Hyperosmotic stress induced inclusion formation of a prionogenic transcriptional corepressor is regulated by glycerol accumulation.

Cory Matthew Nadel

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

Doctor of Philosophy

University of Washington
2018

Reading Committee:
Richard Gardner, Chair
Sandra Bajjalieh
Chris Hague

Program Authorized to Offer Degree:
Pharmacology

University of Washington

Abstract

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Chair of Supervisory Committee:
Richard Gardner
Department of Pharmacology

Environmental stressors cause severe perturbations in homeostasis that, if left unchecked, can severely damage cells. In order to cope with a constantly changing environment, organisms have evolved complex signaling programs that allow for adaptation in the face of stress. These programs require a host of post-translational modifications, including attachment of the small ubiquitin-like modifier SUMO/SMT3. SUMOylation has been shown to be critical for the response to environmental stress, and many targets of SUMOylation have been characterized. What remains unclear, however, are the factors regulating stress-induced SUMOylation and the consequences of these SUMOylation events. It has been shown previously that the major targets of hyperosmotic stress-triggered SUMOylation in the budding yeast *Saccharomyces cerevisiae* are the transcriptional corepressor complex Cyc8-Tup1. Here, I show that the

osmostress responsive MAP kinase Hog1 is a critical regulator of Cyc8-Tup1 SUMOylation via its role in upregulating the biosynthesis of the compatible osmolyte and chemical chaperone glycerol. Mutations that ablate SUMOylation of Cyc8 are sufficient to rescue the osmosensitivity of *hog1*Δ cells, and this is facilitated by inappropriate derepression of glycerol biosynthesis genes in the absence of *HOG1*. We previously showed that Cyc8 forms transient nuclear foci during hyperosmotic stress, and I show here that cells unable to synthesize glycerol display Cyc8 inclusions that are persistent, soluble, and dynamic. These observations unveil a novel intersection between phosphorylation and SUMOylation networks, which are critical for shifting gene expression and metabolic programs during stress adaptation. Moreover, this work provides new insights into metabolic factors regulating the biomolecular condensation of proteins that have important implications for prion biology.

Acknowledgements

Rarely can great accomplishments be attributed solely to a single person, and the culmination of my graduate work is no exception. A number of people have supported, encouraged, and inspired me along this journey, and they deserve recognition here in the pages of this thesis.

To my family, especially my parents, Roger and Debbie: thank you for your endless support and love across all my years of life. You've taught me invaluable lessons on what it means to be a good person, to work hard in the pursuit of a goal, and to maintain perspective during hard times. I love you both and would certainly not be here without you.

To my brother, Adam: thank you for the friendship, laughs, and near-daily conversations during my time in Seattle. Your work ethic is a constant source of inspiration for me and I cherish the relationship we've built into adulthood.

To my friends old and new: thank you for the endless supply of good times. It is truly a gift to be surrounded by such smart, talented, and hilarious people.

To my various climbing partners: thank you for pushing me out of my comfort zone, for encouraging me to try hard, and for literally catching me when I fall. I've learned great lessons from you and our time in the mountains that I'll take with me forever.

To Dr. Richard Gardner and the current and former members of the Gardner Lab: thank you for making work such a wonderful environment to come to every day. Likewise, thank you for listening to me endlessly ramble on about science on a near daily basis; I know it can be exhausting but I appreciate your patience.

To Kasey, thank you for your trust, your friendship, and your love. You took an amazing leap of faith coming with me to Seattle, but I am endlessly grateful for the life we have built together. I love you with all my heart.

This work is dedicated to three people gone but not forgotten:

To my uncle, Ethan, for inspiring me to become a scientist.

To my grandfather, Horace, for teaching me the importance of faith.

To my grandmother, Ruth, for reminding me to always be grateful.

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Chapter 1: Background and Significance

Cellular Stress: Definition, Types, and Biological Significance

Cellular stresses are abiotic perturbations that can severely and irreversibly damage biomolecules and essential cell structures. All organisms experience cellular stress and subsequently must adjust a variety of cellular programs to reestablish homeostasis. Many of these include signal transduction networks, metabolic pathways, gene expression programs, cell-cycle progression, and protein quality control systems (Ananthan, Goldberg et al. 1986, Morimoto 2008, Arsenijevic, Vujovic et al. 2013). Resiliency in the face of stress is crucial to cellular survival with the deterioration of adaptive measures thought to underlie a variety of age-related human diseases such as cancer, heart disease, and neurodegeneration (Samali, Fulda et al. 2010). Moreover, the past two decades have seen a significant shift in the production of fuels, drugs, materials, and other consumables into biological systems (Ro, Paradise et al. 2006, Lam, Ghaderi et al. 2014). A detailed understanding of the adaptive responses to stress in microorganisms will enable us to modify these factories to have greater productive capacities with more robust yields.

Conditions that can elicit cellular stress are wide-ranging. Many of these stressors are exogenous, including major swings in temperature, hyper or hypoosmotic conditions, hypoxia, and exposure to a variety of noxious compounds like heavy metals, toxins, or genotoxic species (Kultz 2005). Conversely, some are endogenous, such as the production of reactive oxygen species produced during mitochondrial respiration (Schieber and Chandel 2014). Alternatively, some stressors come not from exposure, but from lack of exposure. Such stress is observed in organisms undergoing starvation due to lack of utilizable carbon or nitrogen sources (Kang, Pacold et al. 2013).

As a result of this breadth, the biochemical responses that facilitate adaptation to stress are equally diverse and unique to the stresses that initiate

them. Some stressors induce detoxification systems to clear toxic chemical species, while others induce systems involved with repairing damage (Dringen, Brandmann et al. 2015). For example, heat shock causes protein damage by thermally unfolding proteins, resulting in loss-of-function and subsequent aggregation. To deal with this, cells initiate a gene expression program that increases the transcription of chaperone proteins, which assist in maintaining protein structure or triaging terminally unfolded proteins into aggregates that can be sequestered from other cellular components (Zheng, Krakowiak et al. 2016). This is but one example; but generally speaking, these responses are quite intuitive and are directly related to the stress that initiates them.

Cellular stress plays an important role in a number of conditions relevant to human disease. Human cells are constantly under attack from genotoxic and proteotoxic insults; without adaptive measures, a host of disease conditions will arise. This is especially apparent in the case of genotoxic stress, as DNA mutations within somatic cells are both a driver and hallmark of neoplastic conditions (Hosoya and Miyagawa 2014). As we age, homeostatic mechanisms such as protein quality control decline in fidelity, thereby increasing the cellular burden of misfolded or damaged proteins (Taylor and Dillin 2011). Neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, frontotemporal dementia, and others display protein aggregates that, along with significant atrophy of specific brain regions, have become crucial *ex mortem* diagnostic markers (Jucker and Walker 2013). Whether these aggregates are causal or symptomatic remains controversial; what is clear, however, is that the ability to deal with proteotoxic stress declines in patients stricken with these age-related diseases.

Other environmental stressors plague the human body as well. Reactive oxygen species produced during oxidative phosphorylation can cause damage to mitochondria. In response, cells produce antioxidants without which the cell undergoes mitochondrial clearance through mitophagy (Wang, Nartiss et al.

2012). Maintenance of osmotic balance is also crucial, as many critical physiological processes depend on dissolved ionic species. Many human cell types see a spectrum of osmotic conditions as part of their normal physiologic processes, such as the kidney, liver, cornea, gastrointestinal tract, and soft-tissue of the joints and intravertebral discs (Aramburu and Lopez-Rodriguez 2009). Generally speaking, the osmolarity of the fluids surrounding these tissues is maintained within a tight range, and departure outside this range can cause significant alterations in function (Christoph, Beck et al. 2007). Hyperosmolarity has been observed in fluids taken from patients with irritable bowel diseases like Crohn's disease and ulcerative colitis (Schilli, Breuer et al. 1982). Maintenance of plasma osmolarity is critical for normal heart function, and chronic activation of vasoreceptors due to osmotic imbalance can lead to hypertension (Toney and Stocker 2010). Indeed, there is a growing list of disease conditions associated with hyperosmotic stress, including dry eye disease, chronic inflammatory conditions, and diabetes-related clinical issues like peripheral neuropathy and retinopathy (Chiang, Yang et al. 2010, Neuhofer 2010, Lemp, Bron et al. 2011).

Beyond the treatment of human disease, there are other contexts where stress response plays a critical role. For one, there has been a significant shift in the production of complex chemicals and materials away from traditional chemical procedures toward more environmentally conscious and efficient methods (Lee and Na 2013). To do this, researchers have employed genetic approaches to drive plants or microorganisms to create diverse things. While modern synthetic biological approaches now involve cutting-edge laboratory techniques like directed evolution and the creation of entirely synthetic organisms, mankind has been using unicellular organisms like the budding yeast *Saccharomyces cerevisiae* to produce chemicals like ethanol for millennia (Chiao and Sun 2007).

Modern man is amidst a synthetic biology revolution, which will undoubtedly solve many of the problems our species will face in the future.

However, a major barrier to this revolution lies within the so-called factories themselves, as microorganisms lose productive capacity under the stressful conditions that synthetic biologists place them under. Recalling the example of ethanol production in yeast, fermentative capacity decreases significantly as the osmolarity and ethanol concentration of the fermenting environment increases (Ding, Huang et al. 2009). A better understanding of the pathways that influence yeast stress responses will allow us to make more productive and longer lived microbial factories and enable our triumph over significant future adversities like global hunger.

Protein Modification by SUMO: Mechanism, Functions, and Role in Cellular Stress Response

Post-translational modifications are critical for the response to cellular stress. These modifications often occur rapidly upon the onset of stress and can be either chemical or proteinaceous (Bode and Dong 2004, Dai and Gu 2010, Kourtis, Moubarak et al. 2015). One of the major proteinaceous modifications that have been observed to occur during a wide range of cellular stresses is the addition of the small-ubiquitin-like-modifier (SUMO) (Enserink 2015). SUMO is evolutionarily conserved across eukaryotes, with varying degrees of complexity and divergence. In mammals there are four SUMO genes; in lower eukaryotes like *S. cerevisiae*, this system is simplified and the SUMO protein is expressed from a single gene (titled *SMT3* in yeast but here-to-for referred to as *SUMO*) (Kretz-Remy and Tanguay 1999). In yeast, SUMO and specific members of its conjugation/deconjugation machinery are essential, thereby implying their importance for basic biological function (Hayashi, Seki et al. 2002).

Like its cousin, ubiquitin, SUMO is attached to proteins via a complex enzymatic cascade. SUMO is expressed as a nascent preprotein, which must be processed by the SUMO protease Ulp1 to reveal a C-terminal diglycine motif that serves as the attachment point to the target substrate (Elmore, Donaher et al. 2011). Conjugation to targets is initiated first by ATP-dependent activation

through the heterodimeric SUMO activation enzymes Uba2/Aos1 (Gong, Li et al. 1999). Activated SUMO is passed to the sole SUMO E2 Ubc9, which like many ubiquitin E2 enzymes has inherent SUMO ligase activity (Lee, Melchior et al. 1998). Appropriate substrate recognition and subsequent conjugation is achieved through one of four SUMO ligases – Siz1, Siz2, Mms21, and Cst9. Like ubiquitination, SUMOylation is dynamic, and SUMO can be cleaved from substrates by either of two deSUMOylating enzymes Ulp1 or Ulp2 (Schwienhorst, Johnson et al. 2000).

SUMO shares a number of similarities to ubiquitin. While only 18% sequence homolgous to ubiquitin, SUMO is similar in size and shares the "ubiquitin-fold" of three beta sheets with a transverse alpha helix (Taherbhoy, Schulman et al. 2012, Vierstra 2012). Like its cousin, SUMO is covalently attached to lysine residues on target substrates. Furthermore, substrates can be SUMOylated in various manners similar to ubiquitination, including monoSUMOylation, multiSUMOylation (modification by a single SUMO molecule at multiple distinct sites), and polySUMOlyation (modification by multiple SUMO molecules at an individual site) (Hay 2005). SUMO and ubiquitin can be incorporated into heterotypic or "hybrid" chains, further increasing the complexity of ubiquitin and ubiquitin-like conjugation (Hay 2013). Such is seen in the DNA damage response, where the SUMO-targeted ubiquitin ligase (STUbL) RNF4 synthesizes hybrid SUMO-ubiquitin chains that are necessary for the recruitment of RAP80-BRCA1 and subsequent DNA repair (Guzzo, Berndsen et al. 2012).

While SUMO has similarities to ubiquitin, it is functionally diverse. Ubiquitination, amongst other functions, is classically known as a signal for proteosomal degradation; SUMOylation, on the other hand, does not directly target proteins for destruction (Eckermann 2013). Conversely, SUMOylation mediates a wide range of cell biological phenomenon, with SUMOylation substrates being highly enriched in the nucleus (Gill 2004). Some such behaviors include nucleocytoplasmic transport, cell cycle progression, DNA repair and

stabilization of genome integrity, transcriptional regulation, and protein-protein interaction (Enserink 2015).

SUMO-dependent protein-protein interactions are mediated through SUMO Interaction Motifs (SIMs) present in the interacting client protein, which bind SUMO at a discrete beta-strand via hydrophobic interactions (Parker and Ulrich 2012). The multivalent nature of these interactions allow cells to build intricate multi-protein complexes via SUMO-SIM interactions, and the dynamic nature of SUMOylation facilitates spatiotemporal control of these complexes (Li, Stark et al. 2010). This phenomenon has been termed “protein group SUMOylation,” and was first observed as part of the DNA damage response system. Protein group SUMOylation increases the fidelity of crucial cell functions like DNA repair by massively increasing protein complex stoichiometry at a specific site (Jentsch and Psakhye 2013). Further, the multivalent nature of the interactions insulates the response from loss of function by individual mutations, as many SUMO-SIM interactions across many proteins facilitate the eventual assembly of the complex (Psakhye and Jentsch 2016). Not unsurprisingly, many proteins that coalesce into focal structures in the nucleus are SUMOylated, including PML and SP100, and SUMOylation is required to recruit other proteins into these foci (Sternsdorf, Jensen et al. 1997, Zhong, Muller et al. 2000).

As mentioned earlier, SUMOylation has been shown to be an important post-translational modification for the response to cellular stress. Many cellular stresses induce waves of SUMOylation, including oxidative, proteotoxic, genotoxic, chemical, and hyperosmotic stresses (Oeser, Amen et al. 2016). Many of the targets of SUMOylation are specifically modified during distinct stress conditions, and the kinetics of these SUMOylation events are equally unique (Zhou, Ryan et al. 2004, Miller, Scalf et al. 2013, Oeser, Amen et al. 2016). While many studies have documented these targets, the consequences of stress-dependent SUMOylation still remain poorly characterized (Enserink 2015).

Furthermore, little is known about cellular factors that regulate the dynamics of stress-dependent SUMOylation (Wohlschlegel, Johnson et al. 2004).

The Osmoregulatory Mitogen Activated Protein Kinase (MAPK) Hog1

Yeasts subsist primarily by fermenting sugars from decomposing fruit, and are subsequently exposed to a wide-range of salt and sugar concentrations. As those fruits dessicate, the concentrations of external osmolytes increase, frequently beyond the normal physiologic range. In response to hyperosmotic stress, water is rapidly effluxed from cells in an attempt to reestablish osmotic equilibrium. This causes cells to significantly decrease in size and volume, resulting in a number of deleterious effects, including macromolecular crowding, ionic imbalance, oxidative stress, and DNA damage (Hohmann 2015). Without a response, yeast cells would quickly die due to the adverse effects of hyperosmotic stress. Remarkably, yeasts can survive osmotic conditions well outside the physiologic range, indicating a robust adaptive program to hyperosmotic stress (Brion, Pflieger et al. 2016).

In *S. cerevisiae*, the Hog1 (Hyper Osmotic Glycerol) MAPK facilitates the response to hyperosmotic stress through a canonical MAPK pathway (Brewster, de Valoir et al. 1993). First, membrane-bound osmosensors undergo conformational change due to increased turgor pressure within the membrane (Reiser, Raitt et al. 2003). This initiates a phosphorylation cascade, wherein the redundant MAPKKKs Ssk2/Ssk22 and Ste11/Ste50 phosphorylate the MAPKK Pbs2, which then phosphorylates Hog1 at a defined *TXY* motif (Tanaka, Tatebayashi et al. 2014). Dual phosphorylation of Hog1 activates the kinase, triggering import into the nucleus within a few minutes (Ferrigno, Posas et al. 1998). In the nucleus, Hog1 phosphorylates a variety of transcription factors, thereby activating genes associated with stress response (Proft and Struhl 2002). Following adaptation, Hog1 is dephosphorylated by a host of phosphatases, exported from the nucleus, and the system is reset (Mattison and Ota 2000). Along with the aforementioned nuclear targets, Hog1 has a number of other

substrates, including proteins involved in cell cycle progression, mRNA export, translation, metabolism, and membrane transport (Duch, de Nadal et al. 2012, Regot, de Nadal et al. 2013, Bai, Tesker et al. 2015). This pathway is evolutionarily conserved, as Hog1 is considered functionally homologous to the mammalian p38 MAPK (Tao, Sanghera et al. 1996).

A major function of Hog1 during hyperosmotic stress response is to increase the intracellular concentration of compatible osmolytes (San Jose, Monge et al. 1996). Compatible osmolytes are inert solutes synthesized by organisms of all kinds during hyperosmotic stress that function to retain water, reestablish ionic balance, and stabilize native protein structure (Kwon and Handler 1995, Rishi, Anjum et al. 1998). Compatible osmolytes fall broadly under distinct classes: methylamines and methylsulfoniums; amino acids and amino acid derivatives; and polyols and carbohydrate derivatives (Yancey 2005). Generally speaking, increasing the concentration of various compatible osmolytes is achieved through a multifaceted mechanism, including changes in gene expression, increased activity of biosynthetic enzymes, and altered membrane permeability via channels and transporters (Petelenz-Kurdziel, Kuehn et al. 2013).

As its namesake implies, Hog1 primarily drives the biosynthesis and retention of glycerol, the compatible osmolyte of choice for *S. cerevisiae* (San Jose, Monge et al. 1996). This is achieved by modulating glycerol at numerous locations, from the plasma membrane to the nucleus. In the nucleus, Hog1 induces expression of the genes *GPD1 (glyceraldehyde phosphate dehydrogenase* 1) and *GPP2 (glycerol phosphate phosphatase 2)*, which code for enzymes involved in glycerol biosynthesis (Albertyn, Hohmann et al. 1994, Babazadeh, Furukawa et al. 2014). In the cytoplasm, Hog1 modulates the activity of Pfk26 (6-phosphofructo-2-kinase), which helps to shunt precursor molecules out of glycolytic pathways toward glycerol biosynthesis (Kuhn, Petelenz et al. 2008). At the membrane, Hog1 phosphorylates the glycerol efflux pump Fps1,

thereby evicting its positive regulators and closing the channel to glycerol efflux (Lee, Reiter et al. 2013). Also, Hog1 induces expression of the glycerol import protein Stl1, which helps to bring in any available glycerol from the surrounding media (Kayingo, Martins et al. 2009). This aspect of Hog1 function is critical for survival, and cells that are incapable of producing or retaining glycerol are highly osmosensitive (Albertyn, Hohmann et al. 1994, Lee, Reiter et al. 2013). Likewise, cells that have been synthetically rewired to produce glycerol in the absence of Hog1 show rapid volume recovery after exposure to hyperosmotic stress and are able to grow in high osmolarity medium (Babazadeh, Furukawa et al. 2014).

