The effects of modulated global sumoylation levels on gene expression

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A dissertation submitted to the faculty of graduate studies in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Graduate Program in Biology

York University

Toronto, Ontario

January 2026

Abstract

Hundreds of proteins are modified by SUMO (small ubiquitin-like modifier) peptides, the majority of which are nuclear and involved in gene expression. Through SUMO chromatin immunoprecipitation sequencing (ChIP-seq), sumoylated proteins can be readily detected at numerous distinct chromatin sites. This includes promoters of protein-coding genes thus linking sumoylation with transcription regulation. Cells can coordinately modulate levels of sumoylation globally. For example, there is a surge in SUMO conjugation in response to heat shock, an effect that we attribute here to the degradation of the major SUMO protease, Ulp1, in budding yeast. Whereas the effects of sumoylation have been examined for many individual target proteins, not much is known about how coordinated global changes to sumoylation levels impact transcription. To address this, we investigated whether changing cellular sumoylation levels in yeast impacts (1) cell growth in normal and stress conditions, and (2) global transcription and gene expression patterns. Primarily, this was accomplished using strains that express mutant forms of Ubc9, the sole E2 conjugating enzyme, or Ulp1, which harbour constitutively reduced or elevated sumoylation levels, respectively. We find that cells with dramatically reduced levels of sumoylation grow near-normally in optimal, non-stress conditions. While they tolerate multiple stress conditions very well, they show strong sensitivity to heat shock specifically. Both reduced and elevated sumoylation levels lead to widespread changes to transcription, but intriguingly, both result in a gene expression pattern that resembles that of stressed cells. Therefore, heat-shock genes have high levels of expression, even in non-stress conditions. This indicates that both sumoylation and desumoylation are important for suppressing stress response gene expression in non-stress conditions, and paradoxically, our results correlate constitutive expression of stress response genes with temperature sensitivity. Finally, our data implicate activation of the key stress response factors, Msn2 and Hog1, in driving the inappropriate induction of heat-shock genes in Ubc9 and Ulp1 mutants even in the absence of stress.

Acknowledgments

I would like to first thank my supervisor Dr. Emanuel Rosonina for the opportunity to join his lab and for his support and encouragement throughout the years. Your approach to mentoring students clearly exhibits your passion for science and teaching. It has been a pleasure to witness that firsthand over the years and to learn from you all aspects of research. Thank you for encouraging my growth as a scientist and recognizing my curiosity. I would also like to thank my committee members, Dr. Yi Sheng and Dr. Mark Bayfield, for your time and support with my research progress. Thank you for the valuable discussions and feedback that helped me improve and learn from your knowledge as well.

My graduate studies would not have been possible without the support of lab members and other members of LSB that I had the honour to work with over the years. Thank you to Ben, Bryan, Su, Mohammad (Sohaib), Anna, Mahtab, Giovanni, Nujhat, Veroni, Yimo, John, Josh, Atara, Kian, Liz, and Suzy. Each of you enhanced my experience working in the lab and I enjoyed our many conversations. Thank you for your help and support to keep going. Thank you to Akhi (May God rest her soul) for teaching me how to do ChIP for the first time. Thank you to Helen, Anna, Jessica, Myat, Sihat, Sehaj, Gagan, Heyam, Loan, Farnaz, Kyra, Brittney, Gaby, Nour, Ivan, Nick, David, Aneesah, and Ayat for all the fun times and support over the years.

I would not have the privilege of being a doctoral student without the sacrifices of my family. Thank you to my parents for being the first people to encourage my curiosity and modelling continuous learning over their lives whether through formal or informal education. Thank you to my brother, Yousef, for your support and for listening and accidently learning about molecular biology through my descriptions.

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List of Abbreviations

APP: amyloid precursor protein

Arg: Arginine

ATP: Adenosine Triphosphate

BCP: bathocuproine

ChIP: Chromatin Immunoprecipitation

ChIP-seq: Chromatin Immunoprecipitation sequencing

CTD: C-terminal heptapeptide repeat domain

DesI1/ DesI2: deSUMOylating isopeptidases1/2

ESR: environmental stress response

GO: Gene Ontology

GTFs: general transcription factors

HDACs: histone deacetylase complexes

HECT: homologous with E6-associated protein C-terminus

HIFs: hypoxia-inducible factors

HOG: high-osmolarity glycerol

HSE: heat shock elements

IGV: Integrative Genomics Viewer

IP: immunoprecipitation

K/Lys: Lysine

kDa: Kilodaltons

lncRNA: long noncoding RNA

miRNA: microRNA

mRNA: messenger RNA

NEM: *N*-ethylmaleimide

NP40: nonidet P-40

NPC: nuclear pore complex

OD: Optical Densities

PABC1: Poly(A)-Binding Protein Cytoplasmic 1

PAGE: polyacrylamide gel electrophoresis

PIC: pre-initiation complex

PKA: protein kinase A

PMSF: phenylmethylsulphonyl fluoride

pre-mRNA: Precursor Messenger RNA

PTM: post-translational modification

RING: really interesting new gene

RNAPII: RNA polymerase II

RNA-seq: RNA sequencing

RPGs: ribosomal protein genes

rRNAs: ribosomal RNAs

S. cerevisiae*: Saccharomyces cerevisiae*

SC: Synthetic Complete

SIM: SUMO interacting motif

snoRNA: Small Nucleolar RNA

snRNA: Small Nuclear RNA

SSTFs: sequence-specific transcription factors

STRE: stress response elements

STUbLs: SUMO-targeted ubiquitin ligases

SUMO: small ubiquitin-like modifier

TFs: transcription factors

tRNA: Transfer RNA

Uspl1: ubiquitin-specific protease-like 1

WT: wild-type

XPC: xeroderma pigmentosum C

YPD: Yeast Peptone Dextrose

Chapter I: Review of Literature

1.1 Sumoylation

Cells can regulate protein activity through post-translational modifications (PTMs). One such modification is sumoylation, a conserved eukaryotic PTM in which the small ubiquitin-like modifier (SUMO) peptide is covalently attached to lysine residues of target proteins (Johnson 2004; Melchior 2000). The process of sumoylation impacts target proteins by regulating their stability, localization, and activity (Zhao 2018; Flotho and Melchior 2013). SUMO is approximately 12 kDa in size, and while there is a single isoform in yeasts and in *C. elegans*, higher eukaryotes such as mammals have multiple SUMO isoforms (Melchior 2000). Smt3, the single SUMO isoform in the budding yeast, *Saccharomyces cerevisiae*, is essential for viability. The unique distinction of the SUMO peptide that distinguishes it from ubiquitin is an intrinsically disordered region at the N-terminus with a size of roughly 20 amino acids (Bayer et al. 1998). This is observed across all species with a SUMO peptide. Sumoylation requires a diglycine motif at the C-terminus of mature SUMO, which is generated when SUMO precursors are cleaved by specific SUMO proteases (Melchior 2000; Flotho and Melchior 2013). Sumoylation typically occurs at lysine residues within a consensus motif, ΨKx(D/E), where Ψ refers to a large hydrophobic amino acid and x can be any amino acid (Rodriguez et al. 2001). However, lysines outside this motif can also be modified, though it remains unclear how such sites are recognized by the SUMO machinery.

In mammalian cells, sumoylation occurs through the same core mechanism as in yeast. However, while yeast expresses a single SUMO isoform, mammals produce four isoforms: SUMO1, SUMO2, SUMO3, and SUMO4. SUMO2 and SUMO3 share 97% similarity in sequence, whereas SUMO1 is only ~50% similar to SUMO2/3 (Tatham et al. 2001). In contrast to the other isoforms, SUMO4 shows tissue-specific expression, being most abundant in kidney

cells, and it shares 87% similarity with SUMO2/3 (Bohren et al. 2004). SUMO peptides are highly conserved across eukaryotes. For example, human SUMO1 can complement yeast Smt3, and the two proteins share over 50% similarity in sequence (Newman et al. 2017). Yeast Smt3 is itself preferentially sumoylated at lysine residues K11, K15, and K19 (Bylebyl et al. 2003), whereas in mammals, SUMO2, SUMO3, and SUMO4 are predominantly sumoylated at K11.

1.1.1 Sumoylation Pathway

The process of sumoylation is similar to ubiquitination where there are E1 activating enzymes, E2 conjugating enzymes, and E3 ligases to allow for the modification of target proteins (Johnson 2004; Melchior 2000; Flotho and Melchior 2013) (Figure 1). The E1 activating enzyme is a dimeric protein that activates the SUMO peptide by using ATP to form thioester bonds with the peptides. In yeast, the two E1 activating enzyme subunits are Aos1 and Uba2 (Johnson 1997). In mammalian systems, the E1 subunits are homologous with the *S. cerevisiae* forms and they are also called AOS1 and UBA2, although they sometimes referred to as SAE1 and SAE2, respectively (Okuma et al. 1999). The human AOS1 and UBA2 share roughly 33% similarity to the yeast orthologs (Melchior 2000). Aos1 and Uba2 have to be both present to form an active E1 enzyme as the two proteins work together and are not redundant in function.

Unlike ubiquitination where there are multiple E2 conjugating enzymes, there is only one SUMO E2 conjugase across species, called Ubc9 (Seufert et al. 1995; Melchior 2000). Also, unlike ubiquitination, in vitro, Ubc9 alone can catalyze the attachment of SUMO peptides to target proteins without other enzymes of the sumoylation pathway. In vivo, however, E1 and E3 components are also needed (Streich and Lima 2014). Because it is required for SUMO conjugation of target proteins, Ubc9 is essential in budding yeast and at specific developmental stages in mammalian cells (Hayashi et al. 2002). Yeast Ubc9 shares 56% similarity in protein

sequence with the human form, and whereas it is mostly localized to the nucleus, a low level of Ubc9 can be found in the cytoplasm (Watanabe et al. 1996; Melchior 2000). There are many structural similarities between Ubc9 in *S. cerevisiae* and ubiquitin E2 conjugating enzymes Ubc4 and Ubc7, where the similarity is roughly 35% (Giraud et al. 1998). However, Ubc9 contains more positive charges leading to more compatibility with SUMO peptides which is more negatively charged than ubiquitin, leading to specificity between the structurally similar E2 conjugating enzymes of ubiquitin and SUMO to their respective PTM (Liu et al. 1999). Compared to ubiquitination, having one SUMO E2 conjugating enzyme across species could also be why sumoylation is a less common PTM found in cells.

In vivo, E3 ligases are generally required to promote SUMO conjugation to target proteins in cooperation with Ubc9, and they are thought to confer substrate specificity to this process. In yeast, the major E3 ligases include Siz1, Siz2, and Mms1 (Flotho and Melchior 2013; Seeler and Dejean 2003), whereas in humans, there is a broader range, including PIAS1-4 and RanBP2 (Pichler et al. 2004; Rytinki et al. 2009). E3 ligases facilitate SUMO transfer from Ubc9 to the target protein through various mechanisms, including acting as a scaffolds that bring Ubc9 and the substrate into proximity (Gareau and Lima 2010). In budding yeast, Siz1, Siz2, and Mms1 each modify distinct sets of target proteins, although some substrates are shared among these E3 ligases (Reindle et al. 2006).

1.1.2 SUMO Proteases

The process of sumoylation is transient and reversible (Flotho and Melchior 2013; Gareau and Lima 2010). SUMO proteases, also known as desumoylases, are needed for both cleaving precursor forms of SUMO peptides and for removing SUMO from target proteins. In budding yeast, there are two SUMO proteases, Ulp1 and Ulp2, that cleave Smt3 peptides from

conjugated proteins. Ulp1 is essential for viability, while cells can tolerate the absence of Ulp2. (Li and Hochstrasser 1999; 2000). Both SUMO proteases localize to the nucleus, while Ulp1 is specifically enriched at the nuclear envelope and nuclear pore complex (Ryu et al. 2019). Cells with a mutation of *ULP1*, *ulp1-I615N* or *ulp1-mt*, impairs the activity of Ulp1 by impacting the hydrophobic C-terminal domain of Ulp1 and potentially impacting the folding of this region of the protein without impacting its catalytic activity.The *ulp1-mt* cells can still survive however exhibit growth defects, impairments in meiosis, and sensitivity to mild heat shock at 37°C (Soustelle et al. 2004). There is also an accumulation of sumoylated proteins in these cells. When de novo sumoylation events in yeast are rapidly stopped by inhibiting the yeast E1 enzyme with the drug 1,10-phenanthroline, Ulp1 removes almost all existing SUMO conjugations within minutes (McNeil et al. 2024). This indicates that there is normally a rapid turnover of sumoylation which likely enables cells to dynamically adjust global sumoylation levels in response to changing conditions and environment signals (Flotho and Melchior 2013).

SUMO proteases in eukaryotic cells also show homology. In mammalian cells, there are six SUMO proteases called sentrin-specific proteases, SENP1-3, SENP5-7 (Hickey et al. 2012). There are also recently discovered SUMO proteases called deSUMOylating isopeptidases 1 and 2 (DesI1 and DesI2) and ubiquitin-specific protease-like 1 (Uspl1). These proteases are thought to have very specific targets and are relatively low in abundance. The catalytic domains of SENP1-5 share similarity among themselves and with the *S. cerevisae* Ulp1 (Kim and Baek 2009). Meanwhile, SENP6 and SENP7 share similarity with each other and with *S. cerevisae* Ulp2. The two groups of SUMO proteases only share less than 30% similarity and so are grouped this way due to the structural similarities.

1.1.3 SUMO Chain Formation

Like many SUMO targets, SUMO peptides themselves can be sumoylated, forming polySUMO chains in a process referred to as polysumoylation (Flotho and Melchior 2013). This is true for Smt3 in budding yeast and all of the SUMO isoforms in human cells except for SUMO1 which is only sumoylated at non-consesus residues at lower efficiencies which reduces its ability to form polySUMO chains (Tatham et al. 2001). The Smt3 isoform in budding yeast can be sumoylated at lysine residues K11, K15, K19. The human SUMO2-4 peptides can only be sumoylated at K11. In *S. cerevisiae*, Ulp1 primarily removes single SUMO moieties, whereas Ulp2 is thought to deconjugate SUMO from polysumoylated proteins (Bylebyl et al. 2003). In *ulp2*Δ strains where the *ULP2* gene is knocked out, an increase in polysumoylated proteins is observed. This leads to slow growth of these cells compared to wild-type strains and they also exhibit abnormalities in chromosome replication indicating the distinct role polysumoylated chains play in the cell.

