via the Mph1 helicase (Zapatka *et al.*, 2019). The Smc5-Smc6 complex also has been shown to interact with the E3 SUMO ligase Mms21 to promote the sumoylation of the STR helicase complex that acts in the removal of recombination intermediates (Bonner *et al.*, 2016); however I did not find members of the complex Sgs1, Top3, or Rmi1 to undergo increased sumoylation in my MS analysis. I did find that Smc5 and Smc6 are sumoylated during arrest of cells in the G1 and G2/M phases of the cell cycle after acute ethanol exposure (Figure 5.4), but did not observe Smc5 and Smc6 sumoylation during early S-phase arrest induced by addition of HU (Figure 5.5). These data indicate that there are specific windows when acute ethanol-induced Smc5 and Smc6 sumoylation occurs. Considering that Smc5 and Smc6 have nearly identical chromatin localizations during G1 and G2/M phases but

different ones during S phase (Jeppsson *et al.*, 2014), it may be that chromatin context is important for Smc5 and Smc6 sumoylation during ethanol stress.

There was also an observed increase in Smc5 and Smc6 sumoylation after exposure of cells to either ethanol and the DNA alkylating agent MMS (Figure 5.8); however, ethanol-induced sumoylation had a predominant effect over MMS-induced sumoylation. It is possible that acute ethanol stress induced Smc5-Smc6 sumoylation may operate through a different mechanism than MMS stress. I found that both ethanol and MMS exposure induced Rad52 foci to similar extent (Figure 5.10), suggesting that both might lead to Smc5 and Smc6 sumoylation through a chromatin structural change, though the nature of the change might be different. In fact, ethanol exposure did not trigger the intra-S phase checkpoint as seen by the phosphorylation of Rad53 whereas MMS treatment did lead to Rad53 phosphorylation (Figure 5.11). Currently, the function of ethanol-induced Smc5 and Smc6 sumoylation is not clear. Future experiments will be needed to determine if Smc5 and Smc6 sumoylation is due to DNA damage, altered chromatin structure, or a response to protein misfolding that could lead to both DNA damage and/or chromatin structural loss. Consistent with this idea, it is notable that heat shock also induced Smc5 and Smc6 sumoylation, and heat shock can also lead to similar changes in protein/chromatin structure as well as DNA damage (Niskanen and Palvimo, 2017).

Overall, from the data presented in this work there are likely two responses the cell elicits during acute ethanol exposure through sumoylation: one is to protect chromatin structure and the other is to mount an adaptive response through altered gene transcription. Our group previously found that sumoylation modulates a transient phase separation in the Tup1-Cyc8 transcriptional co-repressor complex (Oeser *et al.*, 2016), indicating a chromatin-modifying activity for sumoylation during hyperosmotic stress. From a transcriptional perspective, my groups previous work (Oeser *et al.*, 2016; Nadel *et al.*, 2019) and other studies (Zhou *et al.*, 2004; Stielow *et al.*, 2008; Zhao, 2018) demonstrated that genes involved either directly in transcription or its modulation have been reported to be sumoylated. How stress-induced sumoylation affects protein activity, localization, or stability remains an open question in the field, and it may be dictated by the magnitude and duration of the stress. My studies indicate that sumoylation targets chromatin-associated proteins during ethanol stress adaptation and support the idea that transient sumoylation is a common regulatory phenomenon during stress conditions.

**Acknowledgements**

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

Bradley AI, Marsh NM, Borror HR, Mostoller KM, Gama AI, Gardner RG (2021). Acute ethanol stress induces sumoylation of conserved chromatin structural proteins in Saccharomyces cerevisiae. Mol Biol Cell. 32, 1121-1133.

Thank you to Jeff Moore's group from the University of Colorado for the Rad52 tdTomato strain

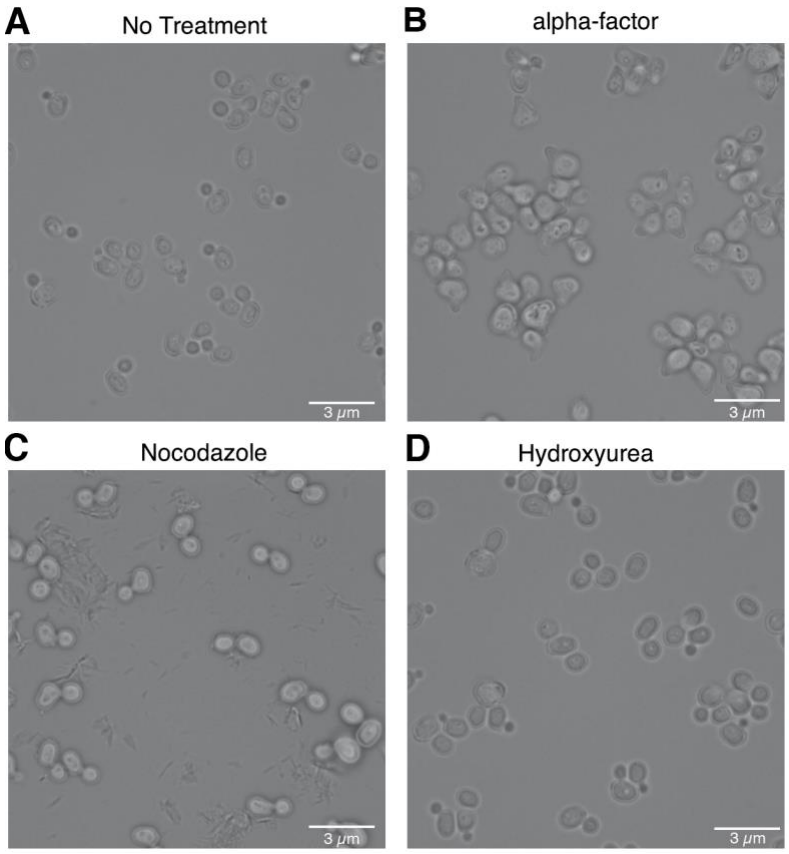


**Figure 5.1 Images of yeast cells during cell cycle arrest**

(A) Cells in asynchronous growth. (B) Cells arrested in G1 with alpha factor. (C) Cells arrested in G2/M with nocodazole. (D) Cells arrested in early S-phase with hydroxyurea.