In *S. cerevisiae*, exposure to hyperosmotic stress initiates a rapid wave of SUMOylation (Abu Irqeba, Li et al. 2014, Lewicki, Srikumar et al. 2015, Oeser, Amen et al. 2016). This signaling event is transient, with maximal SUMOylation peaking within 15 minutes of stress exposure followed by rapid deSUMOylation. Additionally, it has been shown that loss of Hog1 extends the duration of stress-induced SUMOylation (Abu Irqeba, Li et al. 2014). Increasing global SUMOylation by overexpression of the SUMO ligase Siz1 increases the osmosensitivity of *hog1*Δ yeast cells; however, the targets of this extended SUMOylation and a mechanistic understanding of why this occurs has yet to be detailed.

Cyc8-Tup1: A Conserved Transcription Corepressor Complex

We previously discovered that the primary targets of hyperosmotic stress-induced SUMOylation are the transcriptional corepressor proteins Tup1 and Cyc8 (Oeser, Amen et al. 2016). The venerable yeast geneticist Fred Sherman first identified these proteins in 1980 as regulators of cytochrome C expression (Rothstein and Sherman 1980). Since then, they have been implicated in repressing hundreds of components within the yeast genome, including genes involved in carbon source utilization, flocculation, mating behaviors, and responses to cellular stress (Schultz, Marshall-Carlson et al. 1990, Smith, Redd et al. 1995, Friesen, Hepworth et al. 1997, Gounalaki, Tzamarias et al. 2000).

The Cyc8-Tup1 complex is conserved throughout evolution, and is the functional homologue of *Arabidopsis thaliana* TOPLESS, *Drosophila melanogaster* Groucho, and *Homo sapiens* TLE and UTY/UTX proteins (Grbavec, Lo et al. 1999, Liu and Karmarkar 2008). These proteins interact with a diverse mélange of proteins associated with transcription, including histones, transcription factors, the mediator complex, and chromatin modifiers, thereby implying the importance of the Cyc8-Tup1 complex as a scaffolding nexus during transcriptional repression/derepression (Kuchin and Carlson 1998, Watson, Edmondson et al. 2000, Li and Reese 2001).

The Cyc8-Tup1 complex has a unique and modular structure that is critical for its function. The stoichiometry of the corepressor complex consists of four Tup1 molecules per molecule of Cyc8 (Varanasi, Klis et al. 1996). Both proteins are rich in repetitive domain elements that play important roles in context-dependent gene regulation. Tup1 has seven WD repeats, and mutations within these repeats have been shown to affect different regulatory functions (Komachi, Redd et al. 1994, Komachi and Johnson 1997, Zhang, Varanasi et al. 2002). Similarly, Cyc8 bears ten tetratricopeptide repeats (TPRs) that are involved in binding of various transcription factors (Tzamarias and Struhl 1995). These modular structural repeats allow Cyc8-Tup1 to be recruited to a variety of different regulatory genetic elements. Indeed, neither Cyc8 nor Tup1 bind DNA directly (though Tup1 has been shown to interact with modified tails of Histone H3/H4) (Wu, Suka et al. 2001, Fleming, Beggs et al. 2014). Rather, their recruitment to various genes is facilitated through interaction with specific DNA binding transcription factors (Malave and Dent 2006).

On top of containing repetitive domain architectures, both Cyc8 and Tup1 bear long low-complexity sequences. Tup1 has two glutamine-rich regions and one threonine-rich region, while Cyc8 bears an N-terminal polyglutamine stretch and an extremely glutamine rich prion domain (Alberti, Halfmann et al. 2009). Prions are misfolded proteins that induce misfolding of normal cellular proteins,

and can are the root cause of diseases in humans like Creutzfeldt-Jakob disease and fatal familial insomnia (Manuelidis 1985, Urich 1996). Prior to our understanding of them as disease agents, however, prions were first characterized as elements of non-Mendelian inheritance in yeast (Liebman and Sherman 1979, Tuite and Lindquist 1996).

In fungi, prions provide a form of protein-only memory that allows the organism to transmit information to their progeny about previous environmental encounters (Cox 1994, Patino, Liu et al. 1996). Prion forms of proteins fold into an amyloid structure; while this lends prions the extreme stability necessary for transmission, it generally results in loss-of-function of the specific protein (Lindquist, DebBurman et al. 1998). In most cases, such as that of the Sup35 translation termination factor that forms the [PSI+] element, this loss-of-function drives phenotypic diversity, and thereby providing competitive fitness advantages in the face of diverse environmental conditions (DePace and Weissman 2002). Moreover, environmental stressors elicit transcriptional upregulation of a variety of molecular chaperones that are required for nucleation and seeding of prions (Chernoff, Lindquist et al. 1995). Prionogenic switching during stress occurs at a frequency several orders of magnitude greater than genetic mutations and can persist for upwards of seven generations (Doronina, Staniforth et al. 2015, Speldewinde and Grant 2017). Given this, the formation of prions can be seen as a direct response and adaptation to ever changing environmental conditions.

Prions represent an extreme protein-folding response to environmental conditions, but less extreme examples are also common across evolution. Indeed, many aggregate-like non-membrane bound organelles have been characterized in response to myriad cellular stress conditions, including stress granules, P-bodies, Cajal bodies, and others (Platani, Goldberg et al. 2000, Buchan, Muhlrad et al. 2008). These dynamic liquid-like droplets form as a function of multivalent interactions, usually between proteins, RNA, and post-translational modifications (Decker and Parker 2012, Li, Banjade et al. 2012).

These bodies are enriched in proteins bearing low complexity sequences, which are capable of adopting diverse conformations to facilitate separation from their surroundings (Pak, Kosno et al. 2016). Membrane-less organelles are transient, forming in response to stress and dissolving once adaptation has occurred. However, membrane-less organelles can undergo phase shifting as a result of aging, subsequently adopting gel-like or solid-like states (Molliex, Temirov et al. 2015). This shift toward solid aggregates is thought to underlie human neurodegenerative diseases like amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) (Patel, Lee et al. 2015).

Our group previously showed that Cyc8 and Tup1 are rapidly and transiently SUMOylated following exposure to hyperosmotic conditions, and mutations that limit their SUMOylation drastically alter transcriptional patterns during adaptation to stress. Interestingly, we also observed the complex forming reversible nuclear inclusions upon hyperosmotic shock, and the persistence of these inclusions correlates tightly with the duration of SUMOylation (Oeser, Amen et al. 2016). Recent studies have shown that a variety of disordered or prionogenic proteins form dynamic inclusion bodies under stress conditions, but it remains unclear what facilitates a shift from soluble, liquid-like states to insoluble, solid-like states (Kaganovich 2017, Franzmann, Jahnel et al. 2018).

This thesis works to integrate crucial aspects of the topics above in a novel manner. First, I will show that the Hog1 MAPK regulates the SUMOylation dynamics of the Cyc8-Tup1 transcription corepressor complex during hyperosmotic stress, and that this depends on activation and catalytic activity of Hog1. This signaling relationship is important for survival under osmostress, as mutations in Cyc8 that ablate its SUMOylation are sufficient to suppress the osmosensitivity of *hog1*Δ yeast strains during both chronic and acute stress. Following, I will show that Hog1 and Cyc8 SUMOylation work in concert to facilitate the transcription of *GPD1*, and that suppression of *hog1*Δ osmosensitivity by a SUMO-deficient Cyc8 mutant is orchestrated through rescue

of glycerol biosynthesis. Finally, I will show that the accumulation of glycerol is the signal regulating the timing of Cyc8 SUMOylation, and this mediates Cyc8 inclusion formation at the cell biological level. These conclusions shed new light on the interplay of different post-translational modifications during adaptation to stress, thereby detailing one mechanism by which cells accurately induce survival genes in a tightly regulated fashion. Further, this work provides new insights on factors regulating the stress-induced formation of dynamic inclusion bodies, which will inform future studies on this exciting new area of biology.

Chapter 2: The Hog1 MAPK and Cyc8-Tup1 SUMOylation Orchestrate Transcription of Glycerol Biosynthetic Genes During Adaptation to Hyperosmotic Stress

The Hog1 MAPK regulates the duration of Cyc8-Tup1 SUMOylation

It has previously been shown that the Hog1 MAPK limits the accumulation of high molecular weight SUMO conjugates after the induction of hyperosmotic stress (Abu Irqeba, Li et al. 2014, Oeser, Amen et al. 2016), but the protein nature of the Hog1-regulated SUMO conjugates had not been identified. We demonstrated that Hog1 influences the duration of SUMO-deficient Cyc8-Tup1 puncta following stress exposure, but had not directly verified that Hog1 regulates the SUMOylation of the Cyc8-Tup1 complex. To test this, metal affinity purification of SUMOylated proteins was performed on yeast cells expressing *His$_6$-FLAG-SMT3* background after exposure to 1.2 M sorbitol followed by examination of the SUMOylation state for Cyc8 or Tup1 via Western analysis. While parent cells showed transient SUMOylation of both Cyc8 and Tup1, *hog1*Δ cells showed prolonged SUMOylation of both complex members, persisting up to at least 60 minutes (Figure 2.1, 2.2). Hog1 function depends on both phosphorylation-dependent activation on an established *TXY* motif and catalytic function through a defined kinase domain (Choi, Kang et al. 2008). Because Cyc8 SUMOylation is the initial event for the complex, I wanted to verify that these Hog1 functions were necessary for the regulation of Cyc8 SUMOylation. To do so, plasmids encoding intact (WT), activation-deficient (TGY-AGA), or kinase-deficient (D144A) Hog1 were generated and expressed ectopically in *hog1*Δ cells. Osmosensitivity of the mutants was verified by spot titer assay on synthetic medium containing an elevated concentration of KCl (Figure 2.3). SUMOylated proteins were purified from these strains by metal affinity chromatography before and after exposure to 1.2 M sorbitol, and Cyc8 SUMOylation was examined by Western analysis. While ectopic expression of WT Hog1 rescued the delay in Cyc8 deSUMOylation, both activation-dead and kinase-dead Hog1 showed prolonged Cyc8 SUMOylation similar to that of the complete *HOG1* gene deletion (Figure 2.4, 2.5).

Activation of Hog1 occurs via two distinct branches of the osmotic stress pathway: the “fast” histidine-kinase branch dependent on Ssk2/22 and the “slow” MAPK branch dependent on Ste11 (Posas and Saito, 1998). Upon exposure to osmotic stress, Hog1 feedback inhibits the slow MAPK branch to prevent inappropriate activation of the mating pathway, which shares the upstream MAPKKK Ste11 (Saito and Posas 2012). To ensure that our observations on Cyc8 SUMOylation were not due to the inappropriate crosstalk that occurs in absence of Hog1 function, I generated *ste11*Δ cells and performed western blots to look at the distribution of high molecular weight SUMO conjugates. Compared to the prolonged SUMOylation observed in *hog1*Δ cells, these cells showed the same transient SUMOylation profile as the parent cells, indicating that crosstalk with the mating pathway was not responsible for the delay in Cyc8 deSUMOylation (Figure 2.6).

Loss of Cyc8 SUMOylation Suppresses the Osmosensitivity of *hog1*Δ cells

Specific mutations in Cyc8 have been previously shown to partially suppress the osmosensitivity of *hog1*Δ cells (Kobayashi, Inai et al. 2008). Specifically, this mutation introduced a stop codon following Cyc8’s tenth tetratricopeptide repeat. We previously showed that hyperosmotic stress-dependent Cyc8 SUMOylation occurs at a cluster of lysine residues located C-terminal to this truncation. As such, I chose to investigate whether specific mutation of only these lysines would suppress the osmosensitivity of *hog1*Δ via spot titer assay (Figure 2.7). While mutation of our previously identified SUMO sites did not alter growth on plates containing 0.8 M KCl when *HOG1* was present, loss of Cyc8 SUMOylation in *hog1*Δ modestly suppressed its osmosensitivity. I verified the appropriate reduction of Cyc8 SUMOylation in Cyc8^{4KtoR} mutant cells by performing metal affinity purification of SUMOylated proteins and probing for Cyc8-3HSV by Western analysis (Figure 2.8). Spot titer assays are limited in their inability to offer accurate quantitative growth rates, so I generated growth curves in rich medium with and without 1M sorbitol using a

Bioscreen C automated growth curve analyzer (Figure 2.9). With *HOG1* present, inhibition of Cyc8 SUMOylation had little effect on the ability to grow under stress. In the absence of *HOG1*, however, loss of Cyc8 SUMOylation greatly increased the cells' growth rate compared to WT-Cyc8. With these measurements, I calculated doubling times for the various yeast strains using the Yeast Outgrowth Data Analyzer software (Olsen, Murakami et al. 2010), and found that loss of Cyc8 SUMOylation significantly increased the relative growth rate of *hog1*Δ cell when grown in the presence of an osmotic stressor (Figure 2.10).

I also investigated the ability of Cyc8 SUMOylation to suppress the osmosensitivity of *hog1*Δ under acute stress. *Cyc8*Δ or *cyc8*Δ*hog1*Δ mutants were transformed with plasmids encoding Cyc8 or the SUMO-deficient Cyc8^{4KtoR} variant, grown to mid-log phase in complete synthetic medium, and then exposed to increasing concentrations of sorbitol in liquid medium for two hours. After hyperosmotic exposure, cells were plated on complete synthetic medium and grown for two days at 30C. Similar to long-term growth on solid medium, loss of Cyc8 SUMOylation promoted growth in *hog1*Δ after acute exposure to high concentrations of the osmotic stressor (Figure 2.11).

Blockade of Cyc8 SUMOylation restores stress-induced glycerol biosynthesis in *hog1*Δ cells

While Hog1 activates the expression of a variety of genes upon hyperosmotic stress, only the glycerol biosynthetic enzyme *GPD1* is required for survival (Babazadeh, Furukawa et al. 2014). This gene encodes the rate-limiting enzyme in glycerol biosynthesis and is a known target of Cyc8-dependent repression (Marquez, Pascual-Ahuir et al. 1998). My observations employing acute stress indicated that there must be rapid production of a stress-protective factor. Given this, I hypothesized that loss of Cyc8 SUMOylation alters expression of *GPD1*, thereby allowing for increased glycerol production in the absence of Hog1. To test this, I first looked for changes in WT Cyc8 or non-

SUMOylateable Cyc8^{4KtoR} at the *GPD1* promoter by chromatin immunoprecipitation and quantitative PCR (ChIP-qPCR) with or without *HOG1* present (Figure 2.12). Cells were exposed to a time course of hyperosmotic stress and DNA-protein complexes were crosslinked with formaldehyde. Cells were then lysed and Cyc8-DNA complexes were immunoprecipitated with antibodies for the 3HSV epitope attached to Cyc8. Enrichment of Cyc8 at the *GPD1* promoter was probed by qPCR, normalized to enrichment at the non-specific *ACT1* locus, and then expressed relative to the parent strain at time 0. In cells bearing WT Cyc8 and Hog1, Cyc8 exhibited approximately 3.5-fold enrichment at the *GPD1* promoter within 10 minutes of stress exposure, which then returned close to baseline levels after 60 minutes. In *hog1*Δ cells, WT Cyc8 showed nearly identical enrichment at *GPD1* prior to stress onset and during the initial phase of adaptation (10 minutes). However, Cyc8 was still highly enriched at the *GPD1* promoter 60 minutes after stress onset in *hog1*Δ. Regardless of the presence of *HOG1*, cells bearing non-SUMOylateable Cyc8^{4KtoR} showed increased promoter occupancy prior to the onset of stress. During the initial phase of stress exposure, however, these strains showed significantly reduced enrichment at *GPD1*. After 60 minutes of stress exposure, Cyc8^{4KtoR} levels at the *GPD1* promoter returned to baseline, independent of *HOG1*. Taken together, these results imply separate roles for SUMOylation and *HOG1* in the expression of glycerol biosynthesis genes during hyperosmotic stress. On one hand, SUMOylation of Cyc8 is important for accurately positioning Cyc8 at specific genes like *GPD1* during conditions of induction; conversely, *HOG1* is dispensable for the enrichment of Cyc8 at *GPD1*, but is necessary for eviction of WT Cyc8 following adaptation to hyperosmotic stress.

I wanted to test whether our observations on Cyc8 promoter occupancy correlated with alterations in glycerol production. To do this, I generated lysates from the various cells across a time course of hyperosmotic stress and analyzed the levels of glycerol present by a colorimetric assay (Figure 2.13). Both WT and SUMO-deficient Cyc8 cells showed rapid, robust glycerol accumulation upon the

onset of hyperosmotic stress. Cells that were *hog1*Δ exhibited reduced glycerol content, but the additional mutation of Cyc8's SUMO sites partially rescued glycerol accumulation during exposure to hyperosmotic stress. This mutant accumulated approximately 50% of the total glycerol of parent cells but is still able to survive hyperosmotic stress conditions.

While I had observed elevated intracellular glycerol in the *Cyc8*^{4KtoR}/*hog1*Δ mutant, I wanted to ensure that glycerol biosynthesis was indeed necessary for the observed suppression of osmosensitivity. Therefore, I deleted *GPD1* from *Cyc8*^{4KtoR}/*hog1*Δ cells and compared its growth to control cells by spot titer assay (Figure 2.14). The additional deletion of *GPD1* from *Cyc8*^{4KtoR}/*hog1*Δ cells did not affect its growth on rich media; however, *Cyc8*^{4KtoR}/*hog1*Δ*gpd1*Δ cells were highly osmosensitive when grown in the presence of 0.8 M KCl. Taken together, these data indicate that Hog1 and Cyc8 SUMOylation orchestrate a stress-dependent transcriptional program surrounding glycerol biosynthesis. Furthermore, I conclude that the capacity to synthesize glycerol in the absence of Hog1 is necessary for the partial suppression of osmosensitivity in *Cyc8*^{4KtoR}/*hog1*Δ cells.

Discussion

Previous work has identified a role for Hog1 in limiting the levels of SUMOylated proteins during adaptation to hyperosmotic stress, and that increasing the levels of stress-dependent SUMOylation via overexpression of the SUMO ligase Siz1 enhanced the osmosensitivity of *hog1*Δ cells (Abu Irqeba, Li et al. 2014). What was unclear, however, was a mechanistic understanding of how poly-SUMOylated species promoted osmosensitivity. We previously identified Tup1 and Cyc8 as the major targets of SUMOylation during osmotic stress, and showed that deletion of either member of the corepressor complex ablates hyperosmotic stress-dependent increases in global SUMOylation. I show here that Tup1-Cyc8 SUMOylation is prolonged in the absence of Hog1, and that maintenance of Cyc8 SUMOylation requires activation and kinase activity of Hog1. Further, reduction of Cyc8 SUMOylation alone is sufficient to partially

reestablish osmoadaptation in *hog1*Δ. Given this, we conclude that SUMOylated species are unlikely to be toxic themselves. Instead, it appears that Hog1, Cyc8, and the cycle of SUMOylation/deSUMOylation are involved in coordinating gene expression at stress-inducible genes that are necessary for survival. Indeed, we saw that loss of Cyc8 SUMOylation alters Cyc8 occupancy at the *GPD1* promoter, which in turn facilitates increases in glycerol production sufficient for growth under hyperosmotic stress.

SUMO and the SUMOylation machinery have been elsewhere implicated in the expression of inducible genes. Specifically, Cyc8 and Tup1-SUMOylation have been shown to be important in the targeting and timing of the corepressor complex to inducible genes during conditions of nutrient sensing and starvation (Rosonina, Duncan et al. 2010, Texari, Dieppois et al. 2013, Ng, Akhter et al. 2015). Exactly how SUMO coordinates gene expression at inducible promoters remains mysterious, however. As an important modulator of protein-protein interaction, SUMO may facilitate the assembly of complexes necessary for rapid induction of target genes. Conversely, the yeast SUMO ligase Ulp1 resides at the nuclear pore, and artificial tethering of genes at the nuclear pore complex (NPC) is sufficient to increase the rate of their induction (Texari, Dieppois et al. 2013). Given this, SUMOylation of a scaffold like Cyc8-Tup1 may provide a biomolecular handle to drag specific genes to the NPC, where they are rapidly expressed and their resulting mRNA transcripts are quickly exported to the cytoplasm for translation.