Interestingly, different SENPs display distinct preferences for mono-versus polysumoylated substrates (Kim and Baek 2009). SENP1-5 favour removing conjugated SUMO1, which is typically found in monosumoylated form, consistent with the activity of their yeast homolog Ulp1. On the other hand, SENP6 and SENP7 preferentially act on SUMO2/3-modified proteins and lack precursor SUMO processing activity. Because SUMO2/3 are commonly found in polysumoylated chains, this activity parallels that of Ulp2, which also targets polysumoylated targets (Hickey et al. 2012).

1.1.4 Crosstalk between sumoylation and other PTMs

Beyond sumoylation, a wide range of other PTMs also regulate protein activity, including, but limited to, ubiquitination, phosphorylation, methylation, and acetylation. These modifications are often coordinated to modulate protein function (Boulanger et al. 2021). Such

crosstalk can occur through multiple mechanisms, including competition at target residues, consensus motifs that specifically recruit multiple PTMs, to polypeptide formations involving a combination of PTMs that can serve as a signal that can recruit relevant proteins. Crosstalk between sumoylation and other PTMs provides an extra layer of regulation that can help coordinate cellular responses in different circumstances and provides another way to enhance the specificity of the downstream effects on target proteins.

1.1.4.1 Phosphorylation

One of the most studied PTMs is phosphorylation in which phosphate groups are covalently attached to target proteins, typically at serine, threonine, or tyrosine residues in eukaryotes (Cohen 2002). Phosphorylation, which is catalyzed by kinase enzymes, can alter a protein's activity by either activating or inhibiting its function, and is reversible through the action of phosphatases, which remove the phosphate groups. This modification is highly prevalent and plays a central role in many signalling cascade pathways, where phosphorylation events are essential for triggering downstream components the pathway (Ardito et al. 2017). Such pathways are critical for regulating processes including cell division, signal transduction, development, and growth.

Given the extensive range of proteins that are phosphorylated, it is not surprising that there is an overlap between phosphorylation and sumoylation events. A common mechanism by which this crosstalk occurs is through sumoylation target sequences that include amino acid residues that can be phosphorylated. For example, an extended version of the standard SUMO motif (ψKx(D/E) to ψKx(D/E)xxSP, which includes a serine residue which can become phosphorylated, has been identified in many proteins, and was found to enhance the addition of the SUMO residue after the phosphorylation event takes place (Yang and Grégoire 2006). This

type of phosphorylation-dependent sumoylation has been observed in multiple pathways, but is more commonly detected on nuclear proteins, including mammalian transcription factors KLF8, MEF2, HSF1, and GATA1 (Hietakangas et al. 2006).

There are also instances where phosphorylation inhibits sumoylation, although these are less well characterized than phosphorylation-dependent sumoylation events. One example is the tumour suppressor transcription factor p53, in which phosphorylation impedes its interaction with UBC9, and thereby blocks its subsequent sumoylation (Müller et al. 2000). In other cases, phosphorylation is thought to expose lysine residue to SUMO proteases promoting faster SUMO removal and thus preventing the protein from remaining sumoylated (Yang et al. 2003).

1.1.4.2 Ubiquitination

Another common PTM is ubiquitination, a process that is mechanistically similar to sumoylation, in which a ubiquitin peptide is covalently attached to lysine residues on target proteins through the sequential action of E1 activating, E2 conjugating, and E3 ligase enzymes (Hershko and Ciechanover 1998). However, ubiquitination is more complex, involving multiple E2 conjugating enzymes and over 600 E3 ligases. These E3 ligases can be categorized as either containing RING (really interesting new gene) domains or HECT (homologous with E6-associated protein C-terminus) domains (Ebadi et al. 2025). HECT-containing ligases mediate ubiquitin transfer in two steps. First, ubiquitin is transferred from the E2 enzyme to an active-site cysteine residue on the HECT ligase through a transthiolation. Then, the ubiquitin-loaded HECT ligase binds to the target protein and attaches the ubiquitin through an isopeptide bond to a lysine residue on the target. RING-containing ligases, however, bind to both E2 conjugating enzymes and target proteins at the same time and transfer the ubiquitin in a single step (Deshaies and Joazeiro 2009).

The ubiquitin polypeptide is 76 amino acid residues long, including seven lysine residues that themselves can be ubiquitinated, resulting in polyubiquitin chains (Hershko and Ciechanover 1998). Monoubiquitination, the attachment of a single ubiquitin molecule to a target protein, often regulates protein trafficking and function, whereas polyubiquitination serves as a signal for other intracellular processes. In particular, polyubiquitination through lysine residue 48 of ubiquitin targets proteins for degradation by the 26S proteasome. This ubiquitin-proteasome pathway is well studied and is a key focus of therapeutic drug development (Deshaies and Joazeiro 2009; Pickart and Fushman 2004). Given the mechanistic similarities between the ubiquitination and sumoylation pathways, and the presence of multiple lysine residues on both SUMO and ubiquitin peptides, multiple studies have uncovered extensive crosstalk between these two PTMs.

One type of crosstalk between sumoylation and ubiquitination is mediated through SUMO-targeted ubiquitin ligases (STUbLs). STUbLs, which are conserved among eukaryotes, recognize specific sumoylated substrates and mediate their subsequent ubiquitination (Kumar et al. 2017. All STUbLs contain at least one SUMO interacting motif (SIM), which is necessary for binding to their targets, and they contain a RING domain (Sriramachandran and Dohmen 2014) . STUbLs were first discovered in *S. cerevisiae* where a yeast two-hybrid assay identified E3 ubiquitin ligases that interacted with SUMO (Uzunova et al. 2007). One STUbL identified this way is the Uls2 complex, which consists of Slx5 and Slx8 RING domain-containing subunits (Uzunova et al. 2007). When either Slx5 or Slx8 is mutated in combination with mutations of SUMO conjugation pathway components, the cells are not viable. The Slx5-Slx8 complex specifically recognizes and attaches ubiquitin to polySUMO chains, a process that is mediated by the ubiquitin E2 conjugation enzyme Ubc4, which facilitates the targeted termination of

polySUMO chains (Uzunova et al. 2007). This example illustrates the functional crosstalk between sumoylation and ubiquitination, where one modification pathway signals for the action of the other, to modulate protein activity through either degradation, relocation, or change in protein binding.

STUbL-mediated ubiquitination of sumoylated substrates creates a combination of PTMs that influence the activity and stability of target proteins. A common outcome of a STUbL activity is the promotion of target protein degradation. Many transcription-related proteins are sumoylated, and the STUbL RNF4 has been implicated in the ubiquitination and subsequent degradation of several transcription factors (Sriramachandran and Dohmen 2014). For example, during hypoxic conditions, hypoxia-inducible factors (HIFs) such as HIF1α and HIF2α are sumoylated in mammalian cells (van Hagen et al. 2010). These transcription activators, which play key roles in fetal development and tumour angiogenesis, exhibit increased stability and transcriptional activity upon sumoylation. In RNF4-deficient cells, HIF1α and HIF2α accumulate in polySUMOylated forms conjugated with SUMO2 and SUMO3, demonstrating that RNF4 regulates HIF levels and, consequently, transcriptional responses to hypoxia (van Hagen et al. 2010).

Polyubiquitin chains do not always target proteins for degradation through the 26S proteosome. In particular, polyubiquitin chains formed through modification of lysine 63 residues on ubiquitin serve as regulatory signals for diverse cellular processes. Poulsen et al. (2013) showed that the STUbL RNF111 ubiquitinates specific sumoylated proteins involved in the DNA damage response. One such substrate is XPC (xeroderma pigmentosum C), a key DNA damage sensor required for nucleotide excision repair. Upon UV-induced DNA damage, XPC becomes sumoylated, after which RNF111 catalyzes its ubiquitination. Ubiquitinated XPC

promotes its recruitment to damaged chromatin, facilitating efficient nucleotide excision repair. These findings reveal that RNF111 mediates non-proteolytic ubiquitination and demonstrate another regulatory layer between sumoylation and ubiquitination.

1.2 Regulation of multiple cellular pathways by SUMO

More than 3000 proteins have been identified as targets of sumoylation in humans and over 1000 in *S. cerevisiae* (Hendriks and Vertegaal 2016). Targets of sumoylation are involved in various cellular pathways including transcription, mRNA export, ribosome biogenesis and assembly, translation, cell division, DNA replication, and chromatin remodelling (Hendriks and Vertegaal 2016; Makhnevych et al. 2009) (Figure 2). Most SUMO targets, as well as components of the sumoylation pathway are localized in the nucleus and frequently associated with chromatin (Seeler and Dejean 2003; Baig et al. 2021). Although cytoplasmic SUMO targets have been identified, they are considerably fewer than nuclear ones. Certain SUMO targets only appear at specific times stages of cell cycle. For example, septins are sumoylated only during mitosis (Ribet et al. 2017). Identifying all SUMO targets through proteomic approaches remains challenging, as only a small fraction of proteins are sumoylated under optimal growth conditions. However, the extent of sumoylation varies in response to environmental or physiological changes, and many studies have examined SUMO targets under different stress or growth conditions (Enserink 2015; Tempé et al. 2008). Collectively, these observations indicate that sumoylation is a highly regulated and dynamic process that modulates a broad range of biological pathways in a spatially and temporally controlled manner and facilitates adaptation to changing conditions.

1.2.1 Transcription

Transcription by RNA polymerase II (RNAPII) is a fundamental cellular process that converts genetic information stored in DNA into RNAs required for all aspects of cell function (Compe and Egly 2021; Archuleta et al. 2024). Because transcription is resource and energetically demanding, it is tightly regulated in all living organisms. Cells have systems in place to monitor external and internal environmental changes and adjust transcription accordingly (Lee and Young 2013). RNAPII is responsible for producing all protein-coding RNAs (mRNAs) and many non-coding RNAs (Compe and Egly 2021). Proper transcription requires the coordinated action of numerous proteins, including components of the pre-initiation complex (PIC) that enable RNAPII to bind promoters and initiate transcription. Regulation can occur at multiple levels, for example through transcription factors binding to chromatin or through the activity of chromatin remodelling factors.

Although a broad range of proteins have been shown to undergo sumoylation the largest fraction of SUMO targets are involved in transcription and gene expression (Boulanger et al. 2021; Makhnevych et al. 2009). This includes subunits of RNAPII, general transcription factors (GTFs), chromatin remodelling factors, and hundreds of sequence-specific transcription factors (Baig et al. 2021; Makhnevych et al. 2009; Rosonina et al. 2017). While it is now recognized that the effects of sumoylation on transcription are highly target- and gene-specific, the modification was initially linked to transcriptional repression through several mechanisms. For instance, sumoylation of some transcription factors can inhibit their activity by altering their localization, promoting their degradation, or through the recruitment of other factors to repress gene expression. At the chromatin level, sumoylation facilitates the recruitment of histone deacetylase complexes (HDACs) which remove activating histone acetyl marks, leading to gene repression (Gill 2005; Ouyang and Gill 2009).

1.2.1.1 RNAPII and the PIC

RNAPII is responsible for transcribing all protein-coding genes as well as some non-protein coding genes including snoRNAs, snRNAs, miRNAs (not found in *S. cerevisiae*), and lncRNAs (Compe and Egly 2021). RNAPII is comprised of 12 subunits and its largest subunit, Rpb1, contains a large C-terminal heptapeptide repeat domain (CTD) that is subject to dynamic phosphorylation as RNAPII moves along gene bodies during transcription (Phatnani and Greenleaf 2006). A study by Chen et. al (2009) was first the first to report that *S. cerevisiae* RNAPII becomes sumoylated during UV radiation-induced DNA damage. During DNA damage, elongating RNAPII complexes typically stall at lesions, and these polymerases must be removed to allow repair and transcription recovery. Rpb1 species, specifically those that are phosphorylated on Ser2 residues of the CTD repeat, which is a marker of transcription elongation, were found to become sumoylated upon DNA damage (Heckmann et al. 2019). This rapid increase of Rpb1 sumoylation followed by its ubiquitination by the STUbL Sxl5-Slx8, leading to Rpb1 degradation by the 26S proteosome. Together, these findings demonstrate that the largest subunit of RNAPII can be sumoylated and reveal a specific context in which sumoylation plays a vital role in the cellular response to DNA damage.

More generally, sumoylation has been found to play diverse, context-dependent roles in regulating transcription by RNAPII. Prior to initiating transcription, RNAPII assembles with a set of general transcription factors (GTFs) to form a pre-initiation complex (PIC) at active promoters (Compe and Egly 2021). Mutant yeast with defective Ubc9, and therefore decreased overall sumoylation, exhibit reduced RNAPII levels at constitutive promoters, but they also show inefficient shut-down of activated inducible genes, reflecting how sumoylation can play both positive and negative roles in gene expression (Rosonina et al. 2010). Identifying specific targets

of sumoylation within the general transcription machinery will be necessary for understanding how SUMO's individual impacts affect transcription more generally. Along these lines, multiple GTF components have been identified as SUMO targets through proteomic analyses sumoylated proteins are found at promoter regions of actively transcribed genes (Boulanger et al. 2021; Makhnevych et al. 2009). In particular, TFIIF was found to be a major target of sumoylation in yeast (Baig et al. 2021). Notably, both decreasing and increasing TFIIF sumoylation levels were found to reduce RNAPII levels at promoter regions genome-wide. This highlights that the controlled timing of both SUMO conjugation and deconjugation is crucial for its regulation of transcription in normal and stress conditions.