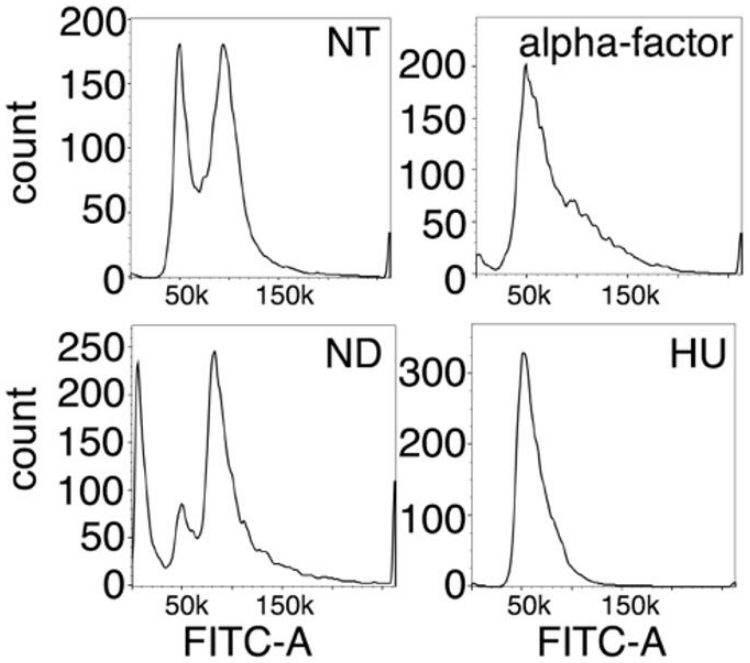


**Figure 5.2 Fluorescence histograms of DNA content**

Asynchronous $His_6$-FLAG-*SMT3* cultures stained with SYTOX green: no treatment (NT), G1 arrest with alpha-factor, GT/M arrest with nocodazole (ND), and S arrest with hydroxyurea (HU)

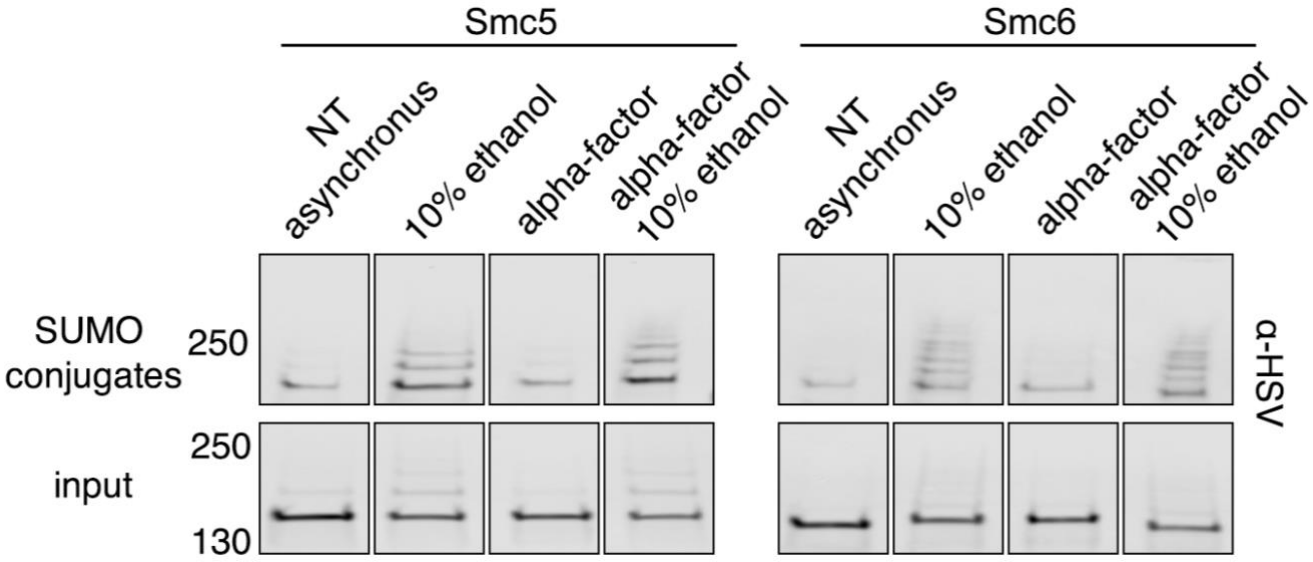


**Figure 5.3 Smc5 and Smc6 are sumoylated during G1**

Asynchronous cells expressing $His_6$-FLAG-*SMT3* and either C-terminally 3xHSV tagged Smc5 or Smc6 from their endogenous promoters were arrested in G1 with alpha factor for 90 minutes prior to treatment with 10% ethanol for 60 minutes. Cell lysates (input) and purified sumoylated proteins (SUMO conjugates) were examined by Western analysis with an anti-HSV antibody.

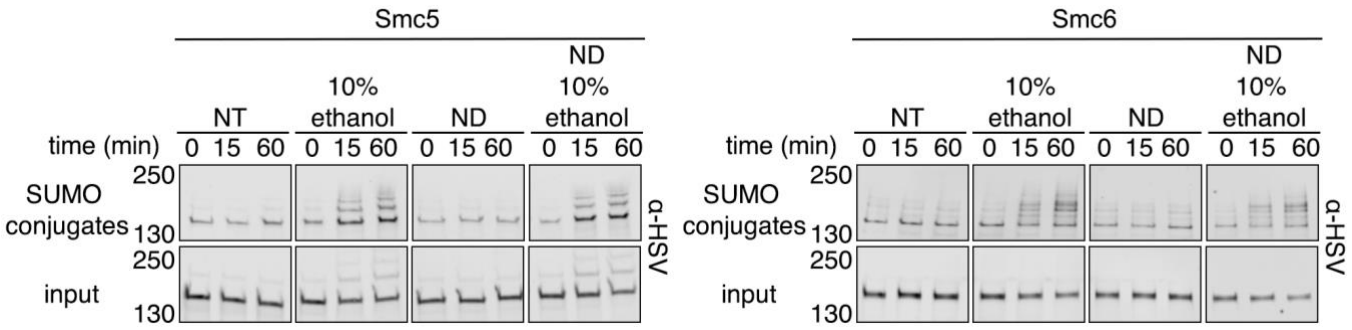


**Figure 5.4 Smc5 and Smc6 are sumoylated during G2/M**

Asynchronous cells expressing $His_6$-FLAG-*SMT3* and either C-terminally 3xHSV tagged Smc5 or Smc6 from their endogenous promoters were arrested in G2/M with nocodazole (ND) for 60 minutes prior to treatment with 10% ethanol for 60 minutes. Cell lysates (input) and purified sumoylated proteins (SUMO conjugates) were examined by Western analysis with an anti-HSV antibody.