Acknowledgements

Thank you to Michelle Oeser for the construction of yeast strains and plasmids used throughout this chapter, Samuel Entwisle for the construction of plasmids used in assessing the role of activation and catalysis in Hog1 regulation of SUMOylation, Mitchell Lee for assistance in use of the Bioscreen C, and Rigney Turnham and Laura Gabrovsek for assistance with qPCR.

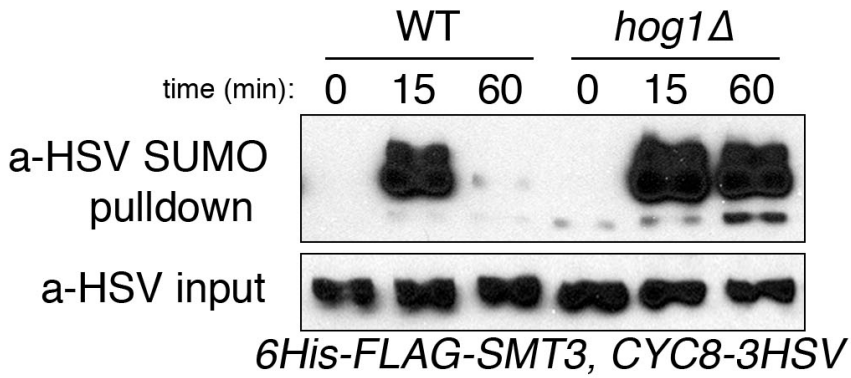


Fig 2.1: Cyc8-SUMOylation is prolonged in *hog1*Δ. Parent or *hog1*Δ cells expressing *6His-FLAG-SMT3* and *CYC8-3HSV* were treated with 1.2 M sorbitol, collected at the indicated time points, and SUMOylated proteins were isolated by metal affinity chromatography. Proteins were separated by SDS-PAGE and Cyc8 was identified by Western analysis using anti-HSV antibodies. Total Cyc8 in the input fraction was used as a loading control.

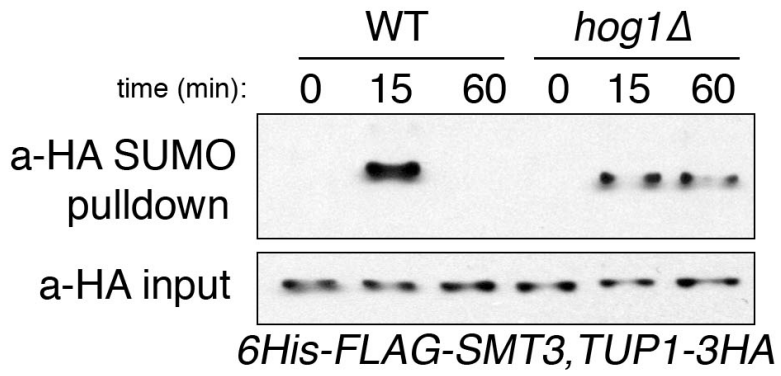


Fig 2.2: Tup1-SUMOylation is prolonged in *hog1*Δ. Parent or *hog1*Δ cells expressing *6His-FLAG-SMT3* and *TUP1-3HA* were treated and analyzed as in Fig 1.1.

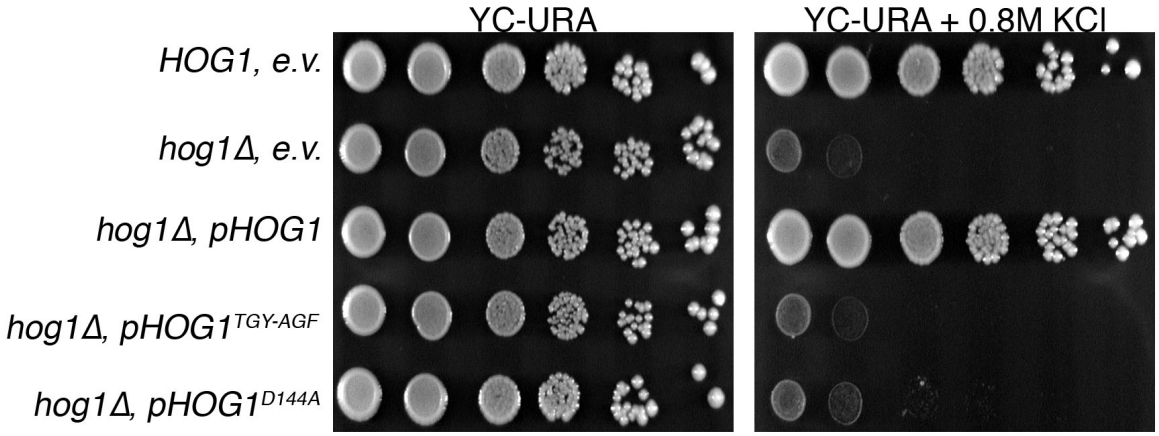


Fig 2.3: Spot titer assay confirming osmosensitivity of the indicated Hog1 mutants. *HOG1* or *hog1*Δ cells were transformed with the indicated constructs and spotted in ten-fold serial dilutions on YC-URA or YC-URA + 0.8 M KCl plates and grown at 30˚C for 2 days.

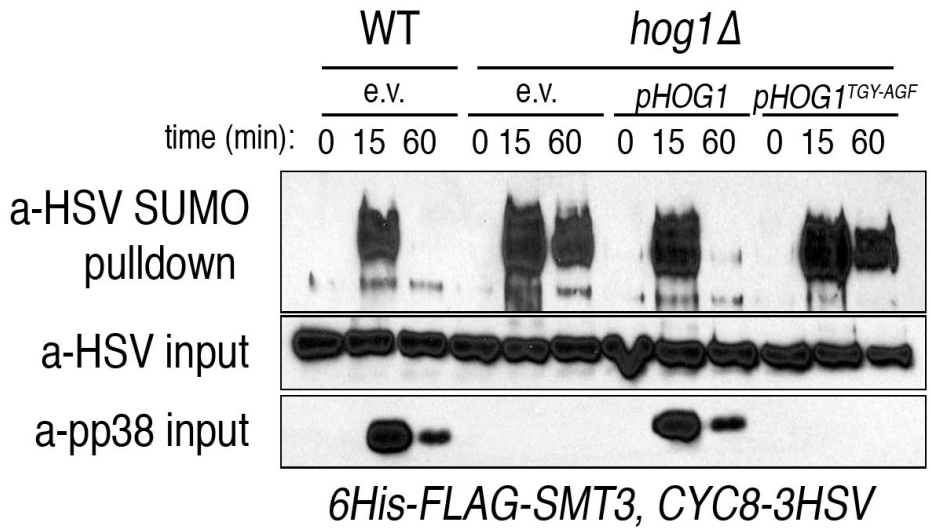


Fig 2.4: Maintenance of normal Cyc8 SUMOylation requires Hog1 activation. Parent or *hog1*Δ cells expressing the indicated constructs, *6His-FLAG-SMT3*, and *CYC8-3HSV* were treated, collected, and analyzed as described in Fig 1.1. Cyc8 was identified by Western analysis using anti-HSV antibodies. Total Cyc8 in the input fraction was used as a loading control, while activated p38 was used as a control for Hog1 presence and activation.

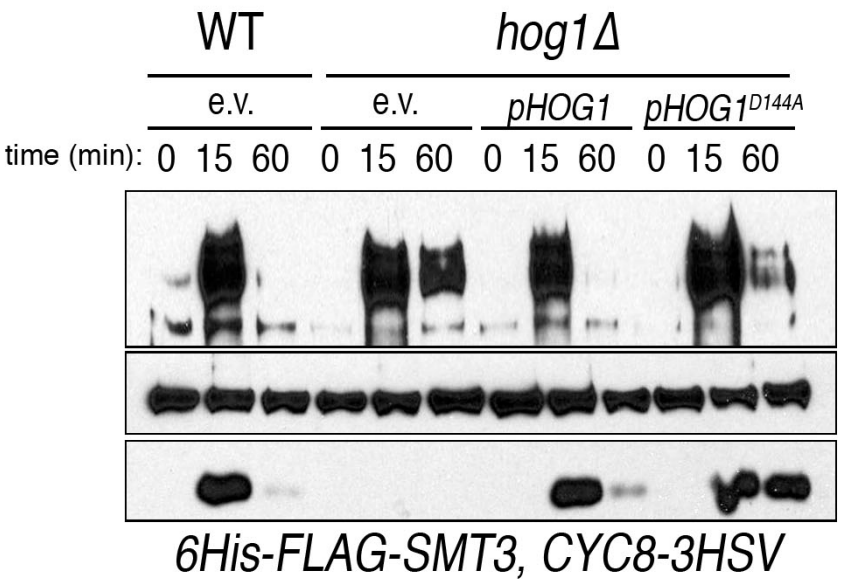


Fig 2.5: Maintenance of normal Cyc8 SUMOylation requires Hog1 catalytic activity. Parent or *hog1*Δ cells expressing the indicated constructs, *6His-FLAG-SMT3*, and *CYC8-3HSV* were treated, collected, and analyzed as described in Fig 1.1. Cyc8 was identified by Western analysis using anti-HSV antibodies. Total Cyc8 in the input fraction was used as a loading control, while activated p38 was used as a control for Hog1 presence and activation.

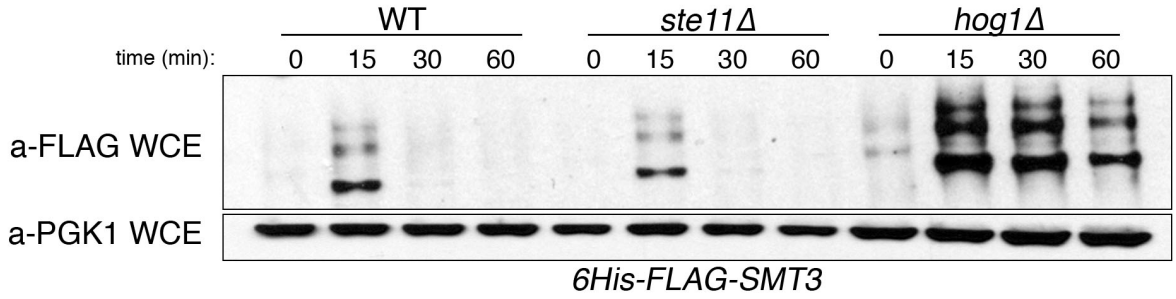


Fig 2.6: Prolonged Cyc8 SUMOylation in the absence of Hog1 is not an artifact of crosstalk. Parent, *ste11*Δ, or *hog1*Δ cells expressing *6His-FLAG-SMT3* were treated with 1.2 M sorbitol and collected at the indicated time points. Whole cell extracts were separated by SDS-PAGE and Western analysis with anti-FLAG antibodies to identify SUMOylated proteins. Anti-PGK1 antibodies were used to detect PGK1 as a loading control.

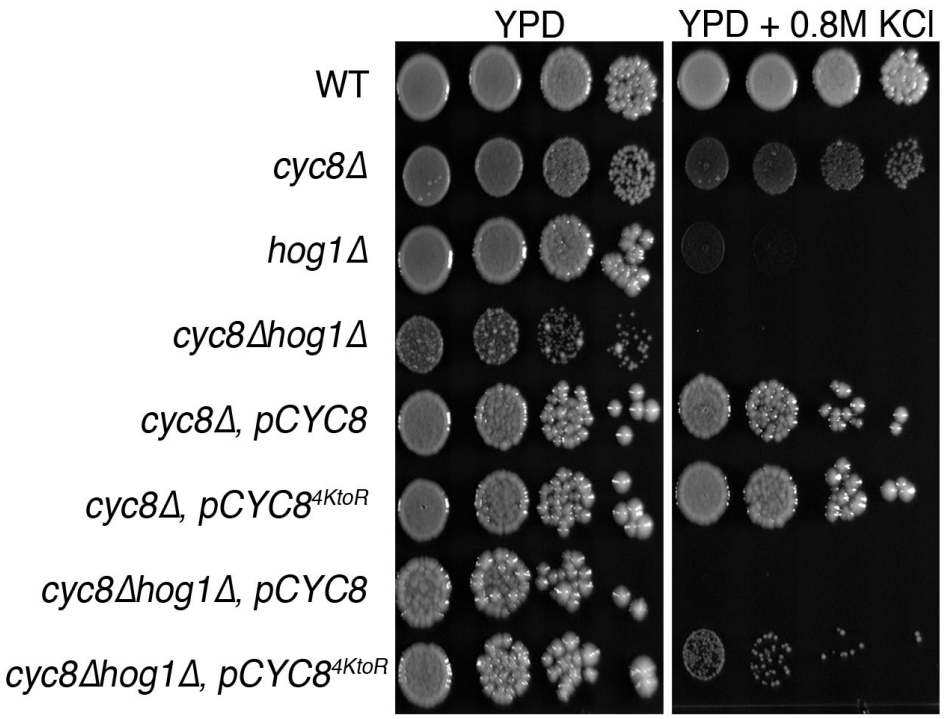


Fig 2.7: Spot titer assay comparing osmosensitivity of various cells. Parent, *cyc8*Δ, *hog1*Δ, or *cyc8*Δ*hog1*Δ yeast cells were transformed with the indicated constructs and spotted in ten-fold serial dilutions on YPD or YPD+0.8 M KCl plates and grown at 30˚C for two days.

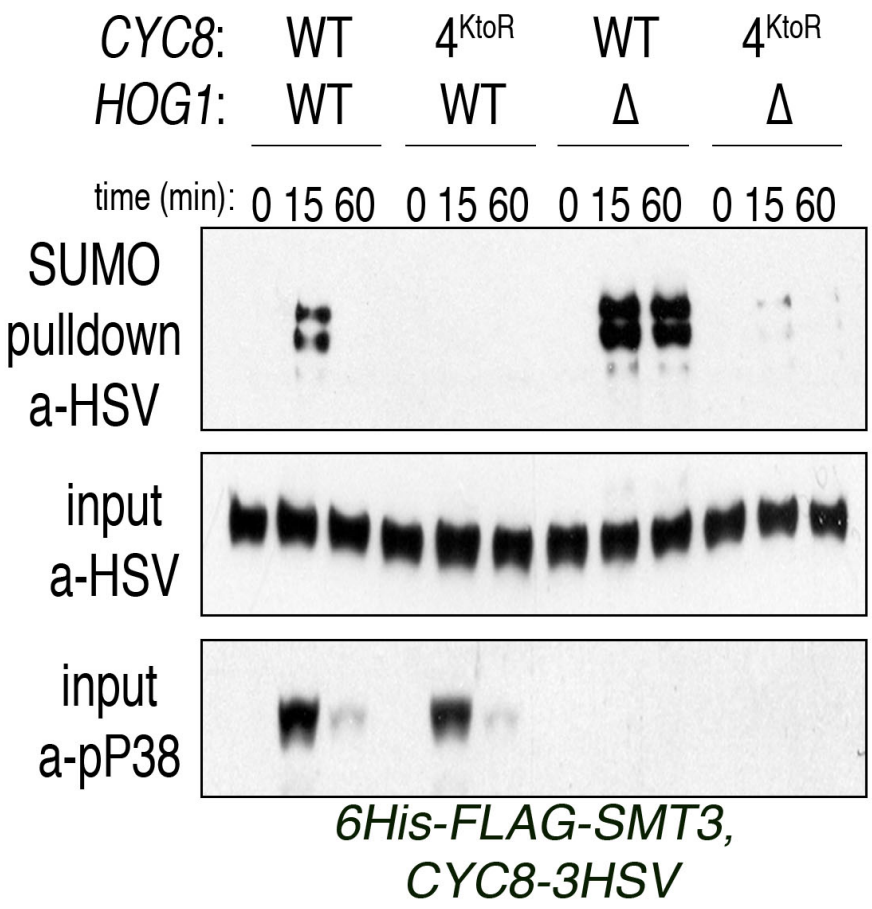


Fig 2.8: Confirmation of reduced Cyc8 SUMOylation in suppressor cells. Indicated cells bearing *6His-FLAG-SMT3* and *3HSV*-tagged *CYC8* were treated with 1.2 M sorbitol and collected at the indicated time points. SUMOylated proteins were collected and analyzed as described in Fig 1.1. SUMOylated-Cyc8 was identified by Western analysis using anti-HSV antibodies. Total Cyc8 in the input fraction was used as a loading control, while activated p38 was used as a control for Hog1 presence and activation.

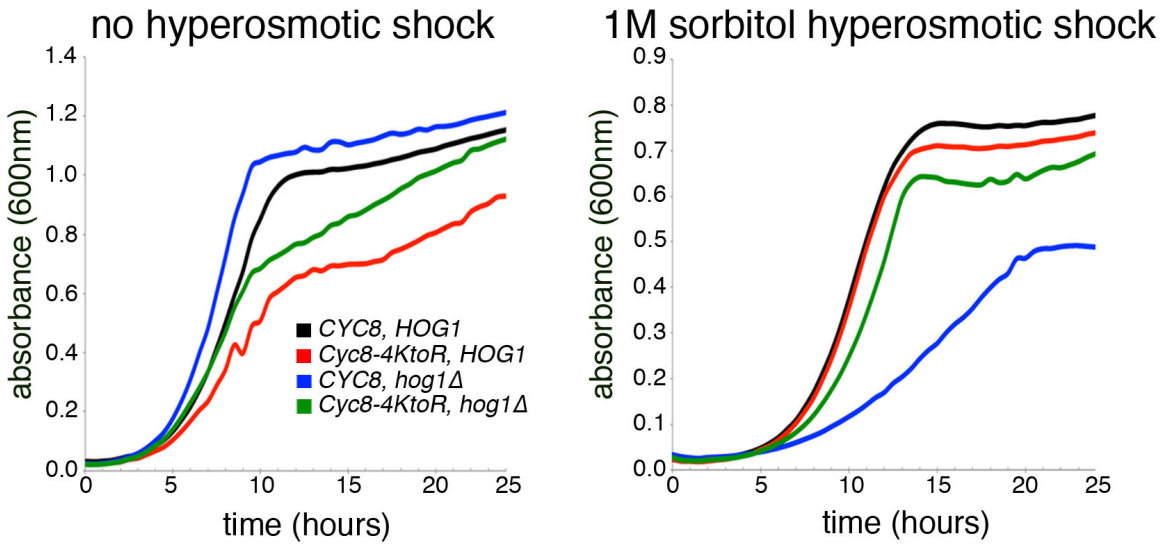


Fig 2.9: Quantitative measure of growth rates generated by Bioscreen C. Indicated cells were grown in triplicate at 30C in YPD or YPD+1M sorbitol for 24 hours with continuous shaking. Absorbance at 600nm was measured every 30 minutes and average absorbance was plotted versus time.