1.2.1.2 Gene-specific transcription factors

In addition to targeting components of the general transcription machinery, hundreds of gene-specific transcription factors (TFs), including most human TFs, have been identified as SUMO targets (Chymkowitch, Nguéa P, and Enserink 2015; Hendriks et al. 2017; Rosonina et al. 2017). In dozens of studies, impairing sumoylation of specific TFs was found to elevate expression of their target genes, suggesting a general negative regulatory function for TF sumoylation (Rosonina et al. 2017; Gill 2005; Traboulsi et al. 2019). In some cases, however, sumoylation is linked with enhanced TF activity. One example is the transcription factor Hsf1 which, in mammalian systems, is sumoylated during heat shock (Hong et al. 2001). Impairing Hsf1 sumoylation prevented its localization to nuclear stress granules and significantly decreased its ability to induce expression of a reporter gene. Another TF that is positively regulated by sumoylation is the yeast Rap1 which activates translation-related and ribosomal protein genes (Chymkowitch et al. 2015). Sumoylation of Rap1 was shown to be required for the efficient

recruitment of the transcription machinery and subsequent activation of its target genes. These examples highlight the versatility of sumoylation as a context-dependent regulator of TF activity.

Besides examining how sumoylation impacts TF activity, some studies have explored whether sumoylation affects the association of target TFs with chromatin. Sko1, a yeast TF with hundreds of target genes, is sumoylated at Lys 567 (Sri Theivakadadcham et al. 2019). In a ChIP-seq study, it was found that mutating this residue to non-sumoylatable Arg resulted in a dramatic increase in the number of Sko1 binding sites genome-wide. This suggested that sumoylation functions to ensure the binding specificity of Sko1 by restricting it to appropriate sites. ChIP-seq studies in human systems also found that impairing sumoylation of certain TFs also resulted in a significant increase in genome-wide binding sites, implying that a role for TF sumoylation in binding site selection is evolutionarily conserved (Rosonina 2019). In another example, sumoylation was found to promote the dissociation of chromatin-bound Gcn4, a yeast TF that functions during nutrient starvation stress (Akhter and Rosonina 2016; Rosonina 2019). Both Sko1 and Gcn4 were found to become sumoylated only after they bind DNA, suggesting that sumoylation acts as a post-DNA-binding regulatory mechanism that fine-tunes TF occupancy to ensure both specificity and timely removal from chromatin.

Targeting sumoylation in a protein-specific manner can be used in a therapeutic manner. One example of this studying the impact of sumoylation in treating breast cancer. ERα is a transcriptional factor responsible for driving breast cancer tumour progression. One possible therapy for breast cancer patients is administering antiestrogen treatments such as fulvestrant. New research has identified that the treatment of fulvestrant leads to the degradation of ERα through increasing its sumoylation which then leads to ubiquitin-targeted proteosome degradation (Vallet et al. 2023). The ability of fulverestrant to induce sumoylation is through the

recruitment of E3 ligases. The sumoylation of ERα was found to occur after its binding to chromatin which then led to its disassociation to chromatin (Traboulsi et al. 2019). Interestingly, in cells with a decreased level of global sumoylation, there was increased binding of ERα to chromatin signifying the importance of sumoylation of regulating transcription factor binding.

1.2.1.3 Histone modifications

In order for transcription to occur, not only must RNAPII and the PIC assemble at promoters, but the underlying chromatin must also be accessible to allow transcription factors and other proteins to interact with DNA (Li et al. 2007). Chromatin accessibility is regulated through a variety of histone PTMs that alter nucleosome structure and recruit effector proteins (Kouzarides 2007). Histones can be modified by acetylation, methylation, ubiquitination, phosphorylation, and sumoylation, each of which can have distinct effects on transcription activity. For example, acetylation of histone lysine residues generally correlates with transcription activation, while other modifications can either promote or repress gene expression depending on their context.

Histone sumoylation has been observed in both human and budding yeast cells (Ryu and Hochstrasser 2021). Although it was initially associated with transcription repression, more recent studies indicate that its effects are context-dependent and influenced by the specific site of modification. For example, in budding yeast, sumoylation of H2B was found to repress transcription, since the substitution of four sumoylated lysine residues with alanines increased transcription (Nathan et al. 2006). Further experiments confirmed this finding by linking an increase in sumoylated H2B to a decrease in histone acetylation at sites that are needed for active transcription, suggesting that sumoylation can counteract the establishment of transcriptionally active chromatin.

Modification of target proteins with SUMO can alter their function, often by modulating interactions with other proteins (Flotho and Melchior 2013; Hendriks and Vertegaal 2016; Chymkowitch, Nguéa P, and Enserink 2015). One way of achieving this is through the interaction of the conjugated SUMO peptide with a SUMO interaction motif (SIM) on the interacting proteins (Kerscher 2007). SIMs contains a hydrophobic core that interacts with a hydrophobic pocket on SUMO peptides. These SUMO-SIM interactions have been identified in both yeast and mammalian systems but have been more thoroughly studied in mammalian systems (Song et al. 2004). One example of sumoylation regulating the interaction of a protein with another through a SIM is the recruitment of histone deacetylases (HDACs) via sumoylated transcription factors. The sumoylation of the p68 transcription factor was shown to facilitate recruitment of SIM-containing HDACs, which leads to transcription repression of p68's target genes (Jacobs et al. 2007). When p68 is not sumoylated however, HDACs are not recruited to the promoter region and do not interact with p68. This example highlights how sumoylation can indirectly impact transcription by regulating histone modifications.

1.2.2 pre-mRNA processing

Sumoylation levels also impact other biological pathways such as pre-mRNA processing including splicing. The processing of 3'end of pre-mRNAs is an important step to produce stable mRNAs. When components of the processing complex are no longer sumoylated, 3'end processing is disturbed in mammalian cells (Vethantham et al. 2007). Transcription and mRNA processing typically occur in coordination with one another and are controlled by the C-terminal domain (CTD) of the largest subunit of RNAPII which recruits different factors as needed. Therefore, sumoylation of proteins involved in modifying any of the proteins involved in either

modifying the CTD or either pathway of transcription or pre-mRNA processing (Mapendano et al. 2010).

Additionally, splicing factors are also known to be sumoylated (Pozzi et al. 2018). Specifically, sumoylated forms of these spliceosome proteins were found to present in active spliceosome complexes. Interestingly, the levels of sumoylation increases when splicing occurs in these complexes indiciating sumoylated proteins are present during splicing. In human cells lines, when SENP1 was inhibited, splicing was also found to be inhibited as well. This indicates that normal sumoylation cycling is needed to assemble the spliceosome, activate splicing activity, and potentially also regulate alternative splicing pathways.

1.2.3 mRNA export

Another process in which sumoylation plays a role is the mRNAs export pathway. In this process, mRNAs are exported from the nucleus to the cytoplasm through the nuclear pore complex (NPC) in a manner that depends on transcription and pre-mRNA processing levels(Lin and Hoelz 2019). The NPC is composed of multiple nucleoporins, and on the nuclear side there is a nuclear basket that prepares and selects mRNAs to be exported. In budding yeast, the NPC is made up of five nucleoporins, Nup1, Nup2, Nup60, Mlp1, and Mlp2 (Singh et al. 2024). Nup60 and Nup2 were both found to be sumoylated at various stages of the cycle and during stress conditions such as osmotic stress (Folz et al. 2019). The exact impact of the sumoylated form of Nup60 and Nup2 remain to be determined, however.

Interestingly, the budding yeast SUMO protease Ulp1 is localized to the NPC on the nuclear side associated with the nuclear basket structure (Palancade and Doye 2008). Nup60 along with Mlp1 and Mlp2 play a role in maintaining the integrity of this structure. In the

absence of Nup60, Mlp1 and Mlp2 dissociate from the NPC along with Ulp1, which is subsequently degraded through the proteosome (Zhao et al. 2004). In a recent study, it was shown that disassociation of Ulp1 from the NPC leads to in turn the degradation of the SUMO E3 ligases Siz1 and Siz2 (Ptak et al. 2025). This coordinated loss of a SUMO protease and SUMO ligases is thought to maintain balanced global levels of protein sumoylation. Therefore, under normal conditions, Nup60-dependent localization of Ulp1 and the NPC contributes to the stability of Siz1 and Siz2 and helps to preserve proper sumoylation homeostasis.

1.2.4 Ribosome Biogenesis

Ribosomes are composed of ribosomal RNAs (rRNAs) and ribosomal proteins (Thomson et al. 2013). The rRNAs are transcribed in the nucleolus, while ribosomal proteins are translated in the cytoplasm, then transported back into the nucleus and delivered to the nucleolus for assembly into ribosome subunits. The ribosome consists of two main subunits, 40S and 60S. Proper ribosome assembly requires coordination among multiple pathways, including transcription of rRNA and ribosome protein genes (Nosrati et al. 2014). Transcription of small nucleolar RNAs (snoRNA) is also critical, as snoRNAs play essential roles ensuring proper rRNA folding and structure.

Multiple biological pathways act in coordination to ensure that each step of ribosome biogenesis proceeds correctly, and PTMs play crucial roles in regulating these pathways. Sumoylation has recently been identified as vital for this process (Ryu 2022). It contributes to the regulation of gene expression for both rRNAs and ribosomal protein-coding genes, and has been proposed to prevent their uncontrolled expression (Neyret-Kahn et al. 2013). However, other studies indicate that sumoylation can positively regulate the transcription of ribosomal protein genes through the transcription factor Rap1 (Chymkowitch, Nguéa P, Aanes, et al. 2015).

Furthermore, loss of the SUMO protease Ulp2 results in overexpression of genes associated with ribosome biogenesis, highlighting the importance of sumoylation in maintaining balanced gene expression critical for proper ribosome production (Ryu et al. 2019).

Beyond regulating gene expression, sumoylation also plays an important role in ribosome assembly. snoRNAs form complexes with nucleolar proteins to facilitate this process (Li et al. 2025). One such protein, Nop58, must be sumoylated to form a complex with snoRNAs and to remain localized in the nucleolus (Westman et al. 2010). In the absence of this modification, Nop58 is targeted for proteasomal degradation. In mammalian systems, loss of the SUMO proteases SENP3 and SENP5 leads to defects in rRNA processing (Dönig et al. 2023). Several other nucleolar proteins have also been found to undergo sumoylation, which is essential for their function. Moreover, studies on Ulp1 have revealed that sumoylation of preribosomal-associated proteins is required for proper 60S subunit assembly, while subsequent removal of SUMO is necessary for export of the assembled ribosomal subunits to the cytoplasm (Panse et al. 2006).

1.2.5 Translation

Translation of mRNAs into proteins occurs in the cytoplasm and represents one of the most energy-demanding processes in the cell (Sonenberg and Hinnebusch 2009). As described in previous sections, translation depends on several interconnected pathways, including transcription, mRNA export, and ribosome biogenesis, to ensure that all required components are available. Translation proceeds through three main stages, initiation, elongation, and translation. Among these, translation initiation is a highly regulated step that requires the coordinated action of translation initiation factors (Jackson et al. 2010). These factors form complexes that recruit mRNAs to ribosomes for translation. The eIF4F complex, composed of the cap-binding protein

eIF4E, the helicase eIF4A, and the scaffolding protein eIF4G, plays a central role in this process. In addition, a variety of other proteins act to regulate the activity and assembly of this complex.

Sumoylation also contributes to the regulation of translation initiation (Watts et al. 2014). Recent proteomic studies have revealed that many proteins involved in this process are sumoylated, although the functional consequences of these modifications are yet to be determined (Matafora et al. 2009). One component of the eIF4F complex, eIF4A, is sumoylated, and in mammalian cells, sumoylation of the eIF4A1 and eIF4A2 subunits is predicted to facilitate mRNA unwinding by removing secondary structures. Another study found that eIF4E in mammalian cells is sumoylated by SUMO1, leading to the activation of the eIF4F complex and enhanced cap-dependent translation (Xu et al. 2010). Overall, sumoylation appears to play a positive role in promoting translation initiation, though further work is needed to fully elucidate its mechanistic contributions.

1.3 Global changes in sumoylation levels

At least in part due to the continuous activity of SUMO proteases, SUMO modifications are highly transient under normal growth conditions, resulting in low overall levels of sumoylation. However, when human, yeast, or plant cells are exposed to certain stress conditions, there is a rapid increase in sumoylation levels (Enserink 2015; Lewicki et al. 2015; Tempé et al. 2008). These stressors include osmotic stress, heat shock, ethanol stress, and exposure to compounds that generate reactive oxygen species. This phenomenon, characterized by a global rise in sumoylation, is known as the SUMO stress response. Although this increase in sumoylation has been well documented, little is known about its functional role or impact on the cell. Interestingly, in many cases, exposure to stress also leads to a general reduction in

transcription, as the cell redirects its resources to the stress response (Himanen and Sistonen 2019).

1.3.1 Budding yeast

A key study examining global changes in sumoylation in response to stress was conducted by Lewicki et. al (2015). This study revealed that the SUMO stress response is rapid and dynamic. By comparing the types of proteins sumoylated under different stress conditions, the investigators identified a common core set of proteins that were consistently sumoylated regardless of the type of stress. Many of these proteins are involved in transcription, including components associated with the TFIID subunit of the general transcription machinery, the Mediator complex, and chromatin remodelling factors. These results were also confirmed in another study by Nguéa P *et al*. (2019) and they identified further proteins which exhibited changes in sumoylation during different stress conditions. This included certain subunits of general transcription factors for RNAPI, RNAPII, and RNAPIII becoming desumoylated during stress while the TFIID subunits showed an increase in sumoylation. This finding further strengthens the connection between sumoylation and the regulation of transcription. Interestingly, this rapid increase in sumoylated proteins was not dependent on the 26S proteasome-mediated degradation or changes in levels of ubiquitination (Lewicki et al. 2015). Inhibition of translation also did not affect the rise in global sumoylation, whereas inhibition of transcription prevented it. Together, these results suggest that that observed increase in sumoylated proteins during stress is not due to new protein synthesis or degradation but is tightly linked to active transcription in the cell.