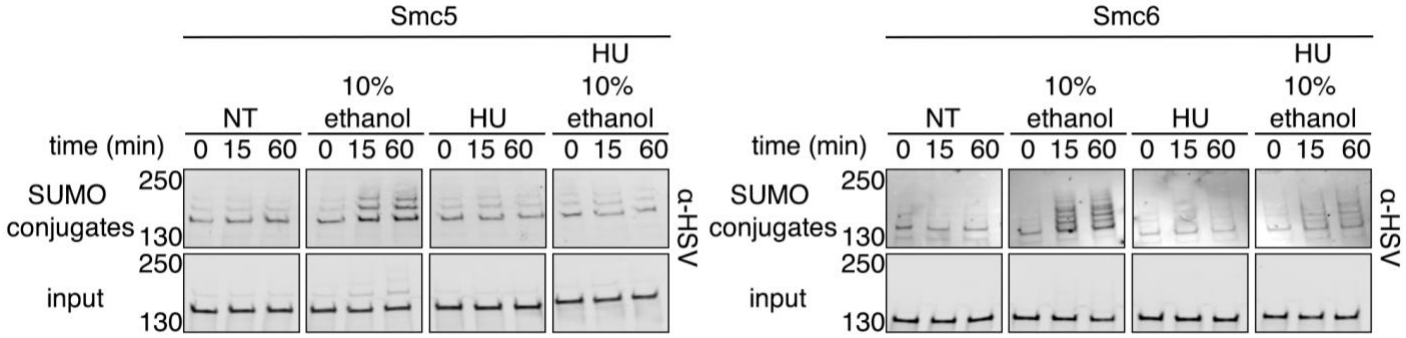


**Figure 5.5 Smc5 and Smc6 sumoylation is reduced in S phase**

Asynchronous cells expressing $His_6$-FLAG-*SMT3* and either C-terminally 3xHSV tagged Smc5 or Smc6 from their endogenous promoters were arrested in S with hydroxyurea (HU) for 60 minutes prior to treatment with 10% ethanol for 60 minutes. Cell lysates (input) and purified sumoylated proteins (SUMO conjugates) were examined by Western analysis with an anti-HSV antibody.

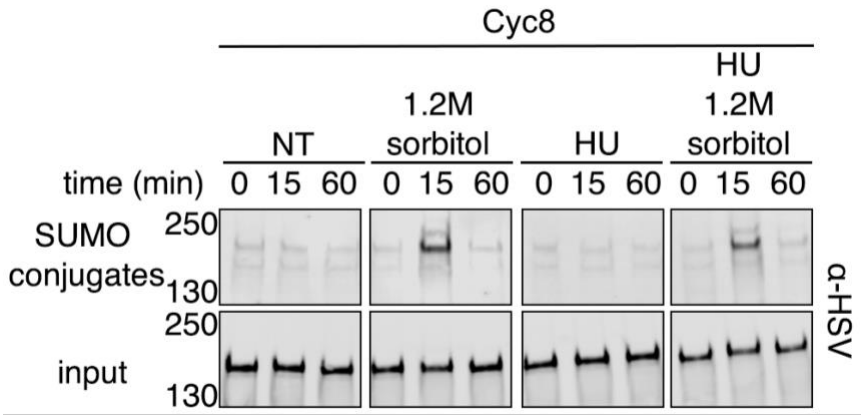


**Figure 5.6 Hydroxyurea does not block global sumoylation**

Asynchronous cells expressing $His_{6}$-FLAG-SMT3 and 3xHSV Cyc8 from its endogenous promoter were arrested in S-phase with HU for 90 minutes prior to 60 minutes of hyperosmotic (1.2M sorbitol) stress. Cell lysates (input) and purified sumoylated proteins (SUMO conjugates) were examined by Western analysis using an anti-HSV antibody.

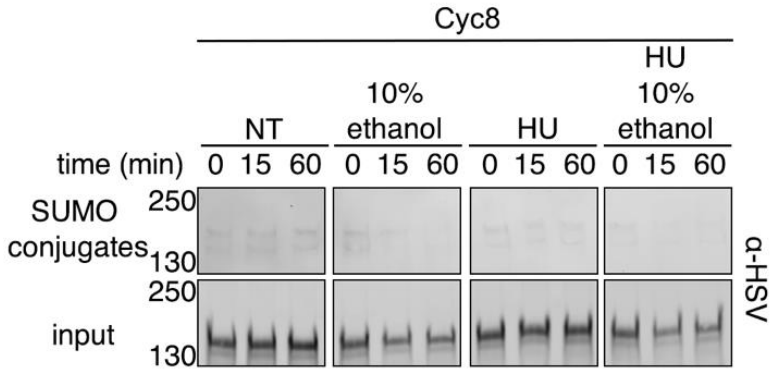


**Figure 5.7 Cyc8 is not sumoylated during acute ethanol exposure under S-phase arrest**
Asynchronous cells expressing $His_6$-FLAG-SMT3 and 3xHSV Cyc8 from its endogenous promoter were arrested in S-phase with HU for 90 minutes prior to 60 minutes of 10% v/v ethanol stress. Cell lysates (input) and purified sumoylated proteins (SUMO conjugates) were examined by Western analysis using an anti-HSV antibody.