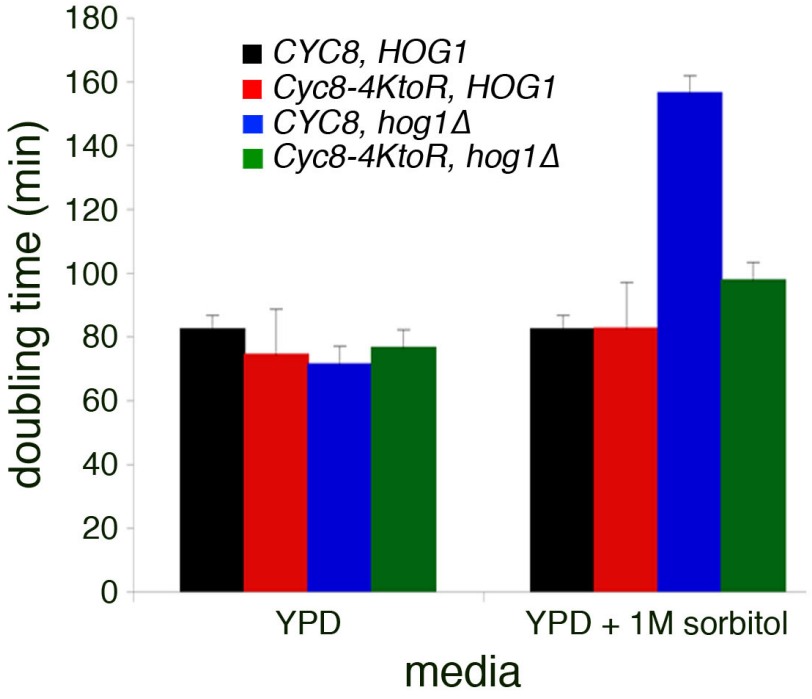


Fig 2.10: Average doubling times in specific media as measured by Bioscreen C. Doubling times for indicated cells were calculated using the yeast outgrowth data analyzer (YODA, (Olsen, Murakami et al. 2010)). Error bars show SD for triplicate samples.

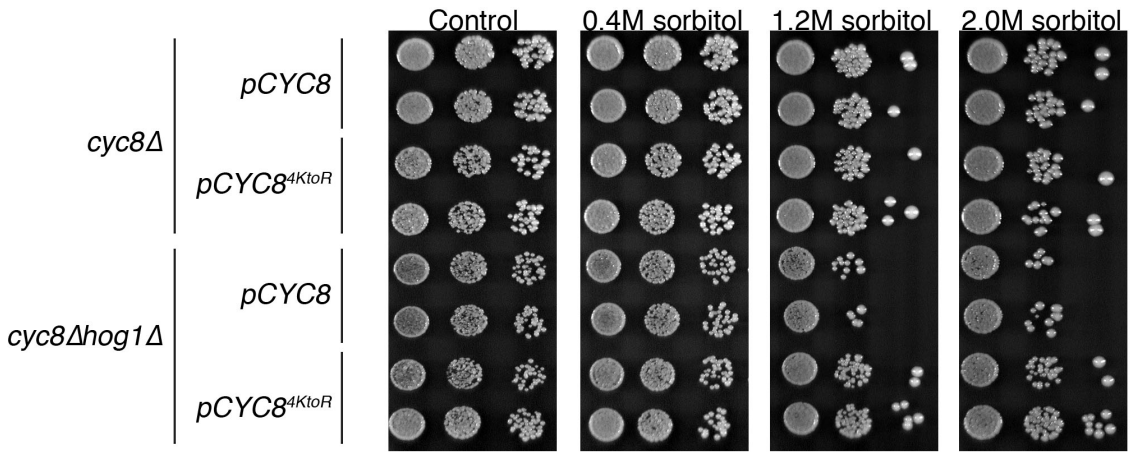


Fig 2.11: Spot titer assay comparing sensitivity to acute hyperosmotic stress. Indicated cells were grown in duplicate in complete synthetic medium to mid-log phase. Cells were incubated with increasing concentrations of sorbitol in liquid medium for two hours at room temperature with mixing. Cells were then spotted in duplicate in ten-fold serial dilutions on complete synthetic plates and grown for two days at 30˚C.

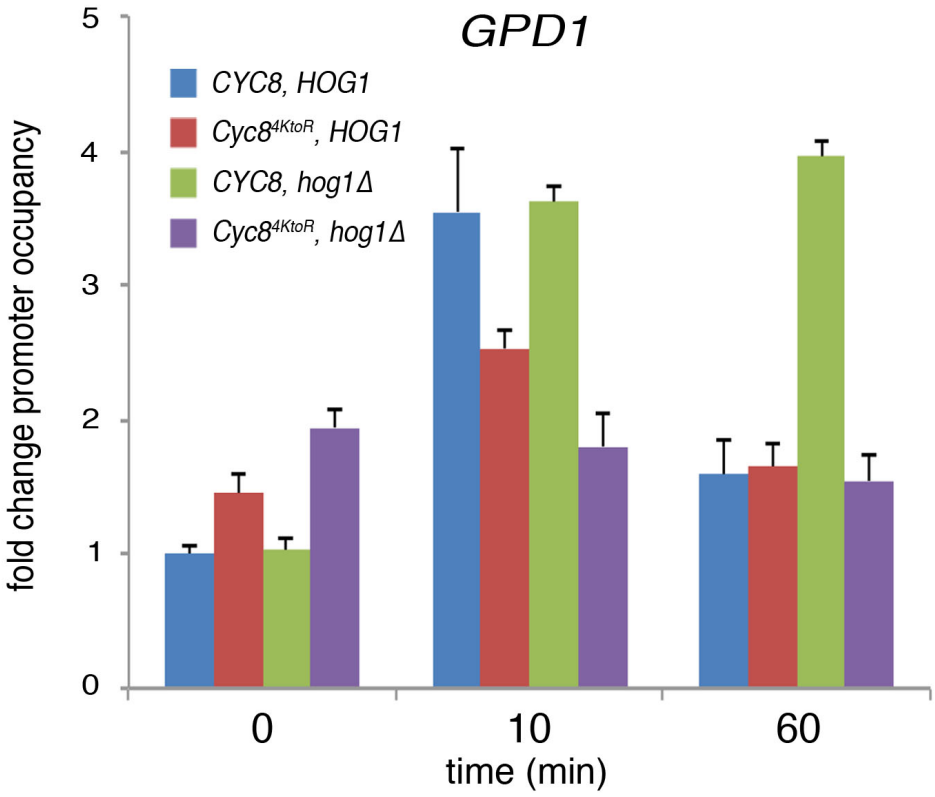


Fig 2.12: Cyc8 transiently associates with the *GPD1* promoter during hyperosmotic stress. Indicated cells were grown in triplicate in rich medium, treated with 1.2 M sorbitol, and collected at the denoted time points. Cyc8-DNA complexes were crosslinked with formaldehyde, immunoprecipitated via incubation with 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 show SD.

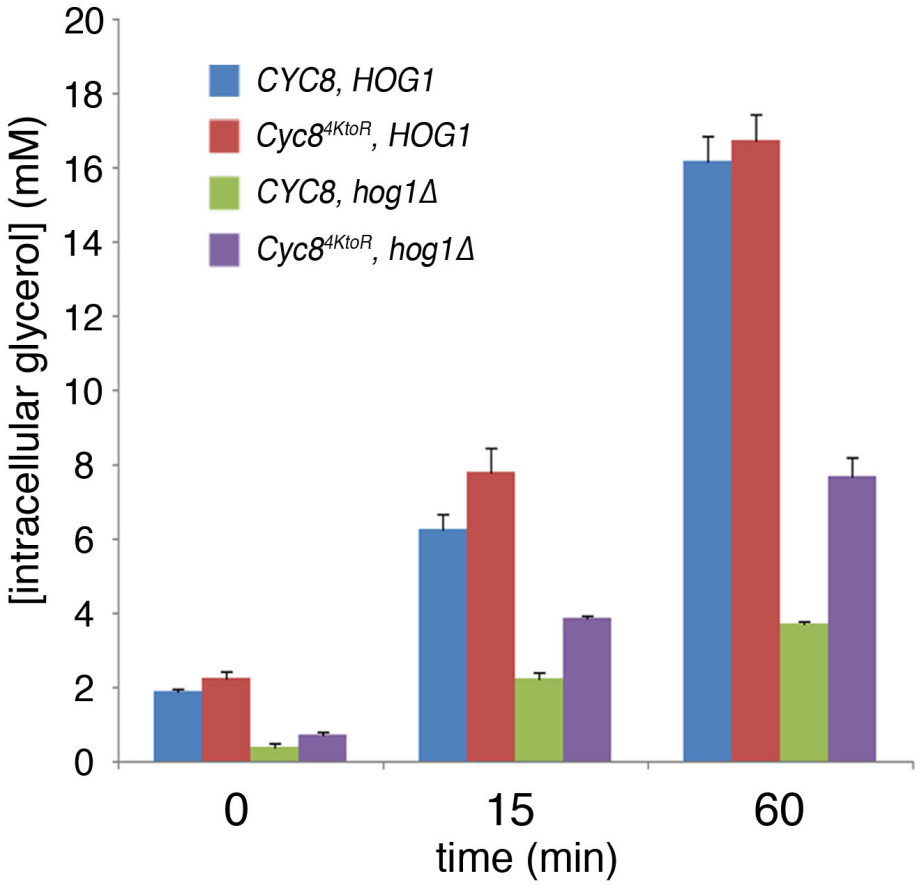


Fig 2.13: Glycerol accumulation assay after exposure to hyperosmotic stress. Indicated cells were grown in triplicate in rich medium, treated with 1.2 M sorbitol, and collected at the denoted time points. Lysates were extracted by boiling at 95˚C for 10 minutes in TBS, clarified by centrifugation, and glycerol was analyzed by colorimetric assay. Error bars show SD.

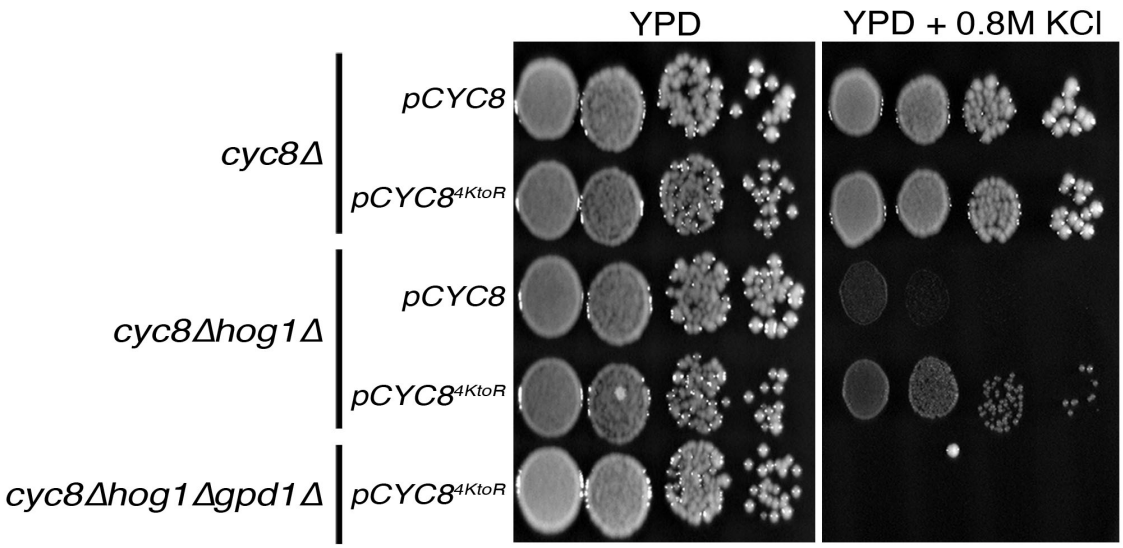


Fig 2.14: Spot titer assay comparing osmosensitivity of various cells. Indicated cells were spotted in ten-fold serial dilutions on YPD or YPD+0.8 M KCl and grown at 30˚C for two days.

Chapter 3: Glycerol is a key regulator of Cyc8 SUMOylation and nuclear foci formation

Glycerol biosynthesis regulates Cyc8 SUMOylation kinetics and foci formation

Loss of Cyc8 SUMOylation partially rescued glycerol biosynthesis in *hog1*Δ cells. Therefore, I chose to investigate whether glycerol biosynthesis regulated Cyc8 SUMOylation. Glycerol biosynthesis is a two-step process in *S. cerevisiae* (Figure 3.1). First, dihydroxyacetone phosphate (DHAP) is reduced to glycerol-3-phosphate (G3P) in the rate-limiting step by the redundant glyceraldehyde-phosphate dehydrogenases 1 and 2 (Gpd1/Gpd2) (Albertyn, Hohmann et al. 1994). Following, G3P is dephosphorylated by glycerol-phosphate phosphatases 1 and 2 (Gpp1/Gpp2) to yield glycerol (Albertyn, Hohmann et al. 1994).

To examine whether glycerol biosynthesis regulated hyperosmotic stress-induced SUMOylation, I deleted components of the glycerol biosynthesis machinery in yeast cells bearing the *6His-FLAG-SMT3* reporter and analyzed SUMOylation kinetics by Western analysis. I found that deletion of individual components of either step of glycerol biosynthesis did not alter SUMOylation kinetics from that of parent cells (Figure 3.2). However, when I functionally crippled the glycerol biosynthesis pathway by combinatorial deletion of *GPD1* and *GPD2*, hyperosmotic stress-induced SUMOylation was prolonged to at least 60 minutes after exposure. G3P is a necessary precursor to the production of lysophosphatidic acid (LPA), a phospholipid derivative with myriad cellular functions (Vancura and Haldar 1994). To verify that my findings were due to a loss of glycerol and not of LPA, I generated cells deleted for the downstream glycerol biogenesis enzymes Gpp1 and Gpp2 (Figure 3.3). As with the upstream Gpd's, only when I deleted both *GPP1* and *GPP2* did I prolong hyperosmotic stress-induced SUMOylation from that of parent cells. Taken together, these data indicate that glycerol biosynthesis is a key regulator of hyperosmotic stress-induced SUMOylation.

To verify that the observed delay in deSUMOylation kinetics was specific to Cyc8, I purified SUMOylated proteins from parent, *hog1*Δ, and *gpd1*Δ*gpd2*Δ cells by metal affinity purification and examined Cyc8 SUMOylation by Western analysis (Figure 3.4). As seen before, parent cells showed a transient pattern of Cyc8 SUMOylation, wherein Cyc8 is specifically SUMOylated upon hyperosmotic stress but only for 15 minutes. Deletion of *HOG1* delays this, resulting in a significant amount of Cyc8 remaining SUMOylated at 60 minutes in *hog1*Δ cells. Cells that are *gpd1*Δ*gpd2*Δ showed robust SUMOylation of Cyc8 at 60 minutes, more so than in *hog1*Δ cells. It has been shown that *hog1*Δ cells accumulate glycerol under hyperosmotic stress, but do so at significantly slower rates than parent cells (Petelenz-Kurdziel, Kuehn et al. 2013). To illustrate the differences in glycerol accumulation between the different deletion cells, I performed colorimetric glycerol assays (Figure 3.5). As expected, parent cells rapidly and robustly accumulated glycerol over the course of an hour under hyperosmotic stress. Cells that are *hog1*Δ accumulated glycerol slowly, with a maximal accumulation of approximately 66% that of parent cells. Cells that are *gpd1*Δ*gpd2*Δ accumulated virtually no glycerol over the observed time course.

Previously, we found that that Cyc8 forms nuclear puncta during hyperosmotic stress, and the persistence of these puncta is influenced by deletion of *HOG1* (Oeser, Amen et al. 2016). Due to our observation of prolonged Cyc8 SUMOylation in the absence of glycerol biosynthesis, I wanted to investigate whether glycerol accumulation regulated the resolution of Cyc8 foci. Using cells expressing Cyc8-eGFP from the endogenous *CYC8* locus, I performed fluorescence microscopy experiments across a time course of hyperosmotic stress (Figure 3.6). As I observed previously, Cyc8 formed nuclear foci in parent cells that are resolved within 15 minutes. By contrast, *hog1*Δ cells showed elevated prevalence of foci and slightly delayed resolution, with puncta persisting out to at least 30 minutes. In cells that are *gpd1*Δ*gpd2*Δ, I observed robust puncta formation with no observable resolution across the entire 90

minutes of observation. By quantifying the relative number of cells with Cyc8 foci for each background across the time course of stress, I observed a significant increase in Cyc8 foci number and lifespan in the absence of glycerol accumulation (Figure 3.7). Given this, I can conclude that glycerol accumulation is necessary to facilitate the resolution of Cyc8 nuclear foci during adaptation to hyperosmotic stress.

Glycerol biosynthesis is sufficient to rescue Cyc8 SUMOylation kinetics in the absence of HOG1

My observation that intracellular glycerol was necessary to maintain normal Cyc8 SUMOylation kinetics during hyperosmotic stress led me to wonder if glycerol biosynthesis was sufficient to rescue the early dysregulation of Cyc8 SUMOylation and foci kinetics observed in *hog1*Δ cells. I therefore generated cells expressing 3HA-tagged *GPP2* alone or *GPD1* and *GPP2* from the constitutive *TDH3* promoter at exogenous genetic loci. After generating these cells, I verified their increased glycerol production during hyperosmotic stress using a colorimetric glycerol assay (Figure 3.8). As expected, only when both *GPD1* and *GPP2* were expressed together did I observe an increase in the levels of intracellular glycerol. This increased glycerol production was not as robust I had initially anticipated. However, studies have shown that prolonged hyperosmotic stress causes preferential shift away from housekeeping genes like *TDH3* toward stress-inducible genes (Vanacloig-Pedros, Bets-Plasencia et al. 2015), and I feel this may account for the difference observed between parent cells and the cells overexpressing glycerol-synthesis enzymes. Nevertheless, I performed SUMO pulldown assays to assess levels of Cyc8 SUMOylation (Figure 3.7). As previously seen, parent cells showed normal Cyc8 SUMOylation kinetics, while *hog1*Δ cells expressing vector or *GPP2* alone showed prolonged Cyc8 SUMOylation. Overexpression of both *GPD1* and *GPP2* in the *hog1*Δ background partially rescued Cyc8 SUMOylation kinetics as seen by the reduced band intensity at 60 minutes after induction of hyperosmotic stress.

Due to this result, I subsequently tested whether overexpression of glycerol biosynthesis machinery would also restore the timing of Cyc8 nuclear foci dissolution. I generated analogous overexpression cells in cells bearing Cyc8-eGFP, and performed fluorescence microscopy experiments across a time course of hyperosmotic stress (Figure 3.9), and then quantified the percentage of cells bearing foci at each time point (Figure 3.9). Similar to my findings on Cyc8 SUMOylation kinetics, I found that overexpression of both *GPD1* and *GPP2* partially rescued the kinetics of Cyc8 foci resolution. Taken together, these results indicate that glycerol biosynthesis is sufficient to rescue the early kinetic deficits observed in *hog1*Δ.

Discussion

Here, I show that glycerol biosynthesis is a key regulator of the persistence of Cyc8 foci and SUMOylation, as the lifetime of both of these is inversely correlated with cellular glycerol content after exposure to hyperosmotic stress. Yeast cells accumulate glycerol following exposure to hyperosmotic stress to reestablish ionic balance, retain water, and counteract molecular crowding (Babazadeh, Adiels et al. 2013). Moreover, glycerol is capable of preventing protein aggregation *in vitro* by altering protein-solvent interactions and promoting a larger radius of hydration (Esposito, Comez et al. 2009). Given this, glycerol may play a role as a chemical chaperone to maintain the proper folding state of Cyc8 during stress.

We previously observed foci formation in Cyc8 after genetically ablating its SUMOylation-sites, and concluded from that observation that SUMO might play a role in preventing aggregation of the Cyc8-Tup1 complex (Oeser, Amen et al. 2016). Here, however, I find that conditions that prolong Cyc8 SUMOylation also prolong the lifespan of Cyc8 foci. It would follow, then, that SUMOylation is unrelated to foci formation; rather, Cyc8 appears to bear some intrinsic quality that facilitates its condensation into foci.