Other studies have sought to distinguish roles of polysumoylated and monosumoylated proteins under stress conditions. In one such study, a yeast strain was engineered so that all

lysine residues in the SUMO peptide were replaced with arginine, preventing the formation of SUMO chains (Srikumar et al. 2013). When exposed to osmotic stress, these mutant cells still exhibited increased mRNA levels of stress response genes comparable to wild-type strains, indicating that polysumoylation is not essential for the stress response and that monosumoylation alone is sufficient to trigger it. However, cells lacking the ability to form SUMO chains displayed elevated basal transcription of stress-related genes, highlighting the importance of normal sumoylation in maintaining proper transcription regulation even under non-stress conditions.

1.3.2 Mammals

In mammalian systems, regulation of SUMO levels is important for survival under varying environmental conditions. For example, animals undergoing hibernation experience reduced blood oxygen levels in the brain (Frerichs and Hallenbeck 1998), a condition similar to that observed in humans experiencing a stroke. In hibernating squirrels, elevated levels of sumoylation were detected in the brain, liver, and kidney, suggesting a protective role under low oxygen and glucose conditions (Lee et al. 2007). To test whether this phenomenon extends to humans, researchers expressed a mutant form of Ubc9 in human nerve cells, which rendered them unable to survive conditions of low glucose and oxygen. These findings demonstrate the importance of elevated SUMO levels as a protective mechanism during environmentally unfavourable conditions.

Global changes in SUMO levels are also associated with numerous human diseases, including cancers, heart disease, neurodegenerative diseases, and autoimmune disorders (Han et al. 2018; Xiao et al. 2021; Shetty et al. 2020; Mandel and Agarwal 2022). A notable example is Alzheimer's disease, which leads to dementia and is characterized by the accumulation of

amyloid plaques in brain cells (Tiraboschi et al. 2004). The proteins responsible for forming these plaques, amyloid precursor protein (APP) and tau, are both SUMO targets (Hoppe 2015). In both mice and humans, during the pre-plaque stages of Alzheimer's disease, global sumoylation levels increase in the brain cells, particularly through SUMO1 modification (Maruyama et al. 2018). Interestingly, as the disease progresses with worsening neurodegenerative symptoms, global sumoylation levels return to baseline during the plaque phase of the disease. These observations suggest that monitoring changes in sumoylation could aid in early diagnosis and provide insights into how the disease progresses.

1.3.3 Plants

Changes in global sumoylation levels under stress conditions are also observed in other species. In *Arabidopsis*, global sumoylation increases under various stresses, including drought, high salinity, heat shock, and oxidative stress (Kurepa et al. 2003). During heat shock, *Arabidopsis* cells exhibit genome-wide redistribution of sumoylated proteins bound to chromatin (Han et al. 2021). Specifically, sumoylated proteins shift from open-reading frames to the promoter regions, with heat shock-related genes showing increased promoter-associated sumoylation. When a specific E3 ligase was deleted in these cells, redistribution of chromatin-bound sumoylated proteins no longer occurred, and the cells became more sensitive to heat shock. These findings show how increased global sumoylation levels during stress is closely linked to transcription changes and enhanced cell survival.

1.4 Heat shock response

Cells must respond in a quick and coordinated manner when exposed to new environmental conditions in order to adapt and survive. The heat shock response is a well-studied

example, conserved across all species, including both prokaryotic and eukaryotic cells (Hu et al. 2022). When cells are exposed to heat stress, cells must initiate this response within a few minutes to prevent death (Richter et al. 2010; Verghese et al. 2012). This systematic reaction involves suppressing energy-demanding biosynthetic processes while inducing the expression of heat shock proteins, which play multiple roles in adjusting to elevated temperatures. The heat shock response influences multiple biological pathways, including transcription, translation, and cell growth.

1.4.1 Cellular response to heat shock

In order to conserve energy and redirect cellular pathways for survival under heat stress, cells undergo a pause in the cell cycle (Rowley et al. 1993). In budding yeast, this pause occurs at the G1 phase. The transition from G_1 phase to S phase is a critical and highly regulated checkpoint (Breter et al. 1983). This cell cycle arrest is thought to result from the downregulation of cell-cycle related genes, including the G_1 and S phase cyclins *CLN1* and *CLN2*. Interestingly, starved cells that have exited the cell cycle and are arrested in the G_0 phase show an increase in temperature stress resistance (Paris and Pringle 1983). This enhanced thermotolerance is attributed to trehalose, a carbohydrate whose levels correlate positively with survival under heat shock (De Virgilio et al. 1994). In cells with arrested cell cycles, trehalose accumulates and contributes to thermotolerance by reducing protein aggregation and facilitating refolding of misfolded proteins (Singer and Lindquist 1998). This process involves the activity of the heat shock protein Hsp104, and both trehalose and Hsp104 are needed to survive heat shock (Elliott et al. 1996).

Another way heat shock stresses the cells is by disrupting the structure and fluidity of cell wall and cell membrane. In response, the cell wall integrity pathway is activated to maintain

proper cell and membrane dynamics (Elliott et al. 1996). This pathway is essential for cell survival, as mutations in its components cause temperature sensitivity. Although the precise mechanism of activation remains unclear, two sensor proteins, Mid2 and Wsc1-4, have been identified as important for activation of the cell wall integrity pathway (Verna et al. 1997). Heat stress is also thought to alter turgor pressure, which activates the high-osmolarity glycerol (HOG) pathway through Sho1, a plasma membrane pressure sensor (Winkler et al. 2002). The impacts of heat shock on the cell wall remain incompletely studied and more work needs to be done to understand the complex signalling pathways involved.

Cells also protect themselves during heat shock by forming cytoplasmic stress granules (Wang et al. 2021). As translation pauses in response to stress, these stress granules sequester untranslated mRNAs, translation initiation factors, and both RNA- and non-RNA binding proteins. Small heat shock proteins are recruited to stress granules and play important roles in their formation (Ganassi et al. 2016). An important component of stress granules in humans is the small heat shock protein HSP22 which is immediately recruited in the stress granule and promotes the removal of misfolded proteins, preventing their accumulation. Stress granules also inhibit apoptosis by sequestering pro-apoptotic signalling factors (Arimoto et al. 2008). By protecting stalled translation components and the retaining survival-related signalling factors, stress granules enable cells to respond rapidly and dynamically to heat.

1.4.2 Transcriptional changes in heat shock response

High temperatures cause immediate transcriptional changes that allow cells to rapidly to swiftly adapt to heat shock. The transcriptional response to heat shock is well-studied in yeast (Verghese et al. 2012). The optimal growth temperature for *S. cerevisiae* is 30°C, with mild heat shock occurring at 37°C and severe heat shock at 42°C. Within minutes of exposure to high

temperatures, hundreds of genes are either upregulated or downregulated (Gasch et al. 2000; Causton et al. 2001). During mild heat shock, gene expression changes peak around 15 min, with roughly 1800 genes showing differential expression (Mühlhofer et al. 2019). Under severe heat shock, roughly 3000 genes are affected. Most upregulated genes encode molecular chaperones such as *HSP26*, while genes involved in protein degradation or metabolic processes are upregulated at later stages. In contrast, downregulated genes are typically associated with translation and ribosome biogenesis.

Several highly conserved master transcription factors are activated during heat shock, including Hsf1, Msn2, and Msn4 (Figure 3). Upon activated through phosphorylation, these transcription factors induce the expression of hundreds through a regulatory cascade. Hsf1 binds to the heat shock elements (HSE), while Msn2 and Msn4 bind to stress response elements (STRE) (Sorger and Pelham 1987; Treger et al. 1998). Msn2 and Msn4 share 41% sequence identity and similar zinc-finger binding motifs, and are therefore often studied together (Estruch and Carlson 1993). Notably, *MSN2* expression does not change with stress conditions, but it is translocated from the cytoplasm to the nucleus, where it activates *MSN4* expression (Gorner et al. 1998). Both Hsf1 and Msn2/4 are activated bind to promoter regions not only during heat shock, but also under other stress conditions such as osmotic stress, carbon source starvation, oxidative stress, and glucose starvation(Amorós and Estruch 2001).

One distinguishing feature between Hsf1 and Msn2/4 is their temporal role in the heat shock response. Hsf1 mediates the immediate, short-term response to acute heat shock, whereas Msn2/4 are more important for recovery and long-term heat stress survival (Yamamoto et al. 2008). Cells lacking both *MSN2* and *MSN4* can survive and grow under normal conditions but fail to survive prolonged heat shock. However, these mutants tolerate brief heat shock (up to 15

min), indicating that Msn2/4 are dispensable for the immediate response. In contrast, Hsf1 undergoes hyperphosphorylation upon heat shock and binds to promoter regions of target genes, contributing to both the acute response and the recovery phase. Hsf1 activation also occurs in response to protein misfolding, whereas Msn2/4 are not. Together, these findings suggest that Hsf1 is important for recovery from heat shock, while Msn2/4 help maintain thermotolerance during ongoing stress conditions (Yamamoto et al. 2008; Amorós and Estruch 2001).

Although Hsf1 is conserved across eukaryotes, a distinguishing feature in yeast Hsf1 is that yeast Hsf1 controls both basal and stress-induced transcription, whereas mammalian HSF1 functions only under stress conditions. Yeast Hsf1 is constitutively nuclear, and deletion of *HSF1* is lethal. Basal transcription of the heat shock chaperones such as Ssa2 (Hsp70) and Hsc82 (Hsp90) depends on Hsf1 activity. The lethality caused by loss or inactivation of Hsf1 can be rescued by the overexpression of those two heat chaperones, demonstrating their essential roles in both normal and stress conditions.

1.5 Sumoylation and heat shock

The heat shock response is a highly coordinated, time-sensitive, and transient response. Sumoylation has emerged as an important PTM that regulates this response, although its role in this context is still a relatively new are of study (Enserink 2015). The rapid global elevation of sumoylation observed during heat shock, along with accompanying cytoplasmic and transcriptional changes, appears to influence multiple aspects of the heat shock response, including transcriptional regulation and stress granule formation.

1.5.1 Human Hsf1 Sumoylation

Many studies have examined the human HSF1 protein and the role of sumoylation in its regulation. An important link between sumoylation and the heat shock response was established with the discovery that HSF1 becomes sumoylated during heat shock (Kmiecik et al. 2021). Under stress conditions, mammalian HSF1 forms a trimeric complex that binds to the genome. HSF1 is primarily mono-sumoylated in response to stress, and this modification occurs when HSF1 is already in a trimeric, DNA-bound form of the protein. Therefore, HSF1 sumoylation takes place while it is bound to promoters of target genes. Although the functional consequences of this modification remain unclear, there is evidence that it does not impact HSF1's DNA-binding capability or its trimeric, active state. It is thought that desumoylation occurs when additional proteins are recruited to displace Hsf1 from the promoter region. This dynamic cycling of transcription factor sumoylation has also been described in yeast, for example with Sko1, as described above (Rosonina 2019). Although there more remains to understand the role of HSF1 sumoylation, these studies show the importance of sumoylation in regulating the heat shock response.

1.5.2 SENP levels

Another link between sumoylation and the heat shock response is the degradation of SUMO proteases during heat shock. In human cells, there are six SUMO proteases, SENP1-3 and SENP5-7, and during heat shock, a rapid accumulation of SUMO2/3-conjugated proteins is observed (Saitoh and Hinchey 2000). All the SENPs, except for SENP6, were found to be heat-inactivated through degradation during heat shock, which likely explains the rapid increase in global sumoylation (Pinto et al. 2012). In *S. cerevisiae* cells, the SUMO protease Ulp1 was found be relocalize from the nuclear periphery to the nucleolus under ethanol stress, further linking the rise in global levels of sumoylation to reduced SUMO protease activity (Sydorskyy et al. 2010).

However, it remains unknown whether heat shock similarly inactivates Ulp1 in budding yeast or by which mechanism this might occur.

1.5.3 Stress Granules

Sumoylation's role in regulating translation initiation also plays a crucial step in stress granule formation (Wang et al. 2021). Proteomic studies have shown that SUMO E2 conjugating enzyme Ubc9, SUMO E3 ligases, and free SUMO peptides are present in stress granules during heat shock (Marmor-Kollet et al. 2020a). The same study further demonstrated that impaired Ubc9 function leads to higher levels of misfolded proteins in the cell. One identified SUMO target in stress granules is Poly(A)-Binding Protein Cytoplasmic 1 (PABC1) in human cells (Huang et al. 2025). Sumoylated PABC1 binds mRNAs containing U-rich regions, and this modification stabilizes its interaction with mRNA. This binding promotes stress granule formation and stabilizes specific mRNAs during heat shock.

1.6 Objectives of study

Sumoylation is an important PTM that plays roles in many biological pathways, particularly in gene regulation. It is well established that many sumoylated proteins are nuclear and involved in gene expression (Zhao 2007; Wohlschlegel et al. 2004; Seeler and Dejean 2003). Many studies previously have focused on the effects of sumoylation on individual target proteins. However, not much is known about how global changes in sumoylation levels affect transcription and other biological pathways related to gene expression under stress conditions. Under normal conditions, global sumoylation levels are typically low, but previous studies have determined that cells can modulate these levels in response to environmental changes, such as the marked increase in global sumoylation observed during heat shock (Zhou et al. 2004;

Sydorskyy et al. 2010; Lewicki et al. 2015). This leads to our hypothesis that cells can make global, coordinated changes to gene expression by modulating the activity of enzymes involved in sumoylation or desumoylation. Using the model organism *S. cerevisiae*, we employed strains that mimic elevated or reduced global sumoylation levels by altering the activity of key proteins involved in the sumoylation pathway, primarily the SUMO E2 conjugating enzyme Ubc9 and the SUMO protease Ulp1. This study aims to advance our understanding of the role and significance of sumoylation and how cells can adjust their global sumoylation status to regulate gene expression.