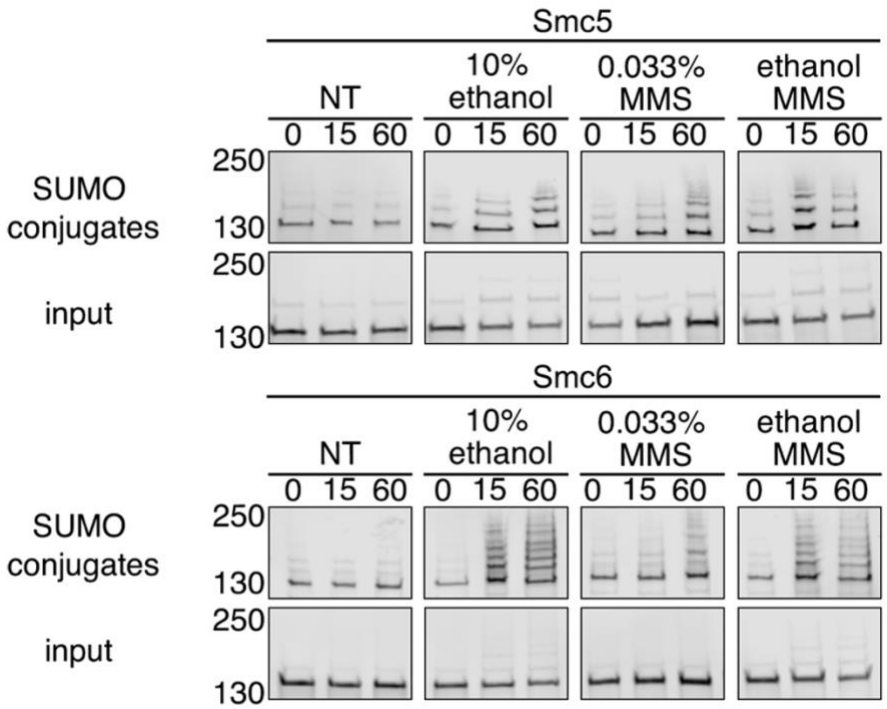


**Figure 5.8 Acute ethanol sumoylation is more predominant than sumoylation induced by DNA damage**

Cells expressing $His_6$-FLAG-*SMT3* and C-terminally 3xHSV epitope tagged Smc5 or Smc6 from their endogenous promoters were exposed to either no treatment, 10% ethanol, 0.033% MMS, or 10% ethanol and 0.33% MMS over 60 minutes. Cell lysates (input) and metal affinity purified sumoylated proteins (SUMO conjugates) were examined by Western analysis using an anti-HSV antibody.

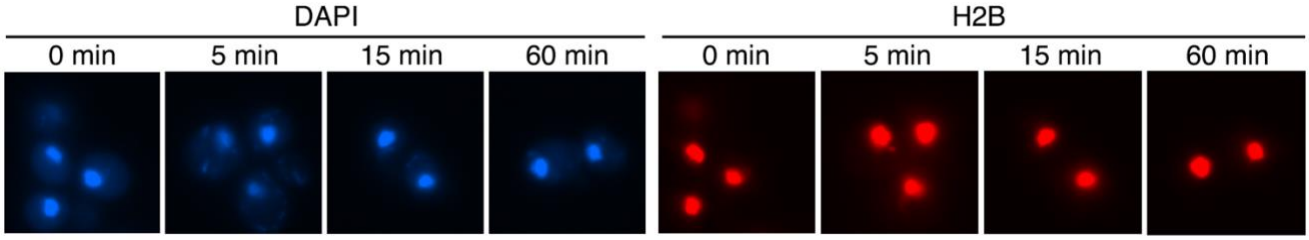


**Figure 5.9 Acute ethanol stress does not affect gross chromatin**

H2B-mCherry cells were exposed to acute ethanol over a 60-minute time course. Cells fixed at indicated timepoints and imaged by fluorescent microscopy.

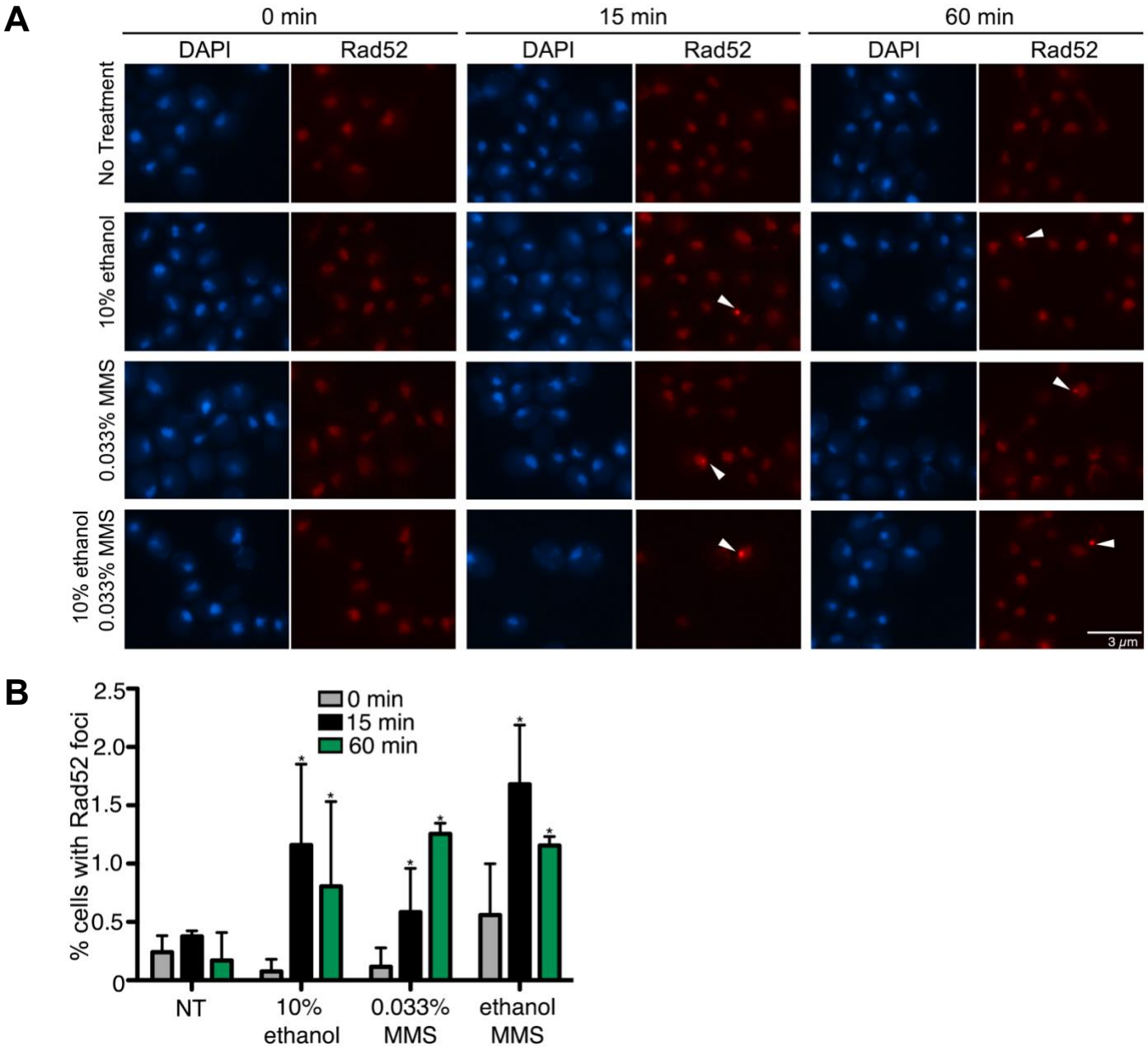


**Figure 5.10 Acute ethanol stress causes Rad52 formation**

(A) Rad52-tdTomato cells were exposed to acute ethanol (10%), 0.033% MMS, or combined ethanol and MMS over a 60-minute time course. Cells fixed at indicated timepoints and imaged by fluorescent microscopy. Five fields of cells for each condition with ≥40 cells/field were counted for the presence of nuclear foci (B).