A wide variety of transcriptional modulators are known to be SUMOylated, and the consequences of these SUMOylation events are equally diverse. Similar to my observations with Cyc8, some of these SUMO substrates are enriched in nuclear foci or have prion-like propensities for aggregation, including *Arabidopsis* TCP proteins and human CPEB3 (Drisaldi, Colnaghi et al. 2015, Mazur, Spears et al. 2017). As such, it seems plausible that biophysically driven foci formation of aggregation prone proteins is thematic throughout nature, and occurs immediately in response to diverse stimuli. Once these initial scaffolds are constructed, post-translational modifications like SUMOylation may allow for the recruitment of necessary interactors that will enact further biological responses (such as the recruitment of transcriptional machinery or chromatin modifiers), after which these foci can be disassembled.

Acknowledgements

Thank you to Michelle Oeser for the construction of yeast strains and plasmids used in this chapter.

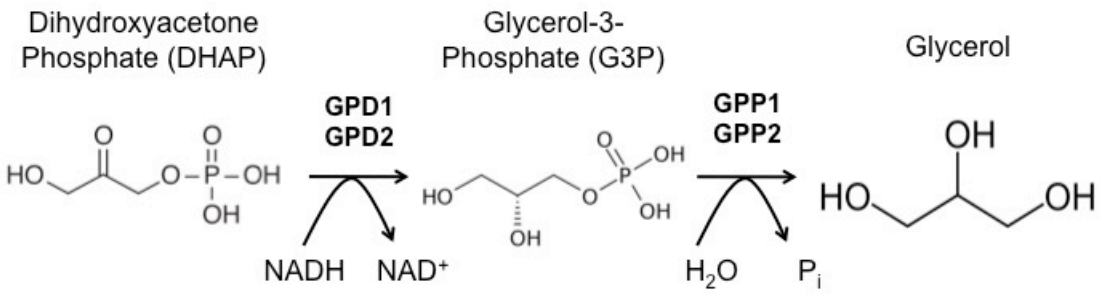


Fig 3.1: Graphic illustration of *S. cerevisiae* glycerol biosynthetic pathway. DHAP is reduced by one of two redundant glycerol phosphate dehydrogenase (GPD) enzymes to G3P, which is dephosphorylated by one of two redundant glycerol phosphate phosphatase (GPP) enzymes to yield glycerol.

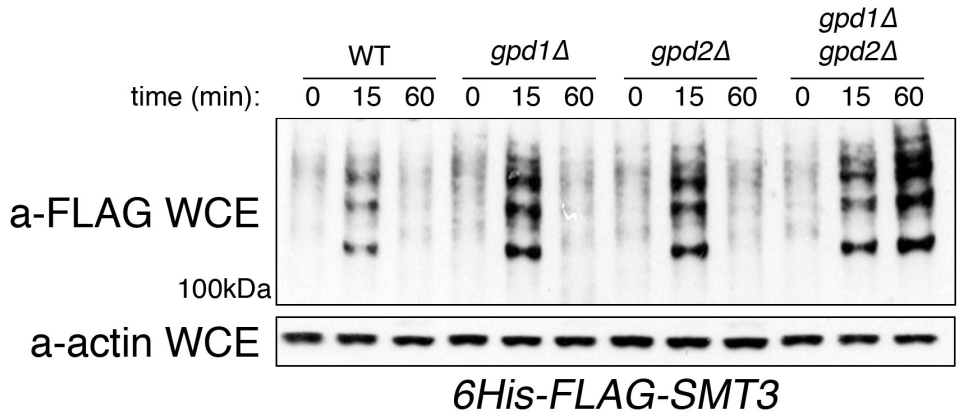


Fig 3.2: Combinatorial deletion of glycerol-3-phosphate dehydrogenases prolongs global SUMOylation during hyperosmotic stress. Indicated cells expressing *6His-FLAG-SMT3* were treated, collected, and analyzed as described in Fig 2.6. SUMOylated species were identified by Western analysis using anti-FLAG antibodies. Anti-actin andibodies were used to detect action as a loading control.

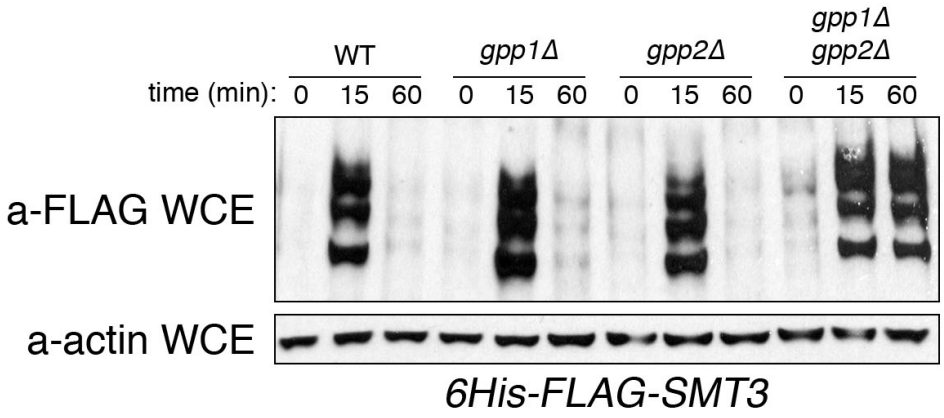


Fig 3.3: Combinatorial deletion of glycerol-3-phosphate phosphatases prolongs global SUMOylation during hyperosmotic stress. Indicated cells were treated, collected, and analyzed as described in Fig 3.2.

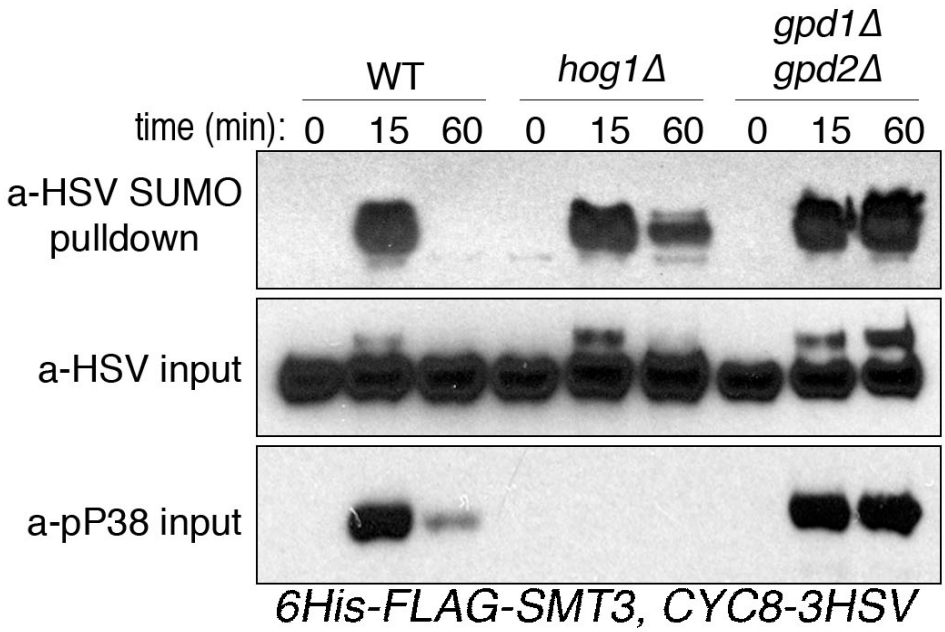


Fig 3.4: Cyc8 SUMOylation is robustly prolonged in the absence of glycerol biosynthesis. Indicated cells were treated, collected, and analyzed as described in Fig 2.1. Cyc8 was identified by Western analysis using anti-HSV antibodies. Total Cyc8 in the input fraction was used as a loading control, while anti-pp38 was used as a control for Hog1 presence and activation.

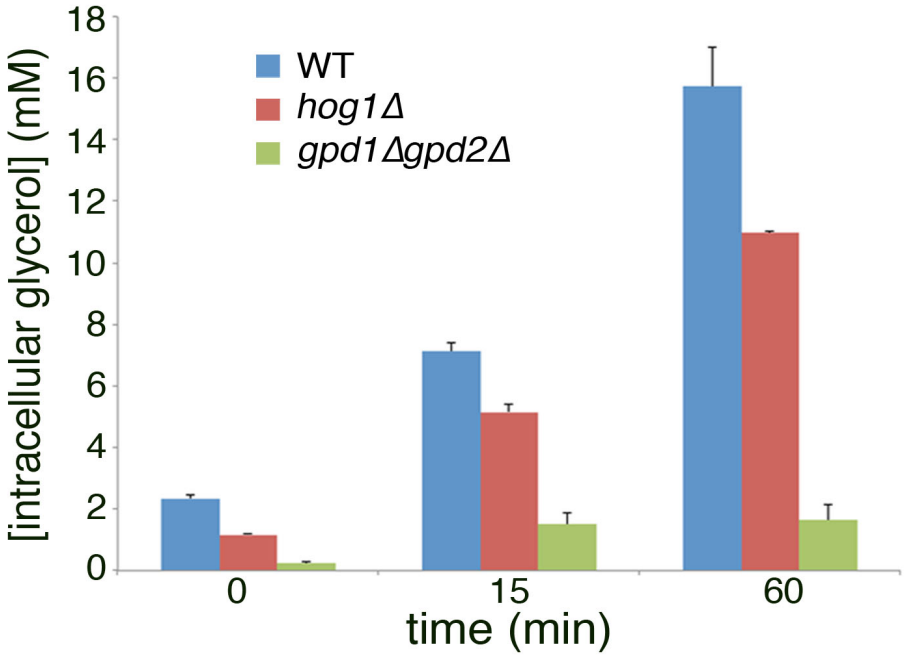


Fig 3.5: Confirmation of reduced glycerol content in glycerol biosynthetic mutant. Indicated cells were analyzed by glycerol assay as described in Fig 2.13. Error bars show SD.

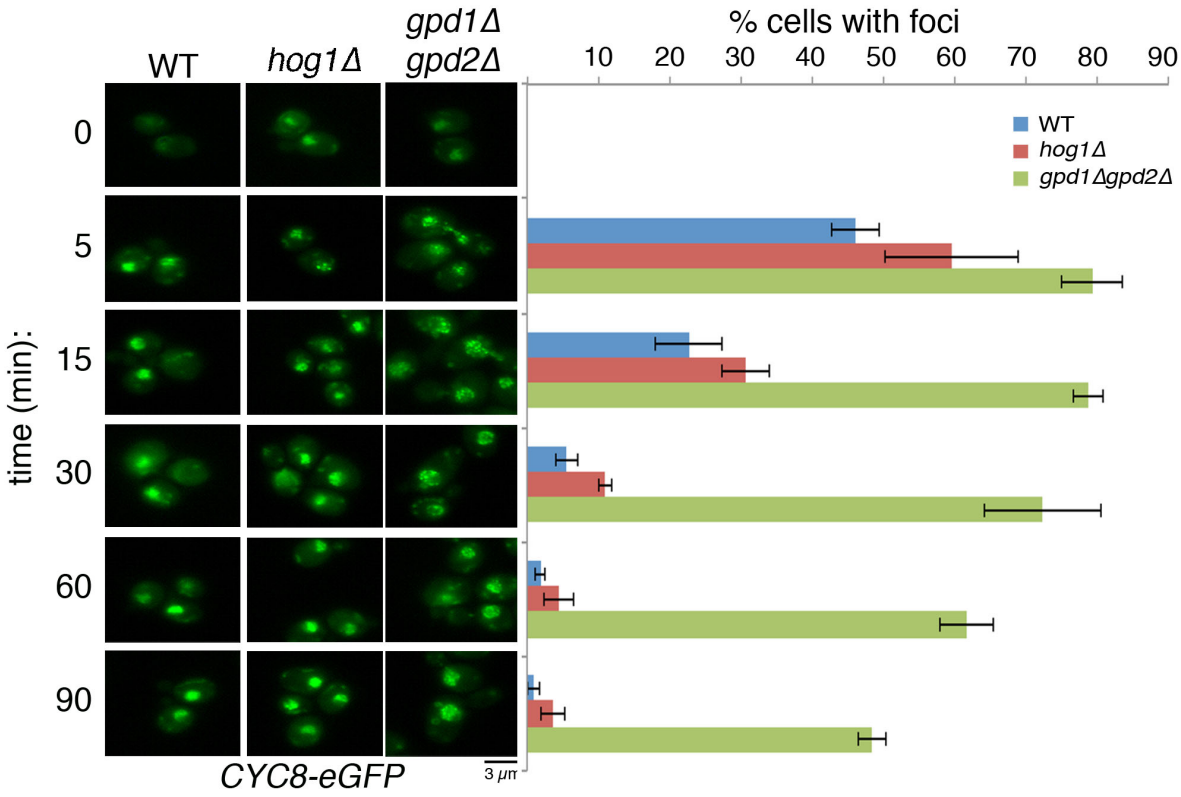


Fig 3.6: Comparison of Cyc8 foci lifetime during exposure to hyperosmotic stress. Parent, *hog1*Δ, or *gpd1*Δ*gpd2*Δ cells expressing *CYC8-eGFP* were grown in complete synthetic medium, treated with 1.2 M sorbitol, and fixed in 4% paraformaldehyde at the denoted time points. Cells were washed in PBS, spotted onto glass slides, and Cyc8 was imaged by fluorescence microscopy. Quantification is shown at right. Foci-bearing cells were counted and represented as percentage of total cells. Error bars show SD.

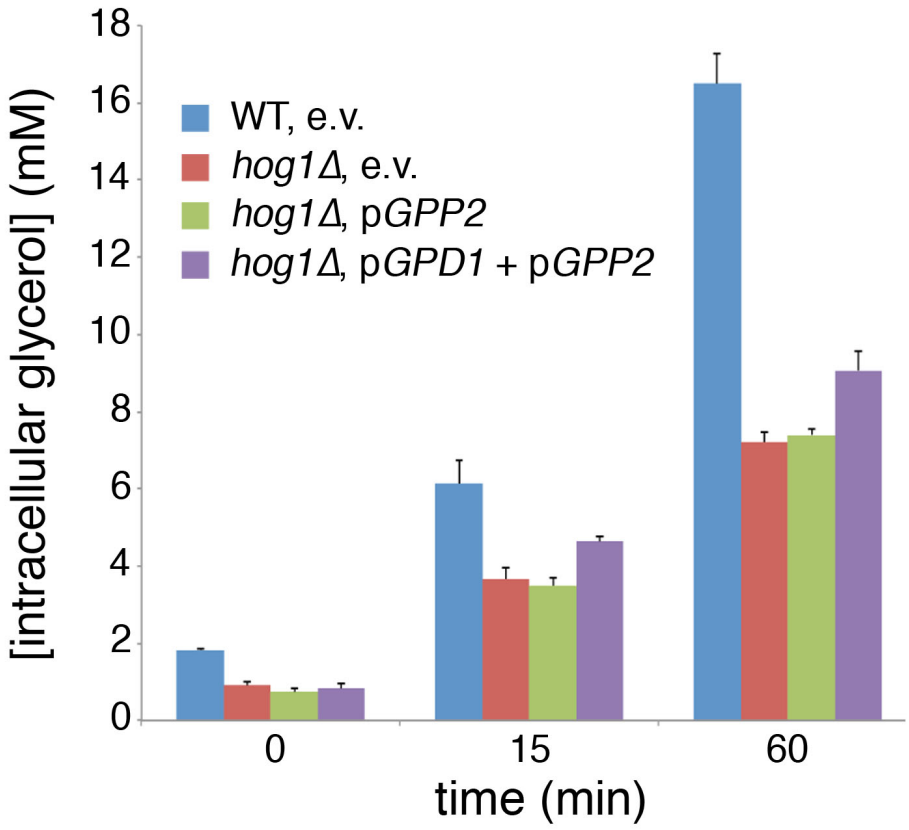


Fig 3.7: Overexpression of glycerol biosynthetic enzymes elevates stress-induced intracellular glycerol in the absence of *HOG1*. Glycerol content from parent or *hog1*Δ cells expressing the indicated constructs was assayed as described in Fig 2.13. Error bars show SD.

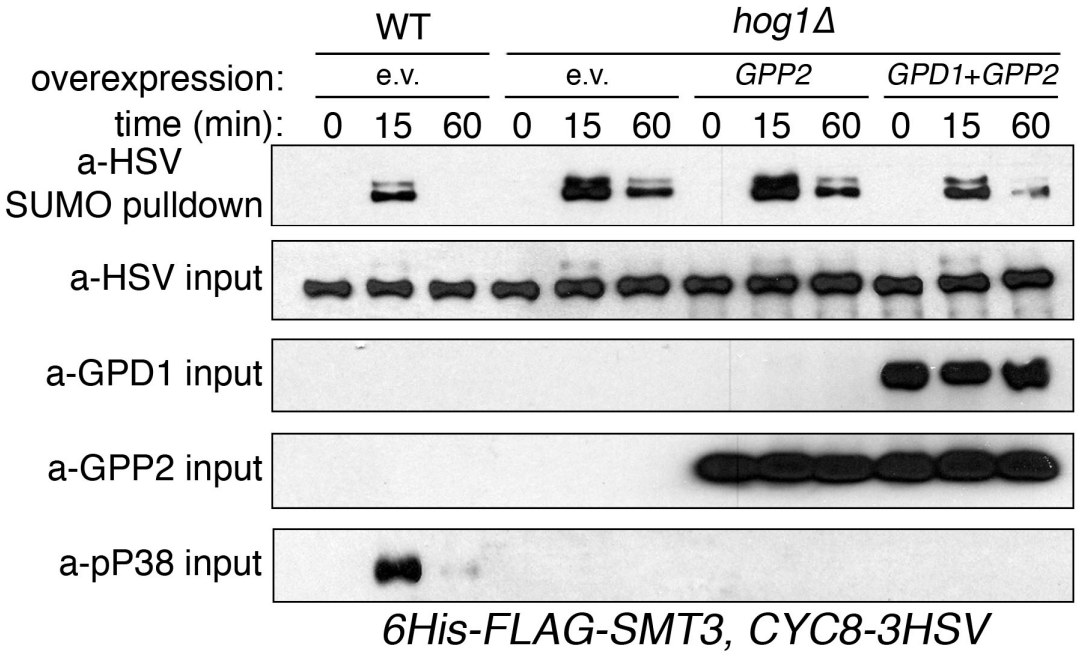


Fig 3.8: Overexpression of glycerol biosynthetic enzymes reduces the duration of stress-induced Cyc8 SUMOylation in the absence of *HOG1*. Parent or *hog1*Δ cells expressing *6His-FLAG-SMT3*, *CYC8-3HSV*, and the indicated constructs were treated, collected, and analyzed as in Fig 2.1. Cyc8 was identified by Western analysis using anti-HSV antibodies. Total Cyc8 in the input fraction was used as a loading control. Gpd1 and Gpp2 overexpression were confirmed using anti-HA antibodies, while anti-pp38 antibodies were used as a control for Hog1 presence and activation.