1.7 Figures

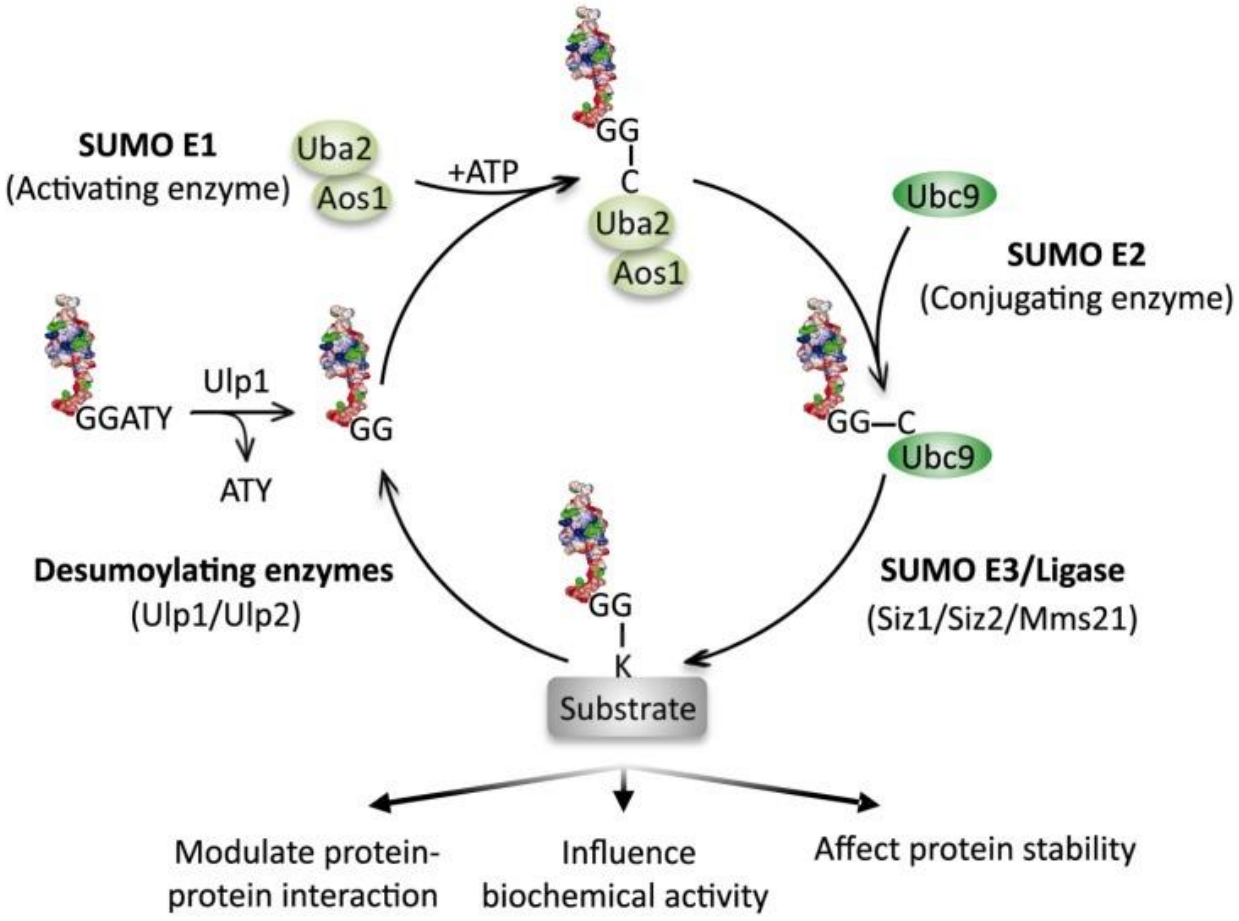

Figure 1: SUMO pathway in *Saccharomyces cerevisiae*, budding yeast. The SUMO pathway requires the dimeric E1 activating enzyme, the E2 conjugating enzyme, and E3 ligases. SUMO proteases are involved in maturation of SUMO peptide and desumoylation of SUMO-conjugated proteins. This figure is reproduced from Cremona *et al.* (2012).

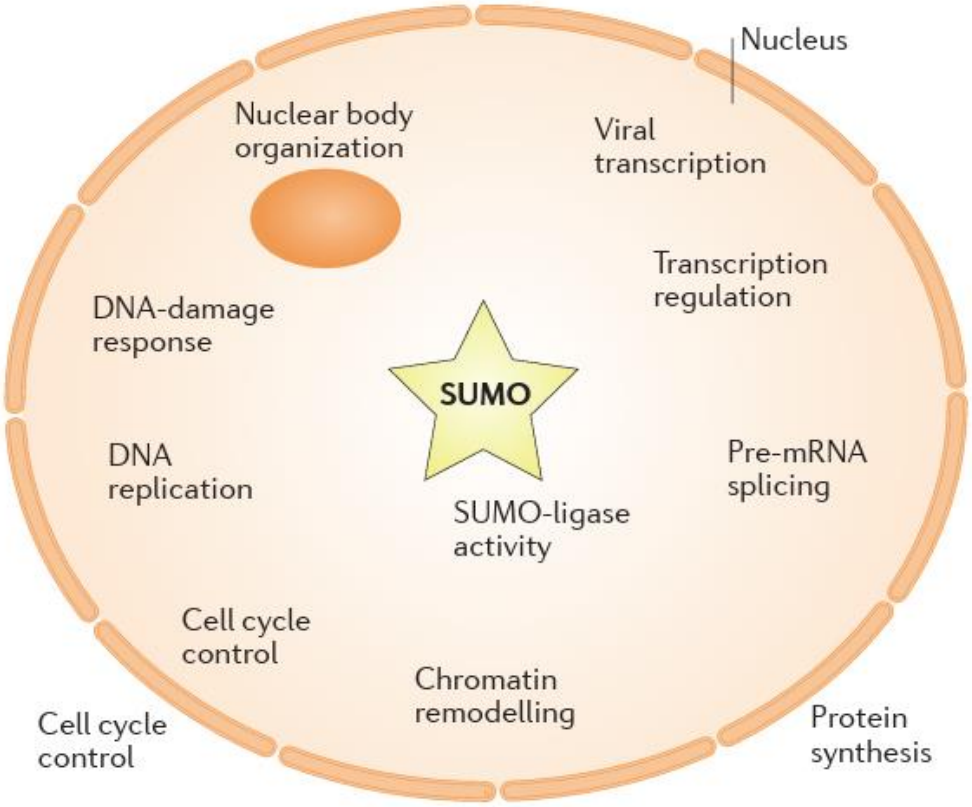

Figure 2: SUMO is involved in many biological pathways. The majority of sumoylation occurs in the nucleus, while some takes place in the cytoplasm. Figure reproduced from Hendriks and Vertegaal (2016) with permission from Springer Nature.

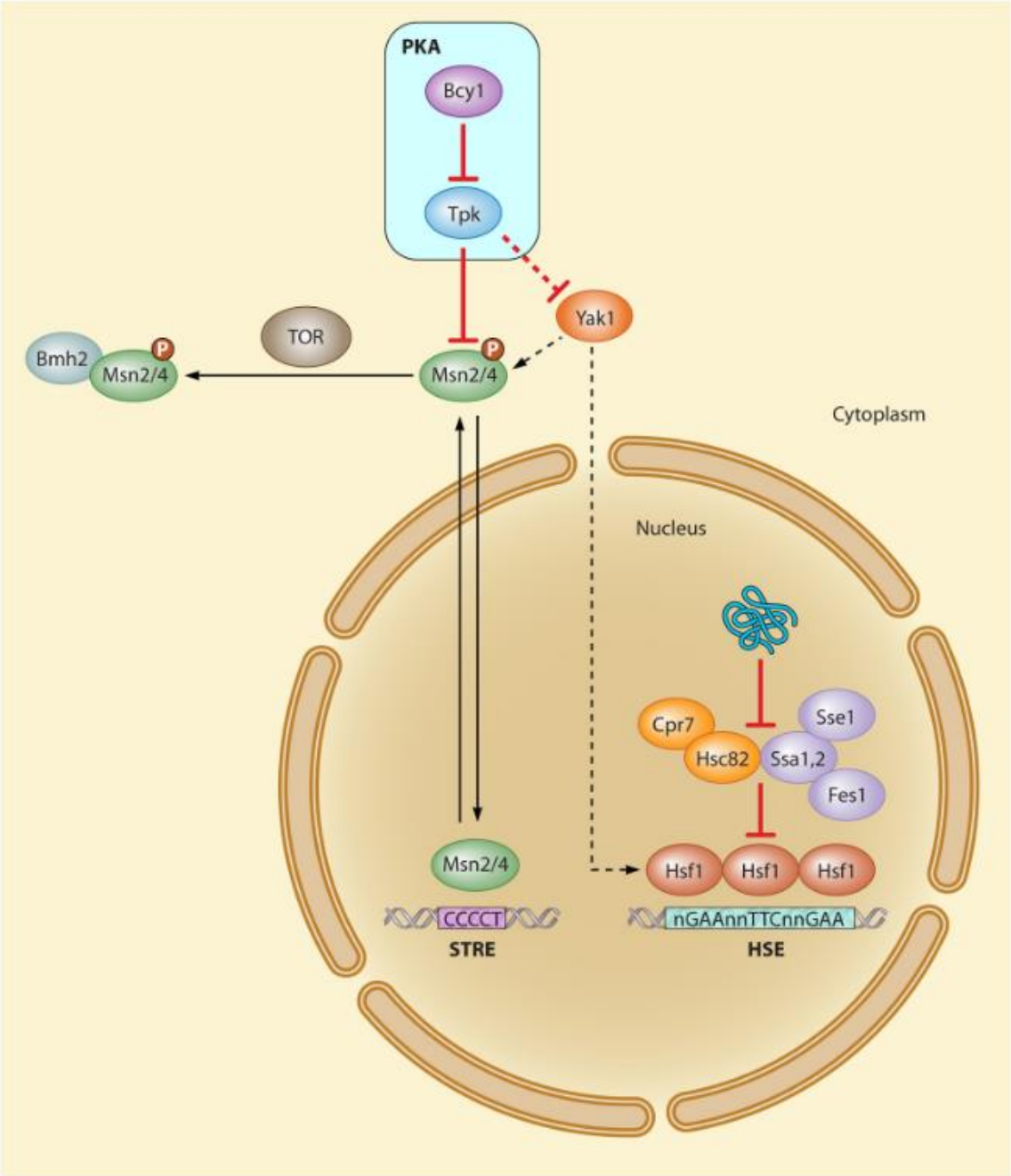

Figure 3: Transcriptional response to heat shock in *S. cerevisiae.* Hsf1 and Msn2/4 are the two main transcription factors that regulate gene expression during heat shock. Figure reproduced from Verghese *et al.* (2012).

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Chapter II: Sumoylation is Largely Dispensable for Normal Growth but Facilitates Heat Tolerance in Yeast

Figures from this Chapter have been published as part of the following publications:

Publication 1: Figs. 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16

Sumoylation is Largely Dispensable for Normal Growth but Facilitates Heat Tolerance in Yeast

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Manuscript published in *Molecular and Cellular Biology* on January 31, 2023

Publication 2: Figs. 1, 2, 3

Dynamic sumoylation of promoter-bound general transcription factors facilitates transcription by RNA polymerase II

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Manuscript published in *PLOS* Genetics on September 29, 2021

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Marjan Moallem conducted RNAPII ChIP-seq and RNA-seq experiments and their validation experiments, assisted in SUMO ChIP-seq experiment, and conducted all Western blots and drop tests. Bejamin Bergey assisted in SUMO-ChIP sequencing experiment and conducted qRT-PCRs with intron-containing RNAs. Dr. Emanuel Rosonina analyzed sequencing data. Farnaz Mansouri-Noori conducted all replicates of polysome profiling.

2.1 Abstract

Sumoylation is a post-translational modification that targets many proteins involved in gene expression, particularly nuclear proteins involved in transcription. Global levels of sumoylation change during stress conditions but not much is known about how this is connected to stress-induced changes to transcription patterns. To understand whether sumoylation levels have an impact on growth, survival, and transcription in optimal (i.e., non-stress) and stress conditions a variety of budding yeast strains with impaired Ubc9 function were studied because they show overall reduced SUMO conjugation levels. In terms of growth, we find that reduced levels of sumoylation are very well tolerated in non-stress conditions, even though they lead to altered RNAPII occupancy at a subset of genes across the genome, as determined by RNAPII ChIP-seq. Low levels of global sumoylation did impact adaptation to elevated temperature specifically, however, as strains with Ubc9 impairments were very sensitive to mild heat shock, but not to other tested stress conditions. During heat shock, RNAPII redistributes across the genome to facilitate the transcriptional response to stress. When sumoylation levels are reduced, however, heat shock-mediated changes to RNAPII occupancy are more severe, implying a role for sumoylation in restraining the re-distribution of the transcriptional apparatus. Additionally, we report the effects of reduced SUMO conjugation levels on the composition of the transcriptome and on translation. Altogether, these results highlight that normal levels of global sumoylation, although largely dispensable during normal growth, are important for cell survival and appropriate RNAPII redistribution during heat shock.

2.2 Introduction

Many eukaryotic organisms can modulate the levels of global sumoylation in their cells in response to environmental stress and disease conditions. Previously described research has shown that levels of SUMO conjugation increase rapidly within minutes when *S. cerevisiae* cells are exposed to osmotic, oxidative, heat shock, and ethanol stress (Lewicki et al. 2015). An increase in SUMO conjugation is also detected in plants experiencing drought and salt stress (Han et al. 2021). Heat shock and certain human illnesses, such as neurodegenerative diseases and multiple cancers, result in increased levels of global sumoylation in the affected cells (Flotho and Melchior 2013), whereas reduced SUMO conjugation has been linked to the progression of bladder and other cancers (Huang et al. 2020). When the Epstein-Barr virus infects human cells, one of its protein kinases inhibits sumoylation globally, which facilitates viral replication while disrupting the normal immune response (Li et al. 2012). There appears, then, to be an evolutionarily conserved link between levels of global sumoylation and the cellular response to changing conditions and illness.