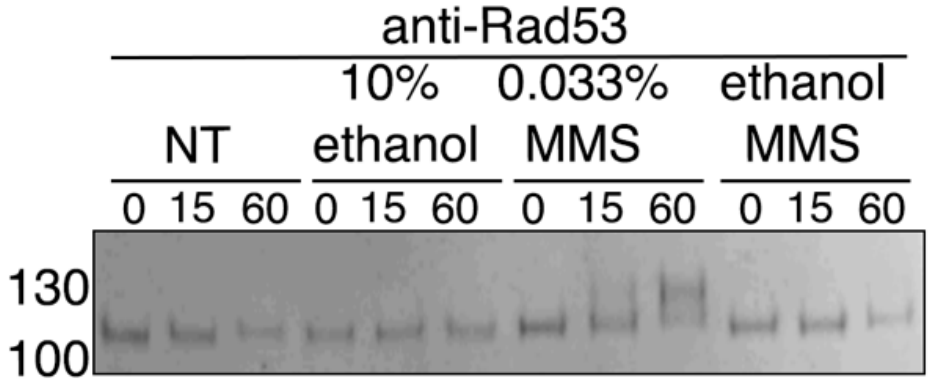


**Figure 5.11 Acute ethanol stress does not induce Rad53 phosphorylation**

Cells were exposed to acute ethanol (10%), 0.033% MMS, or combined ethanol and MMS over a 60-minute time course. Rad53 phosphorylation was observed by Western analysis using an anti-Rad53 antibody.

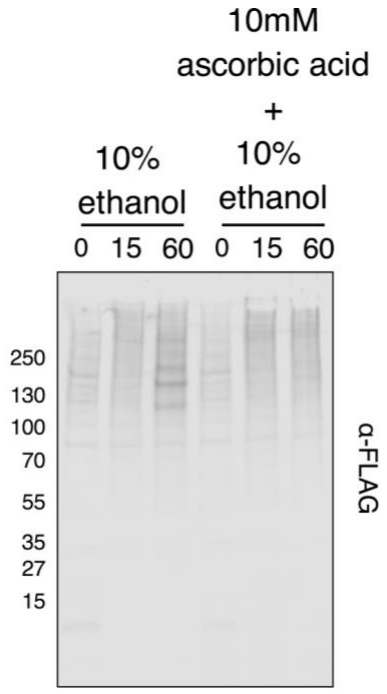


**Figure 5.12 Ascorbic acid treatment decreases acute ethanol induced global sumoylation**
$His_6$-FLAG-*SMT3* sumoylation patterns 10% v/v acute ethanol stress with or without pretreatment with ascorbic acid over 60 minutes. Changes in sumoylation patterns were examined by Western analysis using an anti-FLAG antibody.

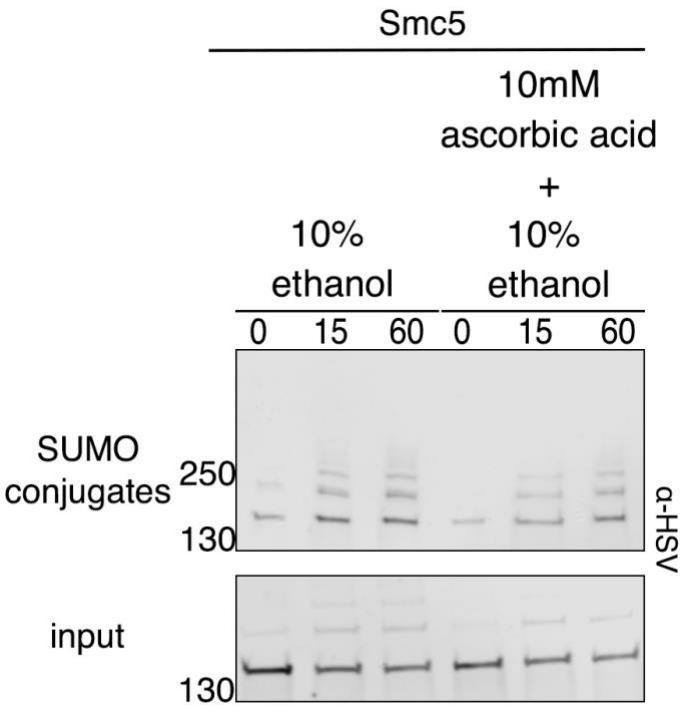


**Figure 5.13 Ascorbic acid treatment does not reduce acute ethanol induced sumoylation in Smc5**

Cells expressing $His_6$-FLAG-*SMT3* and C-terminally 3xHSV epitope tagged Smc5 their endogenous promoters were exposed to 10% ethanol with or without ascorbic acid pretreatment over 60 minutes. Cell lysates (input) and metal affinity purified sumoylated proteins (SUMO conjugates) were examined by Western analysis using an anti-HSV antibody.