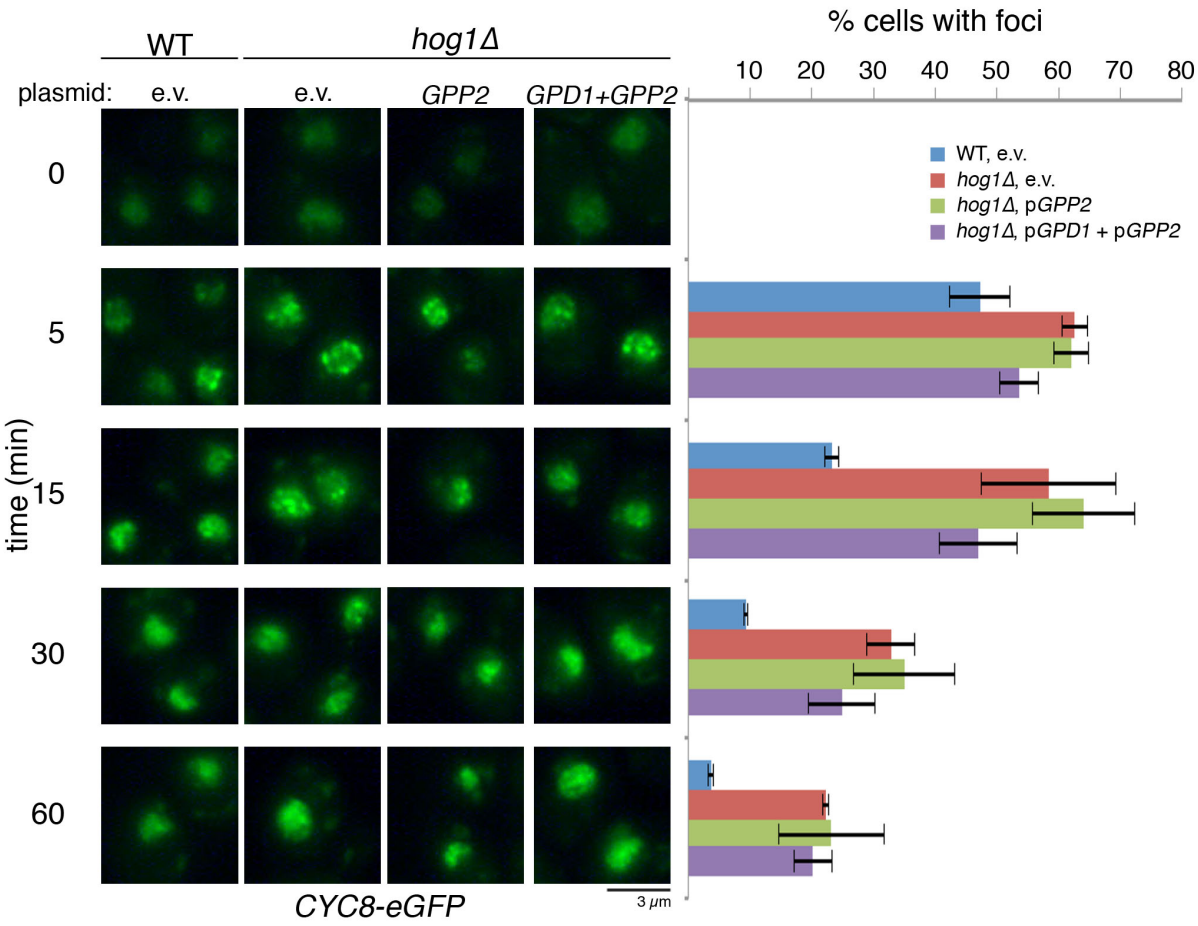


Fig 3.9: Comparison of Cyc8 foci lifetime during exposure to hyperosmotic stress. Parent or *hog1*Δ expressing *CYC8-eGFP* and the indicated constructs were treated, collected, and analyzed as described in Fig 3.6. Quantification is shown at right. Foci-bearing cells were counted and represented as percentage of total cells. Error bars show SD.

Chapter 4: Cyc8 foci are soluble and dynamic

Transcription inhibitors impede glycerol biosynthesis and prolong Cyc8 SUMOylation and foci duration during hyperosmotic stress

To better elucidate the mechanisms regulating Cyc8 SUMOylation and foci formation, I wished to identify tool compounds that could modulate glycerol biosynthesis. While many efforts have been made on this front for the treatment of obesity in humans (Thuresson 2004), few compounds have been identified that potently and specifically inhibit glycerol biosynthesis enzymes in yeast. However, a previous report found that inhibition of transcription prior to the onset of hyperosmotic stress affected the accumulation of global SUMOylated species (Lewicki, Srikumar et al. 2015). To examine if transcription inhibition affects the accumulation of SUMOylated Cyc8, I purified SUMOylated proteins by metal affinity purification from cells pretreated with vehicle or the transcription inhibitors thiolutin (THL) and 1,10-phenanthroline (PHN) (Fig 4.1). These drugs block transcription through unique mechanisms: thiolutin directly inhibits RNA polymerase while 1,10-phenanthroline is potent chelator of metal ions that function as cofactors for RNA polymerase (Tipper 1973, Falchuk, Mazus et al. 1976). I found that both drugs prolonged Cyc8 SUMOylation following the onset of hyperosmotic stress with Cyc8 SUMOylation being maintained at least to 60 minutes (Figure 4.2, 4.3). While both drugs prolonged the duration of Cyc8-SUMOylation, the relative levels of SUMOylation were not consistent, with THL pretreatment showing enhanced SUMOylation over PHN pretreatment. As stated previously, PHN is a metal chelator that forms stable complexes with a variety of divalent metals, including zinc. Zinc is a necessary cofactor for the SUMO E3 Siz1 (Yunus and Lima 2009), but is dispensable for SUMO proteolysis by yeast deSUMOylating enzymes (Li and Hochstrasser 2003). We previously showed Siz1 to be the primary ligase facilitating Cyc8-SUMOylation during hyperosmotic stress (Oeser, Amen et al. 2016). I believe these differences in drug mechanism are responsible for the observed differences in relative SUMOylation.

Consistent with previous conditions that prolonged Cyc8 SUMOylation, I found that inhibition of transcription also prolonged Cyc8 nuclear foci (Figure 4.4). Quantification of the fluorescence microscopy results indicated a significant enrichment of cells with foci following pretreatment with PHN over those treated with THL or vehicle control (Figure 4.4). I further compared the effect of these drugs on glycerol accumulation and found that both THL and PHN significantly reduced the intracellular glycerol concentration during hyperosmotic stress (Figure 4.5). I found that THL slowed glycerol accumulation, while PHN appeared to completely block accumulation, which can likely be attributed to their unique mechanisms of action. I believe this discrepancy in glycerol accumulation explains the differences observed for Cyc8 foci formation.

Cyc8 nuclear foci resolution after transcriptional restart requires glycerol biosynthesis

Both THL and PHN inhibited glycerol accumulation and prolonged Cyc8 foci, but it was unclear whether their action on glycerol biosynthesis was a causative reason for the extended lifespan of Cyc8 foci. To decipher this, I monitored Cyc8 foci by fluorescence microscopy on parent or *gpd1*Δ*gpd2*Δ cells expressing Cyc8-eGFP after washout of the reversible transcription inhibitor PHN (Figure 4.6). Yeast cells were grown to mid-log phase and treated with PHN for 5 minutes to inhibit transcription. As previously seen, this was insufficient to induce significant amounts of Cyc8 foci formation alone (Figure 4.7). Cells were challenged with 1.2 M sorbitol for 30 minutes to form Cyc8 foci, after which cells were moved to media containing 1.2 M sorbitol with or without PHN. Cyc8 foci disappeared over the course of 60 minutes in parent cells post-washout of PHN, but were maintained up to 120 minutes in *gpd1*Δ*gpd2*Δ cells post-washout of PHN. Cyc8 foci did not resolve in either strain background when PHN was maintained in the media (no washout). Quantification of the total number of cells displaying Cyc8 foci following drug washout showed a significant loss of Cyc8 foci only in parent cells by 30 minutes, reaching basal levels by 120 minutes post-washout (Figure 4.8). Cells incapable of producing glycerol, however,

showed no resolution of Cyc8 foci. Under the same experimental design, I monitored intracellular glycerol content of parent or *gpd1*Δ*gpd2*Δ cells after removal of PHN (Figure 4.9). As expected, parent cells showed a sharp accumulation of intracellular glycerol following removal of PHN, which was inversely correlated to the amount of Cyc8 foci seen previously. In line with this, parent cells showed significantly reduced glycerol accumulation when PHN was maintained in the media. As expected, *gpd1*Δ*gpd2*Δ cells showed virtually no intracellular glycerol, regardless of the presence of the inhibitor PHN. Taken together, these results suggest that accumulation of the compatible osmolyte glycerol is the necessary signal for the resolution of Cyc8 foci during exposure to hyperosmotic stress.

Cyc8 foci are not insoluble aggregates

Recent work has shown that a host of proteins that form dynamic liquid droplets can age into solid, insoluble aggregates (Patel, Lee et al. 2015). Many of these proteins are implicated in neurodegenerative disease, including FUS, TDP-43, and hnRNPA2 (Murakami, Qamar et al. 2015, Gopal, Nirschl et al. 2017, Ryan, Dignon et al. 2018). Moreover, disease-associated mutations in these proteins are known to cause aberrant nucleocytoplasmic transport, similar to our previous observations of hyperosmotic stress-induced trafficking of SUMO-deficient Cyc8 to the cytoplasm (Oeser, Amen et al. 2016, Maharana, Wang et al. 2018). Given this, I wanted to see if persistent Cyc8 foci formed in the absence of glycerol biosynthesis would begin to age into insoluble protein aggregates. To do this, I performed sedimentation assays on parent or *gpd1*Δ*gpd2*Δ strains expressing Cyc8-3HSV before and after exposure to 1.2 M sorbitol (Figure 4.10). I found that Cyc8 remains in the soluble fraction, independent of glycerol biosynthesis and across 120 minutes of stress exposure. To support this result, we monitored Cyc8-GFP foci in *gpd1*Δ*gpd2*Δ yeast cells following washout of the osmotic stressor by fluorescence microscopy (Figure 4.11). As previously seen, Cyc8 foci persist in the absence of glycerol biosynthesis as long as the stressor is maintained. When moved back to equiosmolar medium, however, Cyc8 foci

were resolved within 5 minutes. This suggests that Cyc8 foci are not static inclusions; rather, they are dynamic, reversible nuclear bodies.

Discussion

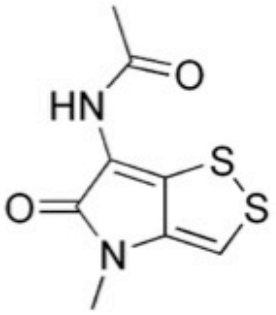
Using the well-established inhibitors thiolutin and 1,10-phenathroline, I show that Cyc8 SUMOylation and foci-formation are prolonged in the absence of ongoing transcription. I show that these drugs block or slow the accumulation of glycerol in response to hyperosmotic stress, closely mirroring our observations using directed genetic perturbations of glycerol biosynthesis. Previous work has indicated that these drugs abolish rapid hyperosmotic stress-induced SUMOylation on a global scale, which is in direct disagreement to my findings here (Lewicki, Srikumar et al. 2015). I think there are some critical differences to the approaches taken previously and my own that may account for this discrepancy. First, my approach employs integrated reporters expressed from endogenous loci on endogenous promoters, which I think is necessary to create the closest recapitulation of normal function. Second, I chose only to look specifically at Cyc8-SUMOylation, rather than global changes in SUMO. Collectively, I feel these two major differences in approach are significant enough to account for altered findings.

Compatible osmolytes have been implicated as regulators of cytoplasmic foci during osmotic stress in multiple organisms, as specific focal structures are persistent in the absence of osmolyte biosynthesis (Bounedjah, Hamon et al. 2012, Davis, Montalbano et al. 2017). Generally speaking, compatible osmolytes are considered chemical chaperones that buffer against protein damage caused by the effects of hyperosmotic stress (Upagupta, Carlisle et al. 2017). If that were the case, one would expect that conditions that prevent the formation of these chaperone-like molecules would deleteriously affect protein structure or homeostasis, subsequently increasing the insolubility of target proteins.

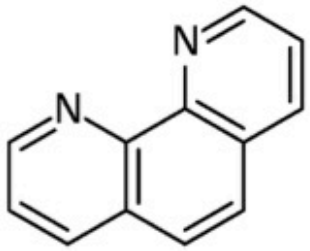
Here, however, I observe no partitioning of Cyc8 into the insoluble protein fraction during hyperosmotic stress, independent of glycerol biosynthesis. Likewise, Cyc8 protein levels do not decrease across hyperosmotic stress, which implies that there is no protein degradation machinery acting on Cyc8 during these stress conditions. Given this, I propose that Cyc8 foci form as a function of cellular water loss and increased crowding during hyperosmotic stress, and that this is facilitated by its aggregation-prone low complexity sequences. This, in turn, allows for derepression of necessary targets that promote volume recovery during stress. Once cellular volume has been recovered via the accumulation of glycerol, Cyc8 foci are resolved and repression is reestablished. In saying, Cyc8 foci are clearly not pathogenic, but rather they play an important role in modulating gene expression during stress.

Acknowledgements

Thank you to Michelle Oeser for the construction of yeast strains and plasmids, and to David Agard for conversations that inspired experiments performed in this chapter.



Thiolutin (THL)



1,10-Phenanthroline (PHN)

Fig 4.1: Structures of the transcription inhibitors used in this chapter.

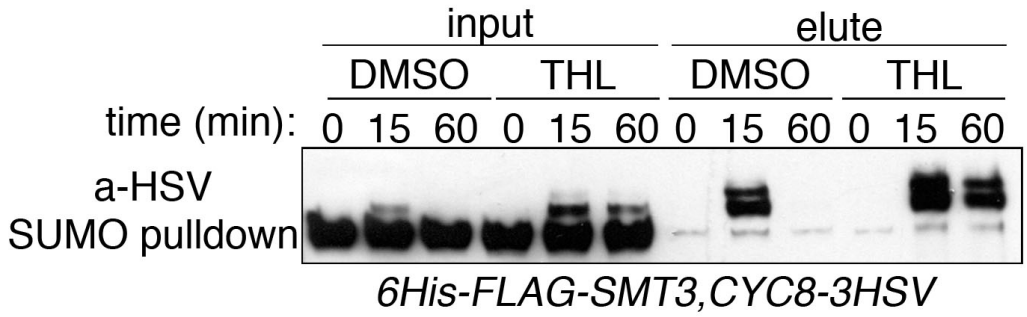


Fig 4.2: Thiolutin (THL) prolongs Cyc8 SUMOylation during hyperosmotic stress. Parent cells expressing *6His-FLAG-SMT3* and *CYC8-3HSV* were grown in complete synthetic medium, treated with 4ug/ml THL or DMSO vehicle control for 5min, and then exposed to 1.2 M sorbitol. Cells were collected and analyzed as described in Fig 2.1. Cyc8 was identified by immunoblotting using anti-HSV antibodies. Total Cyc8 in the input fraction was used as loading control.

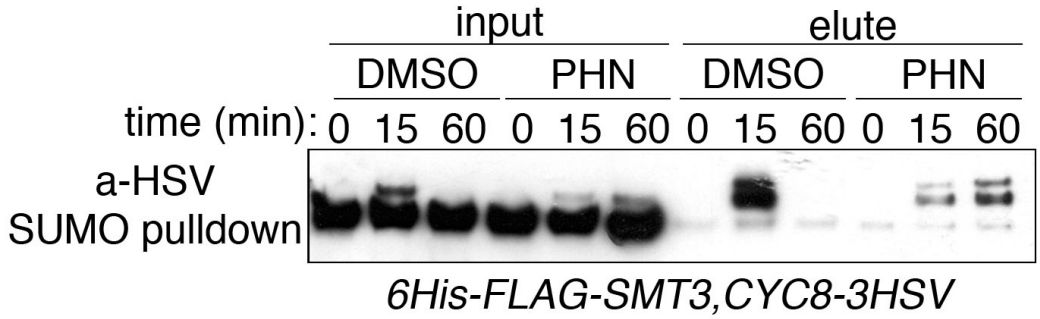


Fig 4.3: 1,10-phenanthroline (PHN) prolongs Cyc8 SUMOylation during hyperosmotic stress. Parent cells expressing *6His-FLAG-SMT3* and *CYC8-3HSV* were grown in complete synthetic medium, treated with 500ug/ml PHN or DMSO vehicle control for 5min, and then exposed to 1.2 M sorbitol. Cells were collected and analyzed as described in Fig 2.1. Cyc8 was identified by immunoblotting using anti-HSV antibodies. Total Cyc8 in the input fraction was used as loading control.

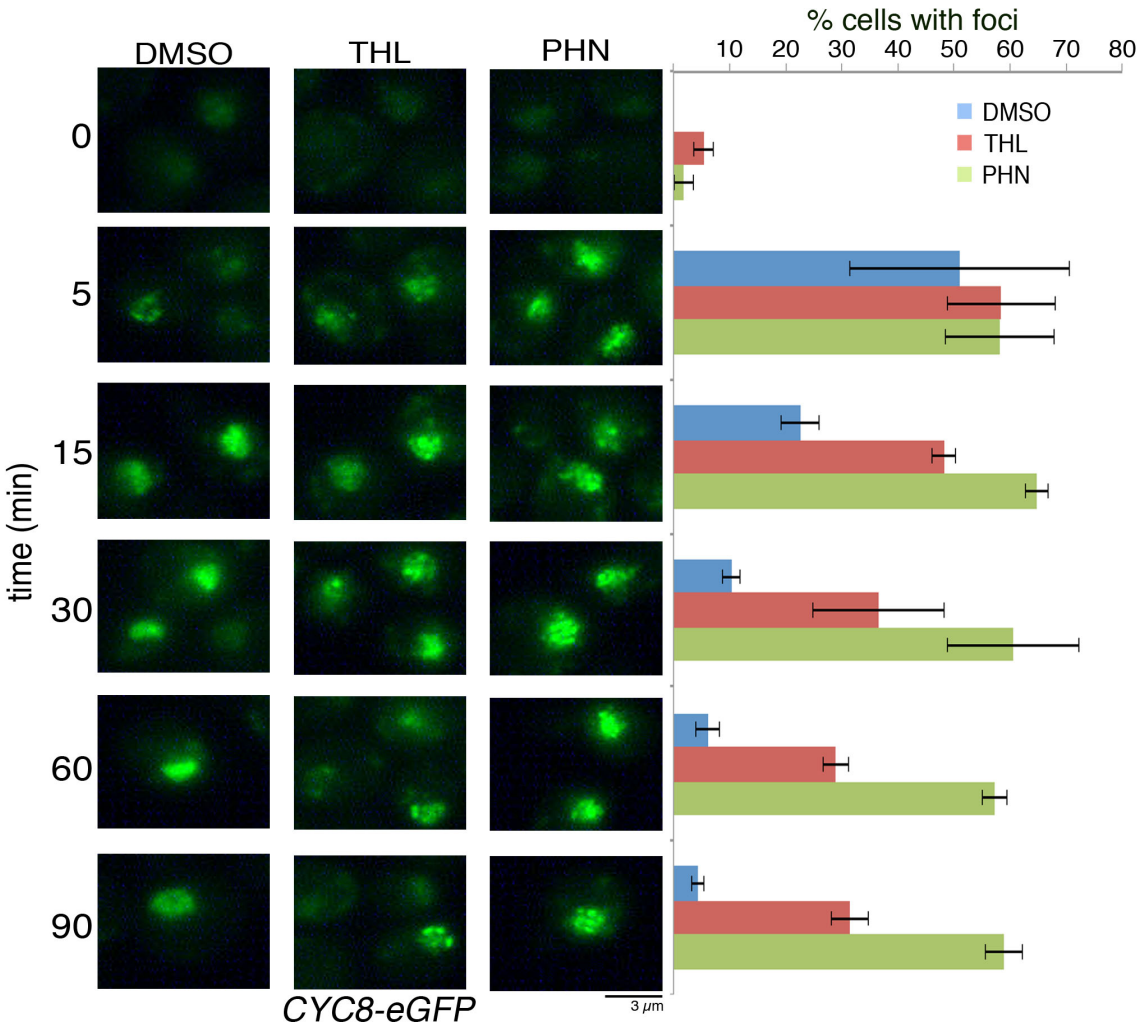


Fig 4.4: Comparison of Cyc8 foci lifetime after transcription inhibition during exposure to hyperosmotic stress. Parent cells expressing *CYC8-eGFP* were grown in complete synthetic medium and treated with the indicated compound for 5 minutes at room temperature. Cells were then collected and analyzed as described in Fig 3.6. Quantification is shown at right. Foci-bearing cells were counted and represented as percentage of total cells. Error bars show SD.