This leads to the question of whether sumoylation is also important during growth in non-stress (i.e., optimal) conditions, and whether roles for sumoylation in these conditions, especially in gene expression, can influence cell survival. Most eukaryotic cells are unable to survive with a complete abolishment of sumoylation. For example, knocking out the gene encoding Ubc9 from the yeast genome and downregulating its expression in chicken cells both lead to cell death (Giaever et al. 2002; Hayashi et al. 2002). Ubc9 is the sole E2 conjugating enzyme in all eukaryotic cells and is essential for conjugating the SUMO peptide to target proteins. Ulp1, the major SUMO protease in yeast, which cleaves the precursor SUMO peptide to the mature form of SUMO, is also essential for cell survival. Even in experimental conditions in which mature

SUMO peptides are produced in the cells without the need for processing, Ulp1 is still essential (Seeler and Dejean 2003). This indicates that a balance of both sumoylation and desumoylation ("sumoylation cycling") is required for cell survival.

Transcription is one of the main biological pathways that is impacted by sumoylation. Many gene-specific transcription factors, general transcription factors, and chromatin remodelling factors are sumoylated (Wohlschlegel et al. 2004; Rosonina et al. 2017). Previous fractionation studies have shown that most sumoylated proteins are associated with chromatin, while SUMO ChIP-seq analysis found sumoylated proteins enriched specifically at highly transcribed genes including tRNA and translation-related genes (Chymkowitch, Nguéa P, Aanes, et al. 2015; Baig et al. 2021). Furthermore, transcription inhibitors were found to reduce cellular sumoylation levels and prevent the surge in SUMO conjugation that is normally observed in response to osmotic stress (Lewicki et al. 2015; McNeil et al. 2024). Although multiple links between sumoylation and transcription have been made, it is not known whether the level of sumoylation that cells maintain in non-stress conditions impacts transcription in an any way, and how elevated sumoylation during stress affects the activity of chromatin-bound SUMO-targeted transcription-related proteins.

To explore the role that the normal levels of SUMO conjugation play in non-stress conditions, and to examine how they impact transcription, we examined yeast strains that have defects in Ubc9 function that lead to reduced levels of global sumoylation. The strains were studied to assess the functions of global sumoylation on growth, transcription and translation. Additionally, we explored whether normal levels of sumoylation in non-stress conditions are needed to facilitate the transcriptional response when stress conditions are encountered. Although cells cannot survive with a complete abolishment of global levels of sumoylation, we determined

that a minimal threshold level of global sumoylation, well below what is seen normally, is sufficient for viability in non-stress conditions, but insufficient for adapting to heat shock. Our experiments also reveal the impact of reduced global levels of sumoylation on transcription, mRNA processing, and translation.

2.3 Materials and Methods

Yeast strains and cloning

Yeast strains used in these experiments are listed in Table 1. RPS20 and RPS27B were tagged with HA tag in both parent (W303a) and *ubc9-6* strains through homologous recombination as described in (Knop et al. 1999).

Yeast growth

Cultures were grown in rich medium (YPD), or synthetic complete medium (SC) where specified, shaking at 30°C until they reached optical densities (ODs) between 0.5 to 0.8 at an absorbance of 595 nm. For heat shock, cultures were transferred to an incubator set to 37°C for the indicated times. To compare growth of different strains, spot assays were performed as follows. Strains were grown overnight in SC then diluted to an optical density (OD at 595 nm) of 0.2. Samples were then spotted side-by-side in five-fold serial dilutions on SC solid-media plates. To test for growth in different conditions, SC plates were made with the addition of 1mM H_2O_2, 1M KCl, and 10% EtOH plates were incubated at 30°C. To test growth under heat shock, SC plates were placed at 37°C. Images were taken after 1 – 3 d for all plates.

Protein lysates

Cultures of 10 mL were harvested through centrifugation at 3000 *g* for 5 min, then pellets were washed with 10 mL of IP buffer, which consists of 50 mM Tris-HCl, pH8; 150 mM NaCl; 0.1% nonidet P-40 (NP40); supplemented with a protease inhibitor cocktail, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 2.5 mg/mL *N*-ethylmaleimide (NEM). Samples were then resuspended in 500 µL of IP buffer. Lysis of cells were performed as described

previously (Sri Theivakadadcham et al. 2019). Samples were then boiled with SDS 2X sample buffer for 5 min at 95°C.

Western blots

Prior to western blotting, protein lysates were analysed by SDS-polyacrylamide gel electrophoresis (PAGE). Wet transfers were performed at 100 V for 1 h using a transfer buffer containing 10% methanol, 50 mM Tris-HCl, and 380 mM glycine. Nitrocellulose was used in all cases for analysis Primary antibody incubation was performed overnight using the following dilutions SUMO 1:1000 (y-84; Santa Cruz, sc-28649), GAPDH 1:3000 (Sigma, G9545), HA 1:1000 (Novus, NB600-363) , Ubc9 1:1000 (Santa Cruz, sc-6721) , 8WG16 1:3000 (Abcam, ab817), Ser2p 1:3000 (Sigma-Aldrich, 3E10), Ser5p 1:3000 (Abcam, ab5131), and Rpb3 1:500 (Abcam, ab20289). Chemiluminescence imaging was performed with a MicroChemi Imager (DNR).

RNA analysis

For isolating RNA, 15-mL cultures were harvested through centrifugation at 3000 *g* for 4 min, washed with 1 mL of ice-cold AE buffer (50 mM sodium acetate, pH 5.2; 10 mM EDTA, pH 8), pelleted again, then resuspended in 400 µL of ice-cold AE and 40 µL of 10% SDS. An equal volume of phenol, pH 4.5 was added, and the samples were mixed thoroughly, placed in a dry ice/ethanol slurry for 5 min, transferred to a 65°C water bath for 5 min, then vortexed for 30 seconds. Another freeze-heat-vortex-freeze cycle was performed, then samples were centrifuged at top microfuge speed for 7 min at room temperature. To the aqueous layer, 600 µL of a 1:1 phenol (pH 4.5)-chloroform mixture was added, samples were vortexed, then centrifuged for 5 min. RNA in the aqueous layer was then precipitated by adding 50 µL of 3 M sodium acetate (pH

5.2) and 1 mL of ice-chilled absolute ethanol, incubating on dry ice for 10 min, then centrifuging for 15 min at 4°C. Pellets were washed with ice-cold 70% ethanol, then resuspended in 100 µL of nuclease-free water. 1 µg of DNase-treated RNA was converted to cDNA through a reverse-transcription reaction using the iScript cDNA synthesis kit (BioRad) for the RT-qPCR experiments. The cDNA was then used for the qPCR reactions using primers as listed were used and results were normalized using 25S RNA levels. qPCR was done with the CFX Opus real-time PCR detection system (Bio-Rad).

For RNA-seq, the RNA was first purified with the RNeasy kit (Qiagen) and diluted to 100 µl using nuclease free water. This purified RNA was then enriched for polyadenylated (polyA) RNA before sequencing. More details on analysis of sequencing data are in Table 5.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously (Sri Theivakadadcham et.al 2019) using 50-mL culture volumes and 2 µg of the SUMO antibody (y-84; Santa Cruz, sc-28649) for the SUMO ChIP experiment and 4 µg of 8WG16 antibody for the RNAPII ChIP experiment (8WG16; Abcam, ab817). For ChIP-qPCR analysis, primers as listed were used for qPCR and results were normalized using inputs. qPCR was done with the CFX Opus real-time PCR detection system (Bio-Rad). The averages of three biological trials were presented and standard deviations are plotted as error bars. Student's t-tests were conducted to compare samples between untreated and heat shock samples. Asterisks indicate a significant difference where the p-value is less than 0.05.

For ChIP-seq experiments, 200 ml culture volumes and 8µg of SUMO antibody was used or 15 µg of 8WG16 (RNAPII) antibody were used for the experiments. Details of library

preparation, sequencing and bioinformatics analysis for the ChIP-seq experiments are listed in Tables 3 and 4. GO term analysis and visualization were performed as previously described (Bonnot et al. 2019).

Polysome Profiling

Wildtype and *ubc9-6* strains were grown in YPD media in 200 ml cultures at 30°C. Treatment with 100 µg/mL cycloheximide was done for 10 min when cell cultures were at a density of 0.5-0.8 OD_{595nm}. Cells were then harvested, lysed and added to sucrose gradients as described in Mansouri-Nouri *et al.* (2023). The solution was then fractioned in a continuous flow and absorbance was measured at 254 nm.

Table 1: Yeast Strains used in this study

Yeast strains				
Name	**Code**	**Background**	**Genotype**	**Source**
W303a			*MAT a ura3-52 trp1Δ2 leu2-3_112 his3-11 ade2-1 can1-100*	Dharmacon
BY4741			*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
Parent-AA	HHY221	W303a	*MATa tor1-1 fpr1::loxP-LEU2-loxP RPL13A-2×FKBP12::loxP*	Euroscarf
Ubc9-AA	YJBa001A	W303a	*UBC9-FRB::kanMX tor1-1 fpr1::loxP-LEU2-loxP RPL13A-2×FKBP12::loxP*	Rosonina lab
ubc9-6	D370	W303a	*ubc9-1:Tadh1:TRP1:ubc9-1*	Rosonina lab
UBC9	YWO1		*Mat α his3-Δ200 leu2-3,2-112 lys2-801 trp1-1(am) ura3-52*	(Seufert et al. 1995)
ubc9-1	YWO102 alpha		*Mat α ubc9Δ::TRP1 leu2::ubc9Pro-Ser::LEU2 his3-Δ200 leu2-3,2-112 lys2-801 trp1-1(am) ura3-52*	(Seufert et al. 1995)
hog1Δ	YVS015a	W303α	*hog1Δ::kanMX Matα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]*	Rosonina lab
ctk1Δ	ERYM021	BY4742	*ctk1Δ-kanMX MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Rosonina lab
yap1Δ		BY4741	*yap1Δ-kanMX*	Horizon Discovery
rpb1-1	DBY120	W303a	*MAT a ura3-52 rpb1-1 trp1::hisG*	(McNeil et al. 1998)
RPL5-HA	YMM026A	W303a	*rpl5-3HA::kanMX*	This study
RPL5-HA *ubc9-6*	YMM028A	W303a	*rpl5-3HA::kanMX ubc9-1:Tadh1:TRP1:ubc9-1*	This study
RPS20-HA	YMM019A	W303a	*rps20-3HA::kanMX*	This study
RPS20-HA *ubc9-6*	YMM029A	W303a	*rps20-3HA::kanMX ubc9-1:Tadh1:TRP1:ubc9-1*	This study

Table 2: Analysis details for SUMO ChIP-seq

SUMO ChIP-seq	
Samples and conditions	Two independent replicates were prepared from cultures grown in SC medium at 30°C. Inputs and SUMO IPs were sequenced. 1 – WT (W303a): untreated 2 – *ubc9-6*: untreated
Library synthesis	NEBNext Ultra II DNA library prep kit (New England Biolabs)
Sequencing	Illumina HiSeq 2500; Paired-end reads; 2x 126 nt; 10 million reads/sample
Quality control	FastQC (0.11.5)
Trimming	Trim Galore (0.4.4_dev) Cutadapt (2.3) Quality Phred score cutoff: 25, Adapter sequence: 'AGATCGGAAGAGC', Minimum required adapter overlap (stringency): 5 bp, Minimum required sequence length for both reads before a sequence pair gets removed: 40 bp, All sequences trimmed by 6 bp from their 5' end.
Genome alignment	Bowtie2 (2.3.5) with sacCer3 reference genome
Peak calling	MACS (2.1.1.20160309) ***Parameters:*** paired-end; input as control; narrow peaks; effective genome size: 1.2e7; *q*-value cut-off: 0.05
Differential binding analysis	DiffBind (2.10.0) ***Parameters:*** Defaults with minMembers=2 for dba.contrast; see notes below
Peak analysis and annotation	ChIPpeakAnno (3.2.0) from Bioconductor ***Parameters:*** TxDb.Scerevisiae.UCSC.sacCer3.sgdGene genome annotation package was used, but each annotation was confirmed visually using IGV.
Composite ("meta-gene") plot	computeMatrix tool from deepTools (3.5.0) ***Parameters:*** Bowtie2-produced BAM files for Replicate 1 (WT and *ubc9-6*) were converted to BED format and analyzed with a custom-made BED regions file containing only SUMO peak-containing non-RPGs .
Motif analysis	MEME suite (5.1.0) tools MEME and DREME were used with sequences surrounding peaks obtained with ChIPpeakAnno tool peakWithSequences.
GO analysis	Gene names for the SUMO peak list of non-RPGs were analyzed by the GO Term Finder (0.86) on the

	Saccharomyces Genome Database (yeastgenome.org) with default parameters.
Notes	To establish SUMO peak list: =WT and *ubc9-6* sets were compared to identify "differentially bound" sites with DiffBind, but all peaks identified in the WT set were considered. Some SUMO peaks, particularly those associated with tRNAs, are stable, even with low sumoylation levels in *ubc9-6*.

Table 3: Analysis details for RNAPII ChIP-seq in WT vs. *ubc9-6* strains

RNAPII ChIP-seq in WT and *ubc9-6* strains	
Samples and conditions	Two independent replicates were prepared from cultures grown in SC medium at 30°C and at 37°C for 12 min. Inputs and 8WG16 (Rpb1 antibody) IPs were sequenced. 1 – WT (W303a) 2 – *ubc9-6*
Library synthesis	NEBNext Ultra II DNA library prep kit (New England Biolabs)
Sequencing	Illumina HiSeq 2500; Paired-end reads; 2x 126 nt; 10 million reads/sample
Quality control	FastQC (0.11.8)
Trimming	Trim Galore (0.4.4_dev) Cutadapt (1.18) Quality Phred score cutoff: 25, Adapter sequence: 'AGATCGGAAGAGC', Minimum required adapter overlap (stringency): 5 bp, Minimum required sequence length for both reads before a sequence pair gets removed: 40 bp, All sequences trimmed by 6 bp from their 5' end.
Genome alignment	Bowtie2 (2.3.5) with sacCer3 reference genome
Peak calling	MACS (2.1.1.20160309) ***Parameters:*** paired-end; input as control; broad peaks; effective genome size: 1.2e7; *q*-value cut-off: 0.1
Differential binding analysis	DiffBind (2.8.0) ***Parameters:*** minMembers=2 for dba.contrast; th=1 for dba.report; see notes below
Peak analysis and annotation	ChIPpeakAnno (3.2.0) from Bioconductor ***Parameters:*** TxDb.Scerevisiae.UCSC.sacCer3.sgdGene genome annotation package was used and the closest feature to the middle of each peak was used for annotation.