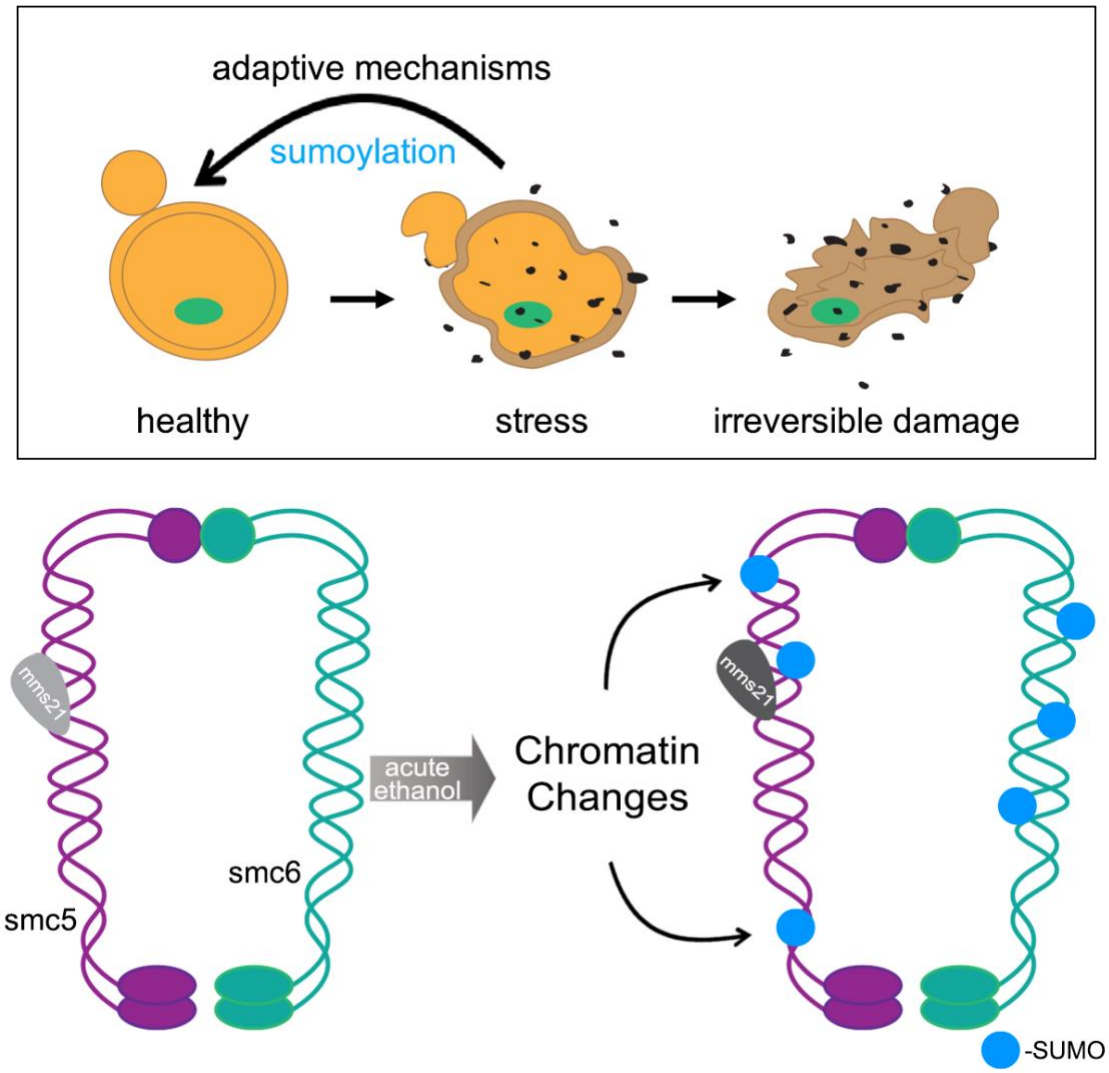


**Figure 5.14 Model for cellular stress and Smc5-Smc6 sumoylation**

## Chapter 6: Conclusions

My initial goal in completing the work described in this thesis was to gain a clearer understanding of the role posttranslational protein modifications. Specifically, the role the small ubiquitin like modifier, SUMO plays in the cellular stress response. Answering this fundamental question was of importance because exposure to stress is inevitable for all living organisms. While there have been hundreds of studies examining the role of SUMO and sumoylation during cellular stress, there remain several open questions in the field. Specifically, what are the mechanisms in which substrates are sumoylated under distinct conditions. One of the reasons there is further work needed to elucidate the effects of stress induced sumoylation on target substrates is due to the sheer volume of targets that are sumoylated under any given condition. To that end, I initially set out to examine the effects hyperosmotic stress, heavy metal stress, and acute ethanol stress had on protein sumoylation and the mechanisms in which sumoylation is occurring.

With respect to hyperosmotic stress, I began to follow up on a previous discovery in our lab. It had been reported that Cyc8, a highly conserved transcriptional co-repressor was transiently sumoylated during hyperosmotic stress that also formed transient inclusions. In our proposed model we suggest that the conserved prion domain of Cyc8 (PrD), is required for inclusion formation and transcriptional de-repression. Through my preliminary studies, I found that the PrD appears to be required for association with chromatin during hyperosmotic stress. Additional preliminary work by me demonstrated that heavy metal stress elicits a global sumoylation response but unlike the global hyperosmotic stress response, accumulation of high molecular weight SUMO conjugates is rapid and stable. While further work is needed to definitively determine both the role of Cyc8's PrD on transcriptional regulation and substrates sumoylated during heavy metal stress both projects demonstrate the vast and complex role of SUMO in the cell.

The majority of this dissertation however, examined the acute ethanol stress response. My work and findings are timely as there have been very few studies that have examined this phenomenon. Additionally, through my mass spectrometry analysis I was the first to report that Smc5-Smc6 are sumoylated after acute ethanol exposure. While my work has revealed the timing of Smc5-Smc6 acute ethanol induced sumoylation and yielded support to my thought that acute ethanol exposure is causing DNA damage it has also led to several additional questions. For example, what is the mechanism of acute ethanol induced sumoylation? Initially I thought

that acute ethanol-induced sumoylation could be occurring in response to formation of reactive oxygen species (ROS). While I found that treatment with an antioxidant prior to acute ethanol exposure decreased global sumoylation, I did not find that antioxidant pretreatment reduced sumoylation of Smc5 induced by acute ethanol. Due to this, and given my results I suspect that acute ethanol may cause DNA damage. While the evidence presented in this thesis is pointing in the direction of DNA damage, this cannot be stated definitively without further study. It would also be interesting to determine if acute ethanol promotes formation of the Smc5-Smc6 complex and if its subsequent sumoylation is enough to protect chromatin before the DNA damage response is fully triggered. While my data shows that acute ethanol does affect chromatin in some capacity, it is likely that it is more subtle compared to that of classical DNA damaging agents like MMS. In this study, I did not examine more in-depth chromatin changes beyond evaluating bulk chromatin which showed no discernable differences when compared to untreated conditions. Another point to consider is that sumoylation of Smc5-Smc6 could be aiding in protecting chromatin during acute ethanol exposure with respect solubility. In a test tube, high concentrations of ethanol precipitate DNA out of solution and it could be possible that sumoylation of Smc5-Smc6 on chromatin prevents this from occurring *in vivo*.