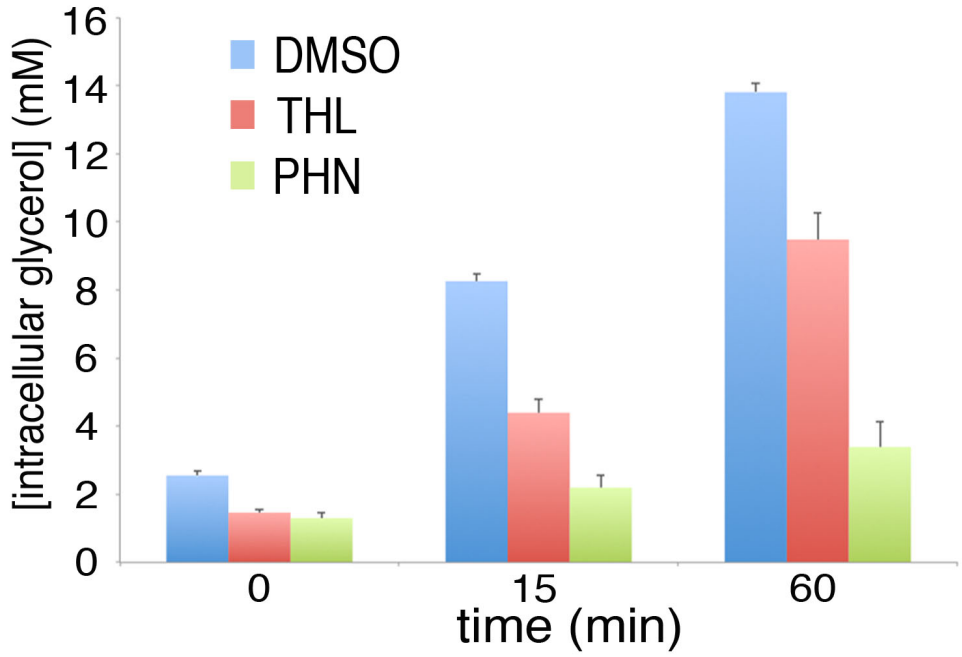


Fig 4.5: Transcription inhibitors slow glycerol accumulation during hyperosmotic stress. Parent cells were grown in triplicate in rich medium and treated with the indicated compound for 5 minutes at room temperature and hyperosmotic stress was initiated by addition of sorbitol to 1.2 M. Cells were collected and glycerol content was analyzed as described in Fig 2.13. Error bars show SD.

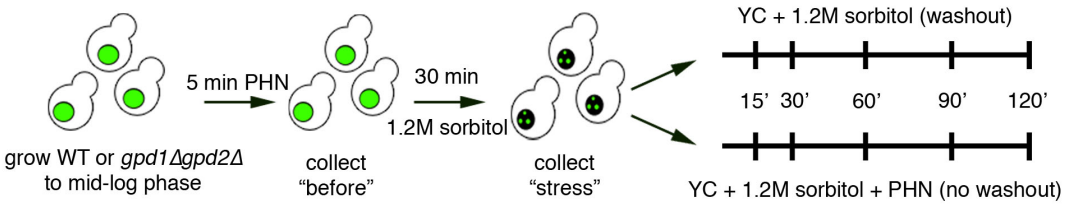


Fig 4.6: Graphical illustration of experimental design of PHN washout experiments.

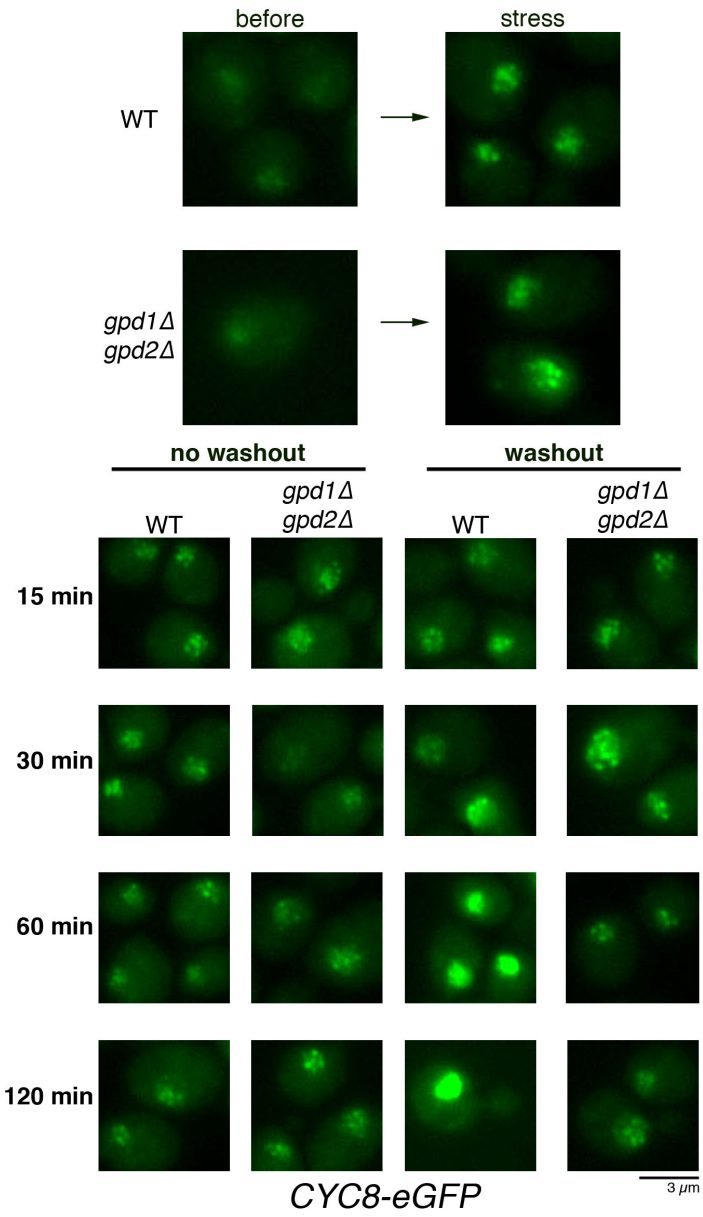


Fig 4.7: Comparison of Cyc8 foci lifetime after washout of PHN. Parent or *gpd1*Δ*gpd2*Δ cells expressing *CYC8-eGFP* were grown in complete synthetic medium, treated with 500ug/ml PHN for 5min, and then challenged with 1.2 M sorbitol. Samples were collected prior to the onset of stress and following 30 minutes of stress to illustrate initiation of Cyc8 foci. Following, cells were collected by centrifugation and resuspended in fresh media containing 1.2 M sorbitol with (no wash) or without (wash) PHN. Cells were collected at the indicated time points following resuspension and fixed in 4% paraformaldehyde. Samples were then collected and fluorescence microscopy was performed as described in Fig 3.6.

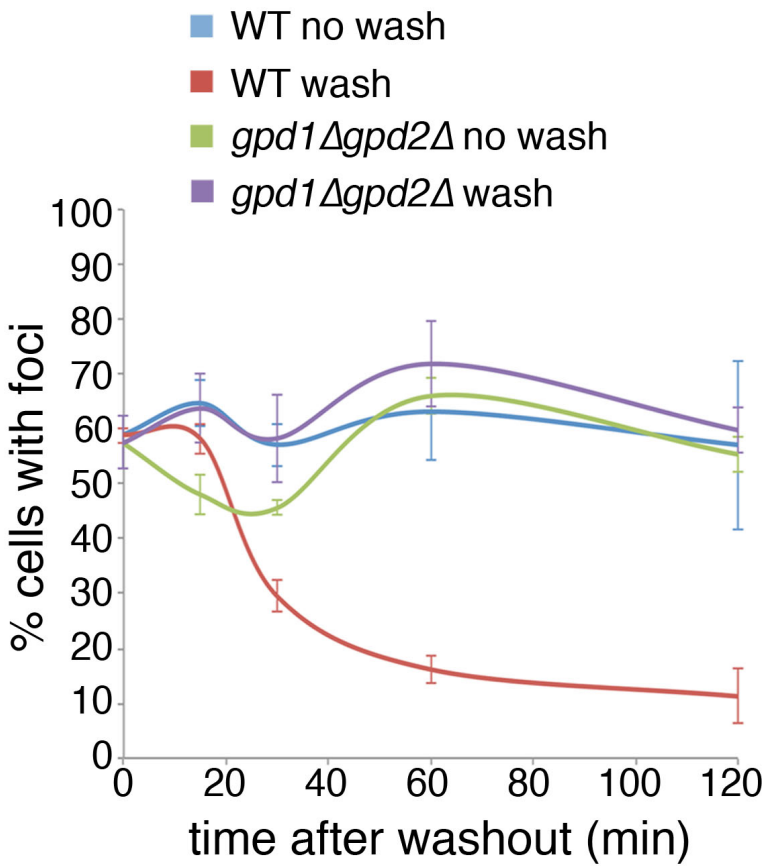


Fig 4.8: Quantification of cells bearing Cyc8 foci. Foci-bearing cells from Fig 4.8 were counted and represented as percentage of total cells. Error bars show SD.

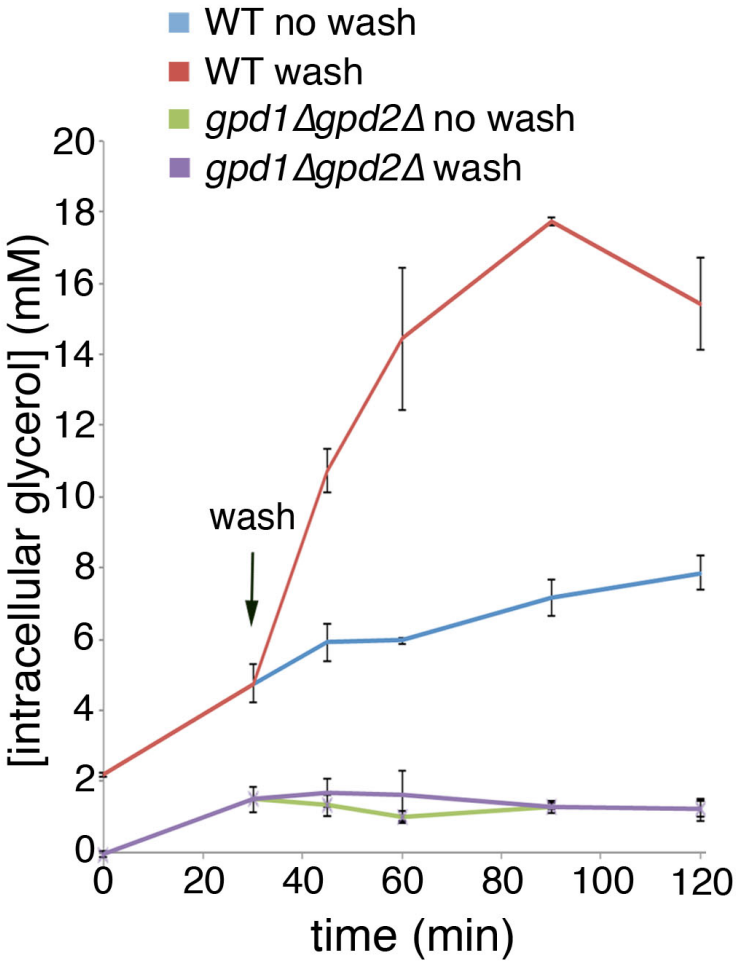


Fig 4.9: Glycerol accumulation assay after removal of PHN. Indicated cells were grown in triplicate in rich medium, treated with 500µg/ml PHN for 5 minutes and then challenged with 1.2 M sorbitol for 30min. Following, cells were collected by centrifugation and resuspended in fresh media containing 1.2 M sorbitol with (no wash) or without (wash) PHN, and collected at the denoted time points. Lysates were extracted and glycerol content was analyzed as described in Fig 2.13. Error bars show SD.

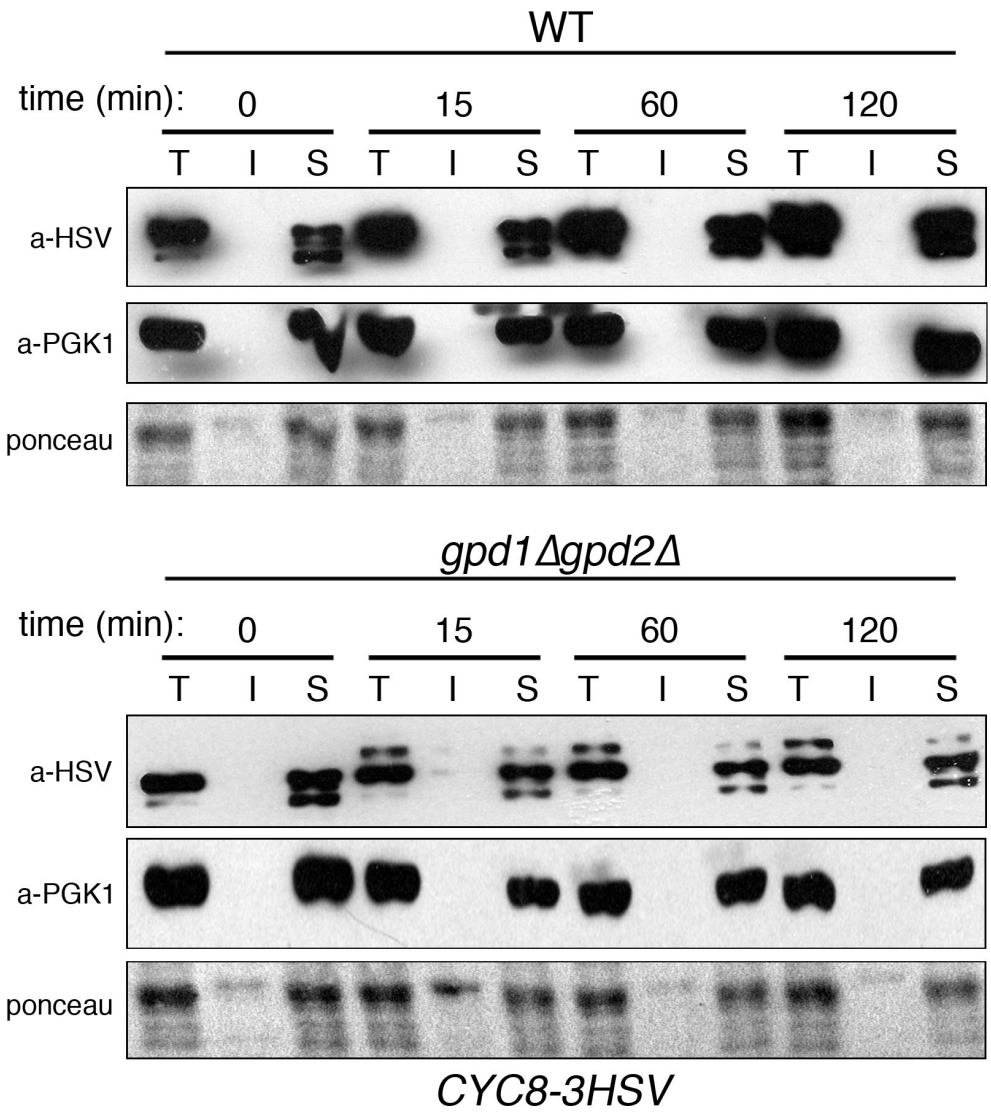


Fig 4.10: Sedimentation assay comparing Cyc8-3HSV solubility in parent or *gpd1*Δ*gpd2*Δ cells. Parent or *gpd1*Δ*gpd2*Δ cells were grown in complete synthetic medium and treated with 1.2 M sorbitol to initiate stress. Cells were collected by centrifugation at the indicated timepoints, lysed in non-denaturing lysis buffer, and subjected to differential centrifugation to separate soluble (S) and insoluble (I) components. Total lysate (T) indicates the total amount of Cyc8-3HSV present in the sample prior to separation of soluble and insoluble components. Anti-HSV antibody was used to probe for Cyc8, anti-PGK1 was used as a control for the soluble fraction, and ponceau was used to indicate the presence of protein in the insoluble fraction.

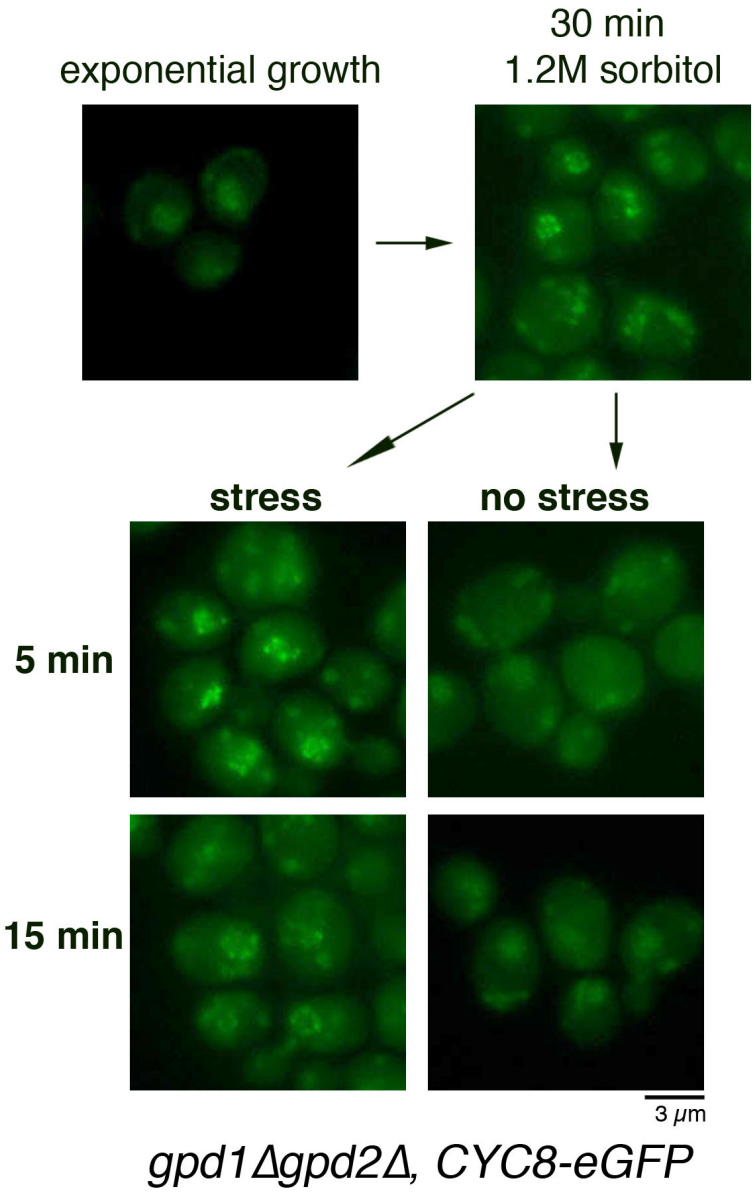


Fig 4.11: Comparison of Cyc8 foci after return to iso-osmotic medium. *gpd1*Δ*gpd2*Δ cells expressing *CYC8-eGFP* were grown in complete synthetic medium and treated with 1.2 M sorbitol to initiate hyperosmotic stress. Cells were incubated for 30 minutes to allow Cyc8 foci to form. Cells were collected by centrifugation and resuspended in synthetic medium with 1.2 M sorbitol (no washout) or in synthetic medium lacking an osmotic stressor (washout). Samples were collected at the indicated time points and fluorescence microscopy was performed as in Fig 3.6.