Notes	To determine RNAPII density at each ORF: DiffBind was applied to the four samples using a pre-defined peak-set that corresponds to ORF regions of all protein-coding genes. The “th=1” parameter was applied to determine the “concentration” (log2 normalized ChIP read counts with control read counts subtracted) of RNAPII at all ORFs in all four conditions. Here, these are referred to as RNAPII densities.

Table 4: RNA-seq Analysis of WT and *ubc9-6* strains

RNA-seq in WT and *ubc9-6* strains	
Samples and conditions	Two independent replicates were prepared from cultures grown in SC medium at 30 °C. 1—W303a (“WT”) 2—*ubc9-6*
Library synthesis	NEB Ultra II directional mRNA library prep (PolyA enrichment)
Sequencing	Illumina HiSeq 2500; Paired-end reads; 2 × 126 nt; 30 million reads/sample
Quality control	FastQC (0.11.9)
Genome alignment	HISAT2 (2.1.0) ***Parameters:*** Default options; UCSC annotation file: sacCer3.ncbiRefSeq.gtf
Transcript counting	Transcripts counted using featureCounts from Subread (2.0.0) ***Parameters:*** Default options; -p (paired)
Differential expression analysis	edgeR (3.32.1) ***Parameters:*** Library sizes were normalized using calcNormFactors; dispersions were estimated with estimateDisp with robust argument; likelihood ratio tests for differential expression were performed with glmFit and glmLRT.
Data availability	NCBI GEO database accession: GSE167427

Table 5: Sequences of Oligonucleotide used in this study

Gene	Oligonucleotide sequence(s)
Primers for qPCR analysis of ChIP samples	
HIS4	Forward: 5'-ACGTTTCACTTGTTGGTCAGG-3'
	Reverse: 5'-CCACTTGGCAAGGACAAAGC-3'
MET6 promoter	Forward: 5'-GCATCTAAGAGCATTGACAACA-3'
	Reverse: 5'-TGGACCGATTCTTGGGAACC-3'
RPS20 promoter	Forward: 5'-CGCGACTAGCCTCAGAGATT-3'
	Reverse: 5'-GCTGAGCTTGAATGAAATAACCC-3'
RPS27B promoter	Forward: 5'-AATTCCCCTCTGCTTCCCG-3'
	Reverse: 5'-ACAGCACACATGAAAGATGAGA-3'
YEF3	Forward: 5'-ACGTTGGTGAAGACGATGCT-3'
	Reverse: 5'-CAATGGCGGCAATGTACTCG-3'
*ChrV (*untranscribed*)*	Forward: 5'-CATTATCCGTAACGCCACTTT-3'
	Reverse: 5'-CGATCTTAGTTCCAATGGTGAAA-3'
Primers for qPCR analysis of RT samples	
HSP26	Forward: 5'-CAACGAAGTTGATGCCTTTAACAG-3'
	Reverse: 5'-CAACTTCCTTGCCAGTAGAATCC-3'
HSP12	Forward: 5'-CTGAAGCTTTGAAGCCAGACTC-3'
	Reverse: 5'-GGTTGAACCTTACCAGCGACC-3'
MET6	Forward: 5'-TTTTGGACTTGCCTGCCAAC-3'
	Reverse: 5'-CAACTGGCAAGCCCTTGATG-3'
IMD2	Forward: 5'-GTCAAGACATCGGCTGTAGGTCG-3'
	Reverse: 5'- CGTAAGAATGTAAATTATGAACG-3'
ADH1	Forward: 5'-CACCGTTTTGGTCGGTATGC-3'
	Reverse: 5'-GACCAAACCTCTGGCGAAGA-3'
RPS20	Forward: 5'- AGGGTCCAGTCAGACTACCA-3'
	Reverse: 5'- CAGGAGCTTCCAAGTCGATGT-3'
RPL5	Forward: 5'- CTGCTGCTTACGCTACTGGT-3'
	Reverse :5'-ACCTTGAATGGACGTGGACC-3'
RPL25 (intron)	Forward: 5'-TTAACGACAGCTTTCTTAGCG-3'
	Reverse: 5'-ACCAACAGCCATTAACAAATCC-3'
RPL25 (exon)	Forward: 5'-CTATGAAGAAGGTTGAAGATGG-3'
	Reverse: 5'-TGGTCTAACCAAAGTGTTAACC-3'
RPS27B (intron)	Forward: 5'-CTGGGTAATGATTTATCCTTC-3'
	Reverse :5'-TGGGTGCAACAAATCTTGAAC-3'
RPS27B (exon)	Forward: 5'-ATGTCAAATGCCCAGGTTGT-3'
	Reverse: 5'-TGACAGCAGTTTGAGCATGA-3'
25S rRNA	Forward: 5'-TCTAGCATTCAAGGTCCCATTC-3'
	Reverse : 5'-CCCTTAGGACATCTGCGTTATC-3'

2.4 Results

2.4.1 Sumoylation on chromatin

Sumoylation is associated with transcription through modification of multiple proteins involved in the process, including numerous transcription factors (Rosonina et al. 2017). To explore whether transcription-related proteins are sumoylated specifically when associated with chromatin, and to map the occupancy of sumoylated proteins across the genome, we conducted a chromatin immunoprecipitation-sequencing (ChIP-seq) experiment in budding yeast with a SUMO antibody (Figure 1, Table S1). The analysis of the sequencing results revealed that sumoylated proteins are indeed associated with chromatin. In all, 607 peaks were identified across the genome and two major groups of genes were found to have SUMO peaks. Most of the SUMO peaks are associated with highly transcribed genes such as tRNA genes and ribosomal protein genes (RPGs). The second group of peaks, with a total of 147 peaks, are associated with protein-coding genes that are not RPGs.

The SUMO peaks detected with this ChIP-seq experiment at protein-coding genes were situated specifically at promoter regions. This indicates that the sumoylated proteins detected are likely transcription factors or proteins that are a part of the transcription pre-initiation complex (PIC). A previous study detailed the binding of sumoylated Rap1, a transcription factor, at the promoter regions of RPGs (Chymkowitch, Nguéa P, Aanes, et al. 2015). This was also observed with our experiment where sumoylated peaks at ribosomal protein genes matched Rap1 binding sites (Figure 2). However, the SUMO peaks at other, non-RPG promoters did not match with a Rap1 binding site. Rap1 does bind some promoters of non-ribosomal protein genes; however, in our experiment, only 14 out of 147 peaks matched with Rap1 binding (Figure 2). At these other promoter regions, the SUMO peak aligned with the binding site for TBP, a crucial component of

the pre-initiation complex that is situated at the core promoter (Figure 2). This indicated for these genes one or more components of the pre-initiation complex are being sumoylated at the promoter region.

Genes associated with SUMO peaks at their core promoter are generally highly transcribed, supporting the idea that sumoylation functions during active transcription (Figure 3). This was determined by comparing the SUMO peaks with another ChIP-sequencing experiment where RNAPII occupation was determined genome-wide in a wild-type strain. The level of RNAPII occupancy on a gene body can be considered an indication of the transcriptional activity level of that gene (Tan and Wong 2019). Although not all genes with SUMO peaks at their core-promoters showed a high level of RNAPII occupation, 51% of SUMO peaks at non-ribosomal protein genes were found at promoters of the top 10% most highly expressed genes, as determined by RNAPII occupation (Figure 3).

The total non-RPG SUMO peaks detected through the SUMO ChIP-seq experiment were 147 peaks, with 14 peaks attributed to Rap1 binding sites and 133 other non-RPGs sites (Figure 1, 2). Out of the 133 non-RPG sites, 111 sites were specific to promoter areas of a single gene and not a promoter that is shared by close divergent genes (Figure 3). These 111 SUMO peaks were distinguished to identify the impact of the sumoylated protein on transcription of a single gene since it is clearer which gene the promoter would affect. In comparison with the rest of the genes, all 111 non-RPG SUMO sites also showed higher levels of RNAPII occupation. These results indicate that there is a correlation between sumoylated proteins at the promoter region and active transcription levels. Additionally, they confirm previous studies that showed the association of sumoylated proteins with tRNA genes and RPG genes and identified a subset of non-RPGs that interact with sumoylated proteins at their promoters.

2.4.2 Levels of sumoylation and its impact on growth

While sumoylation is essential, cells maintain only low levels of SUMO conjugation under normal growth conditions (Wohlschlegel et al. 2004). To determine the role of this basal level of global sumoylation on cell growth and survival, mutant yeast strains with further reduced levels of sumoylation were examined (Figure 4). To achieve this, we examined strains containing a mutation in the *UBC9* gene, which results in lower global levels of sumoylation than wild type cells. Knockout of *UBC9* is lethal, but the P69S mutation, in which codon 69 is changed from proline-encoding to serine-encoding, hinders the catalytic activity of Ubc9 but does not inhibit it completely (Seufert et al. 1995). The *ubc9-1* strain expresses this mutant form of Ubc9 from the *LEU2* gene locus, whereas the *ubc9-6* strain expresses the P69S-mutant Ubc9 as a double-tandem repeat from the natural *UBC9* locus.

We also employed the Ubc9-AA strain, which also exhibits low levels of global sumoylation. This strain expresses Ubc9 with a C-terminal fusion to the Anchor-Away tag, which can be used to re-localize tagged nuclear proteins to the cytoplasm upon the addition of rapamycin (Haruki et al. 2008). After the construction of this strain, however, it was found that even without the presence of rapamycin, the tag itself impacts the normal function of Ubc9 causing low levels of global sumoylation. Each strain was compared to its parental strain because levels of normal global sumoylation depends on the genetic background of the parent strains. Compared to *ubc9-1* and *ubc9-6*, the Ubc9-AA strain shows less reduction in global levels of sumoylation compared to its parent strain (Figure 4).

2.4.3 Impact of stress conditions on growth and global sumoylation levels

The three Ubc9-defective strains were used to examine the effects of reduced SUMO conjugation on growth at optimal temperatures. Spot assays were performed and showed that all three strains can grow at the optimal temperature of 30°C, with only *ubc9-6* showing a modest growth defect compared to its wild-type isogenic parental strain (Figure 5). This indicates that only a low level of global sumoylation is required for cell survival at optimal conditions. Since previous research linked SUMO conjugation and the environmental stress response, the growth of these strains was also assessed under different stress conditions, including exposure to oxidative (H_2O_2), osmotic (KCl), ethanol, and temperature stresses (Figure 5). A separate plate including positive controls for each stress condition was included along with their parent strains while conducting these experiments to make sure that the stress conditions are effective. The following strains were used as positive controls, *yap1*Δ strain was used for oxidative stress (H_2O_2), *hog1*Δ was used for osmotic stress (KCl), *ctk1*Δ and *rpb1-1* were used for ethanol stress, and *rpb1-1* was used for heat shock (Qian et al. 2012; Pascual-Ahuir and Proft 2007; Stephen et al. 1995; Scafe et al. 1990) Interestingly, while the corresponding isogenic parental strains grew at 37°C, none of the three Ubc9-defective strains were viable when exposed to this temperature. However, the none of the strains showed sensitivity to osmotic, ethanol, and oxidative stress (Figure 5). This points to a close link between levels of sumoylation and the heat shock response, specifically.

Overall levels of SUMO conjugation increase rapidly during stress conditions (Lewicki et. al 2015). Considering our observation that Ubc9-defective strains can not survive heat shock specifically, we explored whether a heat shock-triggered surge in sumoylation still takes place in these strains. First, the change in global levels of sumoylation in response to heat shock was assessed in a wild-type strain for comparison. As shown in the western blot in Fig. 6, SUMO

conjugation levels rose rapidly during heat shock in wild-type yeast, with sumoylation increasing within 5 min of the mild 37-degree heat shock (Figure 6). After 30 min at 37°C, global levels of sumoylation had increased by 1.5-fold compared to growth at the optimal temperature of 30°C. Similarly, as expected, the three isogenic wild-type parental strains showed elevated sumoylation when exposed to the mild heat shock for 30 min (Figure 7). Meanwhile, however, no increase in SUMO conjugation levels were detected with any of the Ubc9-defective strains during the heat shock (Figure 7). The inability to sufficiently raise levels of global sumoylation during heat shock may at least in part explain why these strains cannot adapt to higher temperatures. Also, the levels of Ubc9 were assessed in each strain with and without heat shock and the sumoylation levels in these strains were not correlated to the abundance of Ubc9 protein levels (Figure 7).

2.4.4 Impact of reduced cellular sumoylation on transcription

To determine whether global levels of SUMO conjugation impact transcription, RNAPII occupation across the genome was assessed by ChIP-seq in the *ubc9-6* strain and its isogenic parent (Table S2). As shown in Figure 8, 1583 of ~6400 protein-coding genes showed significant differences in RNAPII levels in the two strains. RNAPII occupation increased at a subset of genes while decreasing for another group of genes and so the changes observed in the *ubc9-6* strain were not uniform genome-wide. We performed GO term analysis to determine which categories of genes were most impacted by reduced global sumoylation (Figure 9, Table S3). 683 of the genes that showed a more than 2-fold increase in RNAPII occupancy in the *ubc9-6* strain are associated with translation and ribosome biogenesis. Notably, 110 of 136 ribosomal protein genes (RPGs) showed higher RNAPII occupation levels in the *ubc9-6* strain. Meanwhile, 758 of genes showing a more than 2-fold decrease in RNAPII occupancy in the *ubc9-6* strain are associated with small molecule and amino acid biosynthesis and metabolism. This indicates that

groups of functionally related genes can be coordinately regulated by modulating cellular sumoylation levels.