Overall my findings highlight the versatile features of protein sumoylation during cellular stress conditions. While the range of substrates and their kinetics vary between stress conditions, a common feature in this work and our previous studies illustrate that in response to cellular stress, most substrates have a role in regulating transcription and in the case of acute ethanol stress maintaining genome integrity. My work on acute ethanol stress shines a light onto how we think about stress at the cellular level and how acute stress exposure has been a relatively under studied area in the field.

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## Appendix I:

## Yeast strains used in acute ethanol studies

| Strain | Genotype | Reference |
|---|---|---|
| RGY 5266 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX* | Oeser et al., 2016 |
| RGY 5824 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX, cyc8Δ::NatMX, Cyc8*-3HSV::*LEU2* | Oeser et al., 2016 |
| RGY 5653 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0, H2B-mcherry* | Michelle Oeser |
| RGY 6005 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX*, *Smc6*-3HSV::*KanMX* | this study |
| RGY 6014 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX*, *Smc5*-3HSV::*KanMX* | this study |
| RGY 6339 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX*, *MMS21*-3HSV-IAA1.T10::*KanMX*, *AFB2*::*LEU2*, *Smc5*-3HSV::*URA3* | this study |
| RGY 6340 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX*, MMS21-3HSV-IAA1.T10::*KanMX*, *AFB2*::*LEU2*, Smc6-3HSV::*URA3* | this study |
| RGY 6346 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX*, *siz2Δ*::*KanMX*, *Smc5*-3HSV::*URA3* | this study |
| RGY 6347 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX*, *cst9Δ*::*KanMX*, *Smc6*-3HSV::*URA3* | this study |
| RGY 6361 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX*, *siz1Δ*::*KanMX*, *Smc5*-3HSV::*URA3* | this study |
| RGY 6362 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX*, *siz1Δ*::*KanMX*, *Smc6*-3HSV::*URA3* | this study |
| RGY 6363 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX*, *siz2Δ*::*KanMX*, *Smc6*-3HSV::*URA3* | this study |
| RGY 6344 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX*, *cst9Δ*::*KanMX*, *Smc5*-3HSV::*URA3* | this study |
| yJM2468 | *ura3-52*, *lys2-801*, *leu2-Δ1*, *his3-Δ200*, *trp1-Δ63*, RAD52-tdTomato::*HIS3MX* | Estrem and Moore, 2019 |

## Yeast strains used in hyperosmotic stress studies

| Strain | Genotype | Reference |
|---|---|---|
| RGY 5824 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX, cyc8Δ::NatMX, Cyc8*-3HSV::*LEU2* | Oeser et al., 2016 |
| RGY 5825 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX, cyc8Δ::NatMX, Cyc8(K735R,K736R, K738R, K748R)*-3HSV::*LEU2* | Oeser et al., 2016 |
| RGY 5826 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX, cyc8Δ::NatMX, Cyc8(Δ441-677)*-3HSV::*LEU2* | Oeser et al., 2016 |

| | | |
|---|---|---|
| RGY 5823 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX, cyc8Δ::NatMX, Cyc8(Δ441-677, K735R,K736R, K738R, K748R )*-3HSV::*LEU2* | Oeser et al., 2016 |
| RGY 5957 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX, cyc8Δ::NatMX, Cyc8*-GFP (PrD 22QA 31Q)::*LEU2* | this study |
| RGY 5958 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX, cyc8Δ::NatMX, Cyc8*-GFP (PrD 32QA 31Q)::*LEU2* | this study |
| RGY 5985 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX, cyc8Δ::NatMX, Cyc8*-GFP (K735R, K736R, K738R, K748R,PrD 22QA/31Q)::*LEU2* | this study |
| RGY 5986 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX, cyc8Δ::NatMX, Cyc8*-GFP (K735R, K736R, K738R, K748R,PrD 32QA/31Q)::*LEU2* | this study |
| RGY 5987 | *met15Δ0*, *his3Δ1*, *ura3Δ0*, *leu2Δ0*, 6His-FLAG-*SMT3*::*HIS3MX, cyc8Δ::NatMX, Cyc8*-GFP (K735R, K736R, K738R, K748R,PrD 40QA/31Q)::*LEU2* | this study |

## Appendix II:
## Materials and Methods

### Yeast strains and plasmids

Yeast strains and plasmids used in this study are listed in (Appendix I). Standard yeast genetic methods were used for this study (Guthrie C, 1991). All gene deletions were verified by colony PCR and phenotypic analyses when available.

### Growth and stress conditions

Cells were grown to a density of ~1.5x$10^7$ cells/ml at 30°C in yeast extract peptone dextrose (YPD) media prior to stress induction. All 0 time point samples were collected before stress induction. For ethanol stress, volume per volume amounts were added to cultures for a final concentration of 10% v/v ethanol. For hyperosmotic stress, equal volumes of culture and YPD+2.4M sorbitol were combined for a final concentration of 1.2M sorbitol. For heat stress, cells were pelleted and resuspended in YPD media warmed to 42°C and placed in a shaking platform 42°C incubator.

### Sumoylated protein purification

50ml aliquots of cells were collected at designated time points after stress and flash frozen in liquid nitrogen. Harvested cells were lysed by vortexing with acid washed glass beads at 4°C in 1ml denaturing lysis buffer (8M urea, 50mM Tris pH 8.0, 0.05% SDS with 2mM PMSF and 20mM NEM). Aliquots representing 5% of the input were set aside. Cell lysates were incubated with TALON resin (Novagen) overnight at 4°C. Resin was washed 3x with wash buffer (8M urea, 50mM Tris pH 8.0, 200mM NaCl, 0.05% SDS). Sumoylated proteins were eluted from beads with loading buffer (8M urea, 10mM MOPS, 10mM EDTA, 1% SDS, 0.01% bromophenol blue, pH 6.8) and incubated at 65°C for 10 minutes.

### Western analysis

Total cell lysates and purified sumoylated proteins were resolved by SDS-PAGE using 4-20% gradient gels (BioRad). Western analyses were performed with mouse anti-FLAG (1:2500, Sigma), mouse anti-HSV (1:2500, Novagen), rabbit anti-HSV (1:2000, Abcam), rabbit anti-Rad53 (1:2000, Abcam), and mouse anti-GFP (1:2000).