Chapter 5: Conclusions, Questions, and Future Directions

In this thesis, I have shown a connection between the Hog1 MAPK, the Cyc8-Tup1 transcription corepressor complex, and the response to hyperosmotic stress. This work showcases novel intersections of well-established pathways, linking post-translational modifications, biomolecular condensation events, and the regulation of gene expression during the response to cellular stress. The information provided here is in accordance with existing ideas, which I would like to illustrate further here.

The formation of fungal prions has long thought to be a direct response to ever changing environmental conditions (Chernova, Wilkinson et al. 2014). Prionogenic-switching can inform an organism's progeny about past experience and provide resilience to stressful conditions for several generations (Sindi and Serio 2009). Rather than rely on chance genetic mutations to create fitness advantages, prions provide a tightly regulated mechanism for simple organisms like yeast to promote cellular fitness in the face of stress (Satpute-Krishnan and Serio 2005). This theory explains the diversity in fungal prion species, and why fungal prions frequently confer selective advantages.

Here, I've shown that mutations in Cyc8 that reduce its normal pattern of SUMOylation can actually provide a fitness advantage in the absence of a key genetic activator, Hog1. As Cyc8 is a transcriptional repressor, it makes sense that mutations that inhibit its normal function – or restructuring into a prion form that reduces function – would enhance fitness in the absence of an activator. While I have not shown here that focally condensed Cyc8 is a *bona fide* prion, one could see how loss-of-function at the protein level for proteins of a similar nature could allow yeast strains to survive harsh conditions. Likewise, Cyc8 represses genes involved in myriad functions; temporary loss-of-function of Cyc8 by prionogenic switching could be a generalized mechanism to evade a host of different conditions.

What then, are the focally condensed structures of Cyc8 observed here? Previous studies have shown that Cyc8 can propagate as a prion, but this prionogenic state of Cyc8 required either massive overexpression of its prion

domain or the presence of another existing prion to provide seeding (Patel, Gavin-Smyth et al. 2009). Indeed, no studies to date have shown *de novo* formation of the [OCT+] prion. I have shown that Cyc8 foci formation is dependent on glycerol accumulation, but this is not sufficient to drive Cyc8 toward insolubility or amyloidogenesis. Rather, Cyc8 foci maintain solubility and dynamism independent of glycerol accumulation.

Prion domains are typically enriched in polar amino acids like glutamine and asparagine (Perutz, Pope et al. 2002). Domains bearing mostly glutamine residues tend to be dynamic, and can move in and out of focal structures in a regulated fashion. Those enriched for asparagine, conversely, frequently partition into irreversible amyloid states (Halfmann, Alberti et al. 2011). Perhaps this dynamic structural aspect of Cyc8 is important for its function, and regulated foci formation plays a role in modulating transcription under various contexts. Future experiments are necessary to identify exactly what components of Cyc8 are necessary for foci formation and whether they affect its function. Likewise, compositional mutation of its prion domain may assist in driving Cyc8 into amyloid states, which will be useful to inform on role of dynamic foci vs. amyloid foci in transcriptional regulation.

Non membrane-bound organelles form during stress by virtue of a liquid-liquid phase separation event, wherein salt and protein concentration reach a critical maximum to drive proteins to demix from the bulk solvent (Riback, Katanski et al. 2017). Glycerol is required during hyperosmotic stress to reestablish normal cellular volume by counteracting water efflux and to balance ion gradients (Bremer, Wolff et al. 2017). Indeed, yeast cells can lose nearly half their total volume immediately in response to osmotic shock, greatly increasing macromolecular crowding in an already crowded environment (Petelenz-Kurdziel, Kuehn et al. 2013). It would follow, then, that Cyc8 foci likely undergo phase separation in response to hyperosmotic stress due to increased macromolecular crowding and facilitated by its inherently adhesive low complexity sequences. Glycerol, rather than providing chaperoning to a misfolded protein, is simply required to reestablish normal volume, upon which Cyc8 is released back into a

more diffuse form. Studies have shown that the choice of compatible osmolyte employed to counteract hyperosmotic stress depends on carbon-source, and that yeast cells grown using ethanol as a fermentable carbon source prefer the carbohydrate trehalose over glycerol (Babazadeh, Lahtvee et al. 2017). It would be interesting to test this model under these alternative conditions. Further, it is still under speculation what might initiate [OCT+] formation *de novo*.

Another interesting aspect of Cyc8 biology during hyperosmotic stress is its partial eviction to the cytoplasm when its SUMOylation-sites are ablated. Previous studies have implicated Cyc8 and its SUMOylation in tethering inducible genes to the nuclear pore complex during carbon switching (Texari, Dieppois et al. 2013). While I did not study it in further detail, I frequently found that Cyc8 foci (especially those formed under conditions that prolong their lifespan) accumulate around the nuclear periphery. The lumen of the nuclear pore is composed of FG-nucleoporins that form a liquid phase separated compartment; this is thought to enhance cargo gating at the nuclear pore by requiring nuclear import or export receptors that are capable of interacting within this liquid-like environment (Schmidt and Gorlich 2016). As mentioned previously, I posit that Cyc8 forms liquid-like foci independent of SUMOylation. Perhaps SUMOylation provides a gating function that prevents Cyc8 from inappropriately trafficking out of the nucleus during hyperosmotic stress?

Final Words

Clearly, a number of interesting questions remain from these studies. All of the players in this thesis (Hog1, Cyc8-Tup1, SUMO) are conserved into humans, and are implicated in a number of disease processes, including cancer, neurodegeneration, and developmental disorders. Beyond the human condition, these pathways are paramount for microbial survival during stress conditions. As cellular stressors represent a significant barrier to the production of fuels, foods, and drugs in microbial factories, it is important that we gain greater insight into how these pathways work. Whether these signaling phenomena are conserved through evolution remains to be seen; regardless, their interplay at the nexus of

post-translational modifications, biophysical behaviors, and the response to cellular stress is fascinating. I truly hope that my work will inform future generations of researchers in useful ways, and am excited to see how it is built upon.

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Appendix I: Materials and Methods

Yeast strains and plasmids

Yeast strains and plasmids used in this study are listed below. Standard yeast genetic methods were used for these studies (Guthrie and Fink 1991). All gene deletions were verified by colony PCR.

Growth and stress conditions

Cells were grown to a density of ~1.5x10^7 cells/ml at 30°C in yeast complete (YC) media prior to stress induction. All 0 time point samples were collected before stress induction. For induction of hyperosmotic stress, equal volumes of culture and YC+2.4M sorbitol were combined for a final concentration of 1.2 M sorbitol. For transcription inhibition, cells were treated at room temperature for 5 minutes with either 4ug/ml thiolutin (Sigma Aldrich), 500ug/ml 1,10-phenanthroline (Sigma Aldrich), or an equal volume of DMSO vehicle control prior to the induction of hyperosmotic stress.

SUMOylated protein purification

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

Sedimentation assay

50ml aliquots of cells were collected at each time point after stress and flash frozen in liquid nitrogen. Harvested cells were lysed by vortexing with glass beads at 4°C in non-denaturing lysis buffer (100 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Nonidet P40, 2mM PMSF, 20mM NEM). To remove unlysed cells, lysates were centrifuged at 700 g for 1 minute at 4℃. 50 ul lysate, representing the 'total lysate', was removed and added to 50 ml SUMEB. 100 ul remaining lysate was centrifuged at 12,800 g for 15 minutes at 4℃. 100 ml supernatant, representing the 'soluble fraction', was added to 100 ul SUMEB. The pellet, representing the 'insoluble fraction', was resuspended in 100 ul lysis buffer and 100 ml SUMEB. All samples were incubated at 65℃ for 10 minutes, separated on 8% SDS-PAGE gels, and analyzed by western blot.

Western analysis

SUMOylated proteins were resolved by SDS-PAGE using 4–20% gradient gels. Western analyses were performed with mouse anti-FLAG (1:2500, Sigma), mouse anti-HSV (1:2500, Novagen), mouse anti-HA (1:2500, Sigma), mouse anti-actin (1:2500, Abcam), or rabbit phospho-p38 (1:2500, CST).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as previously described (Gardner, Nelson et al. 2005). Briefly, 50ml aliquots of cells were grown to a density of ~1.5x10^7 cells/ml at 30°C 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 1.2 M, and samples were collected at the indicated times. Protein-DNA crosslinking was initiated by addition of 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 1X TBS, 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 4˚C. 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 4˚C. 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, 2mM PMSF), ChIP wash buffer (10mM Tris pH 8.0, 250mM LiCl, 0.5% NP-40, 0.5% 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 65˚C for 10 minutes. Samples were treated Proteinase K (New England Biolabs) and RNase at 50˚C for one hour, followed by removal of crosslinking by incubation at 65C overnight. Following, input and elute DNA was isolated using QIAprep spin columns (Qiagen) and analyzed by qPCR.

qPCR analyses

Quantitative real-time PCR was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems). Reactions were performed on ChIP inputs and eluates using PowerUP SYBR Green Master Mix (Applied Biosystems) with specific primers for the *GPD1* UAS or *ACT1* control. Primer sequences for *GPD1:* 5'-TCTCACCTCTCACCGCTGAC-3', 5'-AGACTTGCTCAAACCCCAGGAG-3'. Primer sequences for *ACT1*: 5'-TGGCCGGTAGAGATTTGACTGACT-3', 5'-TCGAAGTAAGGCGACGTAACAT-3'. ΔCt values were calculated for each condition and corrected by their respective *ACT1* ΔCt. Results were converted to $\Delta\Delta$Ct and normalized to each cell's respective pre-stress time point.

Glycerol accumulation assays

10ml aliquots of cells were grown to a density of ~1.5x10^7 cells/ml at 30°C in rich medium. A pre-stress sample was collected prior to induction of stress. Hyperosmotic stress was induced by addition of sorbitol to a final concentration of 1.2 M, and samples were collected at the indicated time points. Cells were collected by centrifugation, resuspended in 100uL 1X TBS, and incubated at 95˚C for 10 minutes. Supernatant was collected by centrifugation and glycerol concentration in the resulting fraction was measured using a commercial enzymatic assay kit (Sigma Aldrich).

Fluorescence microscopy

Aliquots of cells at each time point after hyperosmotic stress were removed, fixed in 4% paraformaldehyde solution for 15 minutes at room temperature and then washed with PBS. Cells were imaged on a Nikon Eclipse 90i with a100X objective, filters for GFP (HC HiSN 0 Shift filter set with excitation wavelength (450–490 nm), dichroic mirror (495 nm), and emission filter (500–550 nm)), and a Photometrics Cool Snap HQ2 cooled CCD camera with NIS-Elements acquisition software.

Image processing

All blots were scanned using an Epson Perfection V350 Photo scanner at 300 dpi. All images were processed with a Mac iMac or Pro computer (Apple) using Photoshop CS or CS4 (Adobe).

Appendix II: Yeast Strains and Plasmids

Strain	Genotype	Reference
RGY5266	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6*	(Oeser, Amen et al. 2016)
RGY5645	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ*	(Oeser, Amen et al. 2016)
RGY5654	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, hog1Δ*	(Oeser, Amen et al. 2016)
RGY5708	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1-3HA::URA3*	(Oeser, Amen et al. 2016)
RGY5809	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, tup1Δ::TUP1-3HA::URA3, hog1Δ*	this study
RGY5820	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-eGFP::LEU2*	(Oeser, Amen et al. 2016)
RGY5822	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)-eGFP::LEU2*	(Oeser, Amen et al. 2016)
RGY5824	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-3HSV::LEU2*	(Oeser, Amen et al. 2016)
RGY5825	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)-3HSV::LEU2*	(Oeser, Amen et al. 2016)
RGY5855	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, gpd1Δ*	this study
RGY5859	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, gpp2Δ*	this study
RGY5862	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, gpd1Δ, gpd2Δ*	this study
RGY5863	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, gpd2Δ*	this study
RGY5913	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, gpp1Δ, gpp2Δ*	this study
RGY5921	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, gpp2Δ*	this study
RGY5961	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, ste11Δ*	this study
RGY5962	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ, hog1Δ*	this study
RGY5988	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-eGFP::LEU2, hog1Δ*	this study

RGY5989	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)-eGFP::LEU2, hog1Δ*	this study
RGY5990	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-3HSV::LEU2, hog1Δ*	this study
RGY5991	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)-3HSV::LEU2, hog1Δ*	this study
RGY5996	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-eGFP::LEU2, gpd1Δ, gpd2Δ*	this study
RGY5997	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)-eGFP::LEU2, gpd1Δ, gpd2Δ*	this study
RGY5998	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-3HSV::LEU2, gpd1Δ, gpd2Δ*	this study
RGY5999	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)-3HSV::LEU2, gpd1Δ, gpd2Δ*	this study
RGY6000	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-3HSV::LEU2, pRG919::URA3, pRG951::MET15*	this study
RGY6001	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-3HSV::LEU2, pRG919::URA3, pRG951::MET15, hog1Δ*	this study
RGY6002	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-3HSV::LEU2, pRG919::URA3, pRG4283::MET15, hog1Δ*	this study
RGY6003	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-3HSV::LEU2, pRG4284::URA3, pRG4283::MET15, hog1Δ*	this study
RGY6235	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8(K735R,K736R,K738R,K748R)-3HSV::LEU2, hog1Δ, gpd1Δ*	this study
RGY6236	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-eGFP::LEU2, pRG919::URA3, pRG951::MET15*	this study
RGY6237	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-eGFP::LEU2, pRG919::URA3, pRG951::MET15, hog1Δ*	this study
RGY6238	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-eGFP::LEU2, pRG919::URA3, pRG4283::MET15, hog1Δ*	this study
RGY6239	*met15Δ0, his3Δ1, ura3Δ0, leu2Δ0, 6His-FLAG-SMT3::HIS3MX6, cyc8Δ::CYC8-eGFP::LEU2, pRG4284::URA3, pRG4283::MET15, hog1Δ*	this study

Plasmid	Encoded protein	Parent vector	Reference
pRG4059	*TUP1-3HA*	pRS406	(Oeser, Amen et al. 2016)
pRG4084	*CYC8-3HSV*	pRS405	(Oeser, Amen et al. 2016)
pRG4085	*CYC8-GFP*	pRS405	(Oeser, Amen et al. 2016)
pRG4113	*CYC8(K735R,K736R,K738R,K748R)-3HSV*	pRS405	(Oeser, Amen et al. 2016)
pRG4175	*HOG1*	pRS416	this study
pRG4186	*HOG1-T174A/Y176F*	pRS416	this study
pRG4188	*HOG1-D144A*	pRS416	this study
pRG4283	*PTDH3-GPP2-3HA*	pRS401	this study
pRG4284	*PTDH3-GPD1-3HA*	pRS406	this study

Curriculum Vitae

Education

B.S. Biochemistry *San Francisco State University*, San Francisco, CA (August 2005 – May 2010)
M.S. Chemistry *San Francisco State University*, San Francisco, CA (August 2010 – July 2012)
Ph.D. Pharmacology *University of Washington*, Seattle, WA (September 2012 – June 2018)

Professional Experience

Research Technician *California Pacific Medical Center Research Institute*, San Francisco, CA (January 2010 – July 2012)
Research Associate *BioNidus*, San Francisco, CA (August 2010 – July 2012)

Research Associate *Genomic Systems LLC*, San Francisco, CA (August 2010 – July 2012)

Laboratory Skills

Molecular Biology – plasmid construction, site-directed mutagenesis, quantitative PCR
Protein Biochemistry – protein purification, SDS-PAGE/Western blotting, immunoprecipitation
Yeast Genetics – yeast strain design and construction, genetic screens, chromatin immunoprecipitation
Cell Biology – fluorescence microscopy, FACS, construction and use of luminescent/fluorescent reporters
Tissue Culture – experience with a variety of mammalian cell lines, primary cells, and stem cells
Animal Husbandry – experience with mouse models and a variety of surgical techniques

Conference Presentations and Invited Talks

"Hog1 regulates hyperosmotic stress-induced protein SUMOylation." Speaker, University of Washington Pharmacological Sciences Training Grant Annual Symposium, Seattle, WA. October 2014.

"Exploring the signaling functions of protein SUMOylation during hyperosmotic stress." Speaker, Annual Symposium on Molecular Pharmacology, Leavenworth, WA. September 2015.

"Osmolyte accumulation regulates SUMOylation kinetics in response to osmotic stress." Poster, FASEB SRC on Ubiquitin and Cellular Regulation, Big Sky, MT. June 2016.

"Osmolyte accumulation regulates SUMOylation kinetics in response to osmotic stress." Poster, FASEB SRC on Protein Folding in the Cell, Saxton's River, VT. July 2016.

"Osmolyte accumulation regulates SUMOylation kinetics in response to osmotic stress." Speaker, UW School of Medicine/Metropolitan University of Tokyo Joint Symposium, Seattle, WA. November 2016.

"Stress-induced osmolyte accumulation regulates the folding state of a native polyQ protein in yeast." Poster, University of Washington, University of Kobe, and University of Oslo Joint Symposium on Molecular Pharmacology, Kobe City, Japan. March 2017.

Publications

Michelle L. Oeser, Triana Amen, **Cory M. Nadel**, Amanda I. Bradley, Benjamin J. Reed, Ramon D. Jones, Janani Gopalan, Daniel Kaganovich, and Richard G. Gardner. *Dynamic SUMOylation of a conserved transcription corepressor prevents persistent inclusion formation during hyperosmotic stress*. PLoS Genet. 2016 Jan 22;12(1)

Benjamin Groves, Arjun Khakhar, **Cory M. Nadel**, Richard G. Gardner, and Georg Seelig. *Rewiring MAP Kinases in Saccharomyces cerevisiae to regulate novel targets through ubiquitination.* eLife 2016;5:e15200

Cory M. Nadel and Richard G. Gardner. *Hyperosmotic stress induced inclusion formation of a prionogenic transcriptional corepressor is regulated by glycerol accumulation.* Manuscript submitted for review.

Teaching

Teaching Assistant – Department of Pharmacology, University of Washington, Seattle, WA. September 2013 – March 2014.

- Advised second year Pharm.D. General Pharmacology 401 and 402 students during office hours
- Led regular weekly discussion sections for students
- Graded exams and assignments

Leadership and Extracurricular Activities

UW Pharmacology Student Association Representative. Fall 2013 – Spring 2015

- Organized quarterly meetings to address student needs or desires to be communicated to faculty
- Attended quarterly faculty meetings to serve as student representative
- Arranged regular meetings of students and postdocs to communicate science in an informal setting
- Organized annual recruitment activities

UW Pharmacology Admissions Committee Member. Fall 2014 – Winter 2015

- Reviewed and scored applications for incoming students
- Interviewed recruits during annual recruitment process

Seattle Ubiquitin Research Group Meeting Organizer. Fall 2016 – June 2018

- Arranged bimonthly meetings of the interdisciplinary Seattle Ubiquitin Research Group