We next examined whether heat-shock mediated changes to transcription genome-wide are affected by a global reduction in sumoylation. In wild-type cells, only 6% of genes showed changed RNAPII occupancy after 12 min at 37°C (Table S2). In response to heat shock, we found that the redistribution of RNAPII differed in the parent compared to the *ubc9-6* strain (Figure 10, 11). The *ubc9-6* strain showed a much broader change in RNAPII occupation genome-wide, with roughly 29% of genes having either an increase or decrease in RNAPII levels. Specifically, a total of 1,829 genes showed differences in RNAPII after heat shock compared to 375 genes in the parent strain. Of those genes, there is only an overlap of 259 genes that show similar re-distribution of RNAPII occupation after heat shock. This signifies that the normal re-distribution of RNAPII on the genome is not happening as it normally would and suggests that normal sumoylation levels are important for restraining this re-distribution. This could further explain why cells cannot survive heat shock in conditions of lower sumoylation.

Next, we examined whether the altered RNAPII occupancy levels detected at many genes in the *ubc9-6* strain impacted steady-state mRNA levels. RNA-seq was conducted with two biological replicates comparing mRNA levels of parent and *ubc9-6* strains in SC minimal media grown at the optimal temperature of 30°C (Figure 12, Table S4). The RNA-seq analysis showed that there were significant differences in steady-state of mRNA in the parent and *ubc9-6* strains, with some mRNA levels showing an increase in levels and some showing a decrease. However, there was not a clear correlation of the differences in mRNA levels observed in the ChIP-seq results compared to the RNA-seq (Figure 13, Table S5). Notably, the ribosomal protein steady-state mRNA levels were not significantly different in the *ubc9-6* compared to the wild-type

strain. In some cases, a few of the ribosomal protein mRNAs showed a slight decrease in levels in the *ubc9-6* strain. This is a different result from the RNAPII ChIP-seq experiments where there was a higher RNAPII occupation levels at RPGs. Therefore, although there are changes detected in transcription, those changes are not reflected in the steady-state mRNA levels. This is not unexpected as there is not always a direct correlation between nascent transcription and steady-state RNA levels.

There are known regulatory steps that occur post-transcriptionally to compensate for changes in transcription and buffer between synthesis and decay of RNA (Miller et al. 2011; Timmers and Tora 2018). For example, when cells no longer express mRNA processing and degradation related proteins, transcription rates also decrease to balance out less mRNA decay (Sun et al. 2013). Even though this buffering system is well established, it is unknown exactly how the signalling occurs to balance out the levels of transcription and mRNA processing. One possible mechanism is that the level of ribosome proteins could serve as the signal as they are shuttled between the nucleus and the cytoplasm (Timmers and Tora 2018). The levels of ribosomal protein not only impact translational levels but can also impact mRNA steady levels. For example, a study found that in *S. cerevisiae*, RPL9B which is a ribosomal protein when present in excess can bind to 3'UTR of nascent mRNAs and trigger their degradation co-transcriptionally (Gudipati et al. 2012). This example is one of the five examples of ribosomal proteins that can regulate their own levels through changes in mRNA stability.

2.4.5 The effects of reduced global levels of sumoylation on translation

Our ChIP-seq data that revealed that in conditions of lower global sumoylation levels, more RNAPII occupies ribosomal protein genes (RPG) and other translation-related genes. To determine whether these differences in these genes have any effect on translation, polysome

profiling was conducted. Three biological replicates were performed in *ubc9-6* and its parent strains. Cycloheximide was added to growing cultures when they were at the exponential phase to stop translation from occurring, then pellets were harvested and lysed. Samples were run on sucrose gradients and absorbance measurements determined the amount of free 40S or 60S ribosome subunits, 80S ribosomes and polysomes in the cells.

The absorbance peaks are shown in Figure 14. The absorbances for both strains are shown overlapped to highlight differences between the strains. The major observed difference detected is that there are fewer polysomes in the *ubc9-6* strain, implying that translation levels are reduced when SUMO conjugation levels are low. Given the levels of monosomes remained the same as the parent strain but there were less polysomes, this could indicate a reduction in translation initiation compared to translation elongation in the *ubc9-6* cells leading to less ribosomes binding to the same mRNA (Heyer and Moore 2016). Therefore, although translation related genes are expressed at a higher rate when sumoylation levels are low, translation levels are slightly reduced. Possibly explaining this, through some mechanism, cells might be attempting to compensate for reduced translation by elevating transcription of translation related genes, even though that does not lead to elevated steady state mRNA levels of these genes in the *ubc9-6* strain.

Next, we examined whether reduced overall translation levels, but elevated transcription of RPGs, in the *ubc9-6* strain led to altered ribosomal protein levels in this strain. Derivative strains expressing HA-tagged versions of RPL5 and RPS20 were generated which are representative of two RPGs that had higher RNAPII occupation at those genes in the *ubc9-6* cells, and their protein levels were determined by western blot. As shown in Figure 15, no differences in the levels of these proteins were detected in the two strains, in either SC minimal

or YPD rich media. This is consistent with our analysis of steady state mRNA, and it supports the idea that cells can buffer changes in transcription and translation to maintain appropriate levels of mRNAs and proteins (Miller et al. 2011; Timmers and Tora 2018; Kusnadi et al. 2022). To support the idea that cells buffer elevated RPG transcription in *ubc9-6* cells by balancing steady-state mRNA levels, we examined intron containing RPG RNAs. Nascent pre-mRNAs contain introns which are rapidly removed through splicing and are absent from mRNAs. As such, the level of intron-containing RNAs can be considered a measure of nascent transcription. To assess this, qPCR was performed with primer pairs that span intron-exon junctions for RPL25 and RPS27b RNAs. These two RPGs were selected as they have intron-containing regions and also had higher RNAPII occupation in *ubc9-6* cells. Indeed, as shown in Figure 16, in the *ubc9-6* strain, there is a higher level of intron-containing RNAs compared to the wild type. This supports the idea that there is a higher level of RPG transcription when sumoylation levels are low. The elevated level of transcripts is not being processed into functional mRNAs, however, which aligns with the concept of a buffering mechanism in place to balance mRNA levels when transcription levels are altered.

2.5 Discussion

Sumoylation is an important post-translational modification that can regulate many target proteins (Zhou et al. 2004; Makhnevych et al. 2009). The role of sumoylation in regulating transcription has been described previously, focusing largely on sumoylation of sequence-specific and general transcription factors (Boulanger et al. 2021; Rosonina et al. 2017). Previous research has also described the role of sumoylation in gene expression-related processes such as the regulation of histones and chromatin remodelling factors (Ouyang and Gill 2009; Srikumar et al. 2013). Sumoylation levels in the cell are known to increase in stress conditions from its normally low levels in the cell (Lewicki et al. 2015). The results presented in this chapter focus on determining how this change in global levels of sumoylation impacts transcription. This was done by investigating mutant yeast strains that harbour constitutively reduced levels of sumoylation to determine how this reduction impacts normal transcription.

Sumoylated proteins have been previously detected on chromatin. For example, Baig et al. (2021) detected sumoylation of Tfg1, a subunit of the general transcription factor TFIIF, and Sko1 and Rap1, two sequence-specific transcription factors, have also been determined to be sumoylated (Sri Theivakadadcham et al. 2019; Chymkowitch, Nguéa P, Aanes, et al. 2015). Specifically, sumoylation of Rap1 was shown to be a positive regulator of transcription of its target genes, which includes most RPGs. More generally, Ubc9 has also been found associated with activated genes, leading to the assumption that sumoylation is important for normal transcription (Rosonina et al. 2010). Our SUMO ChIP-sequencing results showed that there are at least 306 genomic sites associated with sumoylated proteins. Most of these peaks were associated with highly transcribed genes, supporting the link between active transcription and sumoylation.

Global levels of sumoylation are low when yeast grow under optimal conditions. Sumoylation is an essential post-translation modification, so abolishing all sumoylation leads to cell death (Hayashi et al. 2002). When Ubc9 activity is impaired and cannot conjugate target proteins with SUMO efficiently, cells have reduced global levels of sumoylation. We demonstrated that this reduced level of sumoylation is sufficient for cells to grow under optimal conditions and in many stress conditions, but not a mild heat shock. Previous studies that have linked elevated global sumoylation with the stress response in cells facing heat shock, osmotic stress, oxidative stress, and ethanol stress (Lewicki et al. 2015). One assumption is that cells need to have a basal level of global sumoylation that can swiftly increase in response to stress. Given that cells with reduced sumoylation could not survive heat shock specifically, it could indicate that the heat shock response in particular is dependent on changes in sumoylation levels.

The question, then, was whether the low, but essential level of sumoylation observed under non-stress conditions plays a role in controlling transcription genome-wide. By using a strain with reduced levels of global sumoylation, *ubc9-6*, we observed through RNAPII ChIP-seq that transcription was vastly altered compared to in a wildtype strain. Interestingly, reduced sumoylation did not have solely a general repressive or activating effect, instead changes in transcription levels (i.e., RNAPII occupancy) were gene specific and did not necessarily correlate with changes to gene expression. That is, in the *ubc9-6* strain, gene expression levels (i.e., steady-state mRNA levels) increased or decreased for many genes, but these effects were not well-coordinated with the changes to transcription of those genes. This indicates that reduced sumoylation of different proteins could alter gene expression in a different way depending on the context of the sumoylation event and the genes that are targeted. This adds to the complexity of the multiple impacts that sumoylation confers when modulating target proteins and its

downstream effects. Sumoylation can for example inhibit one transcription factor's activity thereby decreasing transcription of its target genes, while promoting the chromatin binding of another transcription factor, leading to increased expression of its targets (Rosonina 2019; Lyst and Stancheva 2007). Given that the changes in transcription were gene specific and that some genes were not impacted at all, this indicates that some genes are more sensitive to alteration of cellular sumoylation levels than others.

These results also gave us more insight into the role sumoylation has in response to heat shock, through changes to transcription. Since the reduced levels of sumoylation altered transcription of many genes in non-stress conditions, it was predicted that this may alter the transcription response to heat shock. This prediction was further supported by the growth assays that showed that the Ubc9-defective strains could not survive heat shock, specifically. During heat shock, reduced levels of global sumoylation did alter the transcriptional response to heat shock, as measured by RNAPII ChIP-seq, resulting in a much larger subset of genes to show elevated or reduced RNAPII occupancy during heat shock compared to in a wild-type strain. Previous studies have shown that, at least in some cases, sumoylation of transcription factors helps with the specificity of the protein's binding to the genome (Rosonina 2019). When transcription factors that are typically sumoylated are modified so that they no longer can be sumoylated, the transcription factors bind more sites than usual. This was observed in different species and different transcription factors and so it was proposed that a general, conserved role for sumoylation of transcription factors is in helping to ensure their binding only at specific, appropriate sites. SUMO-mediated modulation of transcription factor binding specificity could at least partly explain how reduced global sumoylation in the *ubc9-6* strain results in widespread

changes to transcription. It also implies a potential mechanism by which the rise in global levels of sumoylation during heat shock can help coordinate the transcriptional response to stress.

Cells have many mechanisms in place to regulate gene expression and to ensure a balance between transcription and RNA decay. Whereas below-basal levels of sumoylation resulted in changes in RNAPII occupation at many genes, this did not always correlate with corresponding changes in mRNA levels. The transcriptome (as measured by RNA-seq) showed fewer changes in conditions of reduced global sumoylation compared to changes in transcription activity (as measured by RNAPII ChIP-seq). Additionally, for many genes, the opposite pattern was observed in the RNA-seq data compared to the ChIP-seq data. This could reflect a protective mechanism that is activated when transcription levels are altered to ensure that steady-state levels of mRNA remain appropriate (Miller et al. 2011). This mechanism consists of a buffering system in which the RNA levels are balanced by adjusting transcription rates and RNA decay rates. This buffering system is important to maintain the balance of the transcriptome and so all cellular functions can be maintained as normal even when transcription levels or RNA decay levels are altered (Timmers and Tora 2018). It is one way that cells can survive different environmental and stress conditions by correcting for different amounts of transcription occurring from normal conditions.

The changes detected in active transcription and the transcriptome in conditions of reduced global levels of sumoylation did not alter the levels of ribosomal proteins in these cells. Buffering systems that are in place to adjust for aberrant transcription and mRNA levels might explain this and why Ubc9-deficient cells grow almost normally under optimal, non-stress conditions. Cells can also adjust translation depending on gene expression levels and specifically mRNA levels. This type of translational buffering alters translation levels to address changing

levels of mRNA in the cell to restore normal protein expression in the cells despite changes in mRNA levels (Kusnadi et al. 2022). Therefore, in the *ubc9-6* strain, we would expect that genes that had higher levels of RNAPII occupation leading toan increase in steady state mRNA levels, translation of those mRNA would decrease. For genes which showed decreased levels of mRNA in the *ubc9-6* strain we would predict that translation of these mRNA would increase in response to the low levels of mRNA. Given that our results revealed a net overall increased levels of gene expression in the *ubc9-6* strain, we would predict reduced levels of translation to respond to this change of mRNA levels. Indeed, this is what is observed in the polysome profiling experiments where a decrease in overall translation is observed in the cells. Future work could be done to assess the types of mRNA that are being translated in the *ubc9-6* strain to determine if there are any gene-specific differences reflected at the translation levels where there is an opposite effect on translation based on the change in mRNA level.

Altogether, the results presented give us a better understanding of the role that basal levels of global sumoylation play in gene expression. By assessing cells with reduced global sumoylation, the impact of sumoylation on transcription, translation, and cell growth were determined. Our results illustrate the importance of maintaining appropriate levels of global sumoylation for coordinating biological pathways in the cell, especially transcription, and how this regulation impacts survival to heat shock.

2.6 Figures