**Mass spectrometry analyses**

Sumoylated proteins from cells exposed to 0, 5, or 60 minutes of 10% (v/v) ethanol stress were enriched by metal affinity chromatography as described above. Samples were run 1 cm into a 4-20% SDS-PAGE gel and bands were excised. Proteins in gel slices were digested with trypsin and digestion products desalted and dried by vacuum centrifugation. Dried peptide mixtures were resuspended in 7ul of 0.1% formic acid. 5μL was analyzed using a LTQ OrbiTrap mass spectrometer (Thermo Scientific). Complete MS methods were performed as previously described (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 (~10% false discover rate). The entire dataset is in Table S1). The significance of the changes in peptide counts between 0, 5, 60 minutes of ethanol stress was determined by a two-tailed, homoscedastic student's t-test. Data was filtered by a p≤0.1 and a +/-3-fold change in summed peptide counts. Final filtered data is in Table 4.1.

**Auxin-degron depletion experiments**

Cells were grown to a density of ~$0.86x10^7$ cells/ml at 30°C in Yeast Complete (YC) media prior to addition of either NT, vehicle (1:1000, 95% EtOH), or 100μM 3-Indoleacetic acid (IAA, Sigma). Cells were then incubated for 90 minutes at 30°C and prior to stress induction. 10% (v/v) ethanol was then added to cultures for an additional hour with 50ml aliquots of cells collected at designated timepoints flash frozen in liquid nitrogen before proceeding with sumoylated protein purification.

**Cell cycle analysis**

Asynchronous cells were diluted back to a density of ~$0.39x10^7$ cells/ml in YPD or YPD+1% DMSO (nocodazole) media prior to cell cycle halt and stress induction. For G1 arrest, 50μg/ml alpha-factor (Sigma) was added immediately to cultures and incubated at 30°C for 90 minutes. For G2/M arrest, cells were incubated at 30°C for 2 hours then treated with 0.05 mg/ml nocodazole (Sigma) for an additional hour. For S arrest, 100mM hydroxyurea (Sigma) was added immediately to cultures and incubated at 30°C for 90 minutes. All cell arrests were

verified by pelleting a 200ul aliquot of culture and examining under a phase contrast microscope (Nikon). ~75-90% of cells per field were observed to be arrested at a given phase (Supplemental Figure 2). For G1 arrest cells were observed to be either large and unbudded or with shmoo morphology. Cells arrested in S-phase were observed to be large with small buds. While G2/M arrested cells were observed as dumbbells.

**Flow cytometry analysis**

Yeast cell-cycle analyses by flow cytometry were conducted similar to those previously described (Richardson *et al.*, 2012). Cells were grown in 5 ml cultures to a density of ~0.8 x $10^7$ and exposed to the following conditions: no treatment, alpha-factor, nocodazole, or hydroxyurea and arrested as described above. After arrest, cells were harvested by centrifugation, fixed in 70% ethanol, washed with 50mM sodium citrate containing 0.25mg/ml RNase A, incubated at 95ºC for 15 minutes and then 37ºC for 2 hours. Fixed cells were resuspended in 50 mM sodium citrate, stained with 2µM SYTOX green (Invitrogen), and sonicated for 1s at an amplitude of 10% immediately before analysis by flow cytometry. Cell cycle analyses were performed with FlowJo software.

**Fluorescence microscopy**

Aliquots of cells at each time point after ethanol stress were removed, fixed in 4% paraformaldehyde solution for 15 minutes at room temperature then washed with 1X PBS. Cells were imaged on a Nikon Eclipse 90i with a 100x objective, filters for GFP [HC HiSN Zero Shift filter set with excitation wavelength (450–490 nm), a dichroic mirror (495 nm) and emission filter (500–550 nm)], tdTomato [HC HiSN Zero Shift filter set with excitation wavelength (530–560 nm), a dichroic mirror (570 nm) and emission filter (590–650 nm)] or DAPI [HC HiSN Zero Shift filter set with excitation wavelength (325–375 nm), a dichroic mirror (400 nm) and emission filter (435–485 nm)], and a Photometrics Cool Snap HQ2 cooled CCD camera with NIS-Elements acquisition software.

**Chromatin immunoprecipitation**

50ml aliquots of cells were grown to a density of ~1.5x$10^7$ cells/ml at 30C in rich medium prior to stress induction. A pre-stress sample was collected prior to induction of stress. Hyperosmotic stress was induced by addition of sorbitol to a final concentration of 1.2M, and samples were collected at indicated times. Protein-DNA crosslinking was done by adding formaldehyde to a concentration of 1% and incubated at room temperature for 15 minutes with continuous swirling.

The reaction was quenched by addition of glycine to 0.125M for 5 minutes at room temperature. Samples were washed in 1XTBS, collected by centrifugation, and flash frozen in liquid nitrogen. Cells were lysed by bead-beating in 5ml breaking buffer (100mM Tris pH 7.9, 20% glycerol, 2mM PMSF) at 4C. Insoluble chromatin was collected by centrifugation and resuspended in 700ul ChIP lysis buffer (50mM HEPES pH 7.5, 140mM NaCl, 1% Triton X-100, 0.1% deoxycholate, 2mM PMSF) and sonicated to shear chromatin. A sample prior to immunoprecipitation was collected to represent input controls. Immunoprecipitation was performed on 200ul sheared chromatin by incubation with mouse anti-HSV bound to protein A-sepharose beads overnight at 4C. Beads were washed twice each with ChIP lysis buffer, high salt ChIP lysis buffer (50mM HEPES pH 7.5, 500mM NaCl, 1% Triton X-100, 0.1% deoxycholate, 1mM EDTA, 2mM PMSF), and 1X TE buffer. Immunoprecipitated protein-DNA complexes were eluted by incubation in ChIP elution buffer (50mM Tris pH 8.0, 1% SDS, 10mM EDTA) at 65C for 10 minutes. Samples were treated with Proteinase K (NEB) and RNase at 50C for one hour, followed by removal of crosslinking by incubation at 65C overnight. Input and eluate DNA was isolated using QIAprep spin columns (Qiagen) and analyzed by qPCR.

**qPCR analyses**

Quantitative real-time PCR was performed using a Quant-Studio 3 Real-Time PCR system (Applied Biosystems). Reactions were performed on ChIP inputs and eluates using PowerUP SYBR Green Master Mix (Invitrogen) with specific primers for HXT6, HXT7, or ACT1 control.

**Image processing**

All blots were scanned using a Licor Odyssey CLx and ImageStudio Lite. All images were processed with a MacBook Pro or iMac computer (Apple) using Photoshop (Adobe).

**Rigor and reproducibility**

All biochemical and microbiological assays were performed in triplicate. For fluorescence microscopy, three separate researchers quantified foci formation. Statistics used were paired students t-tests and two-way ANOVA with Bonferroni *post hoc* test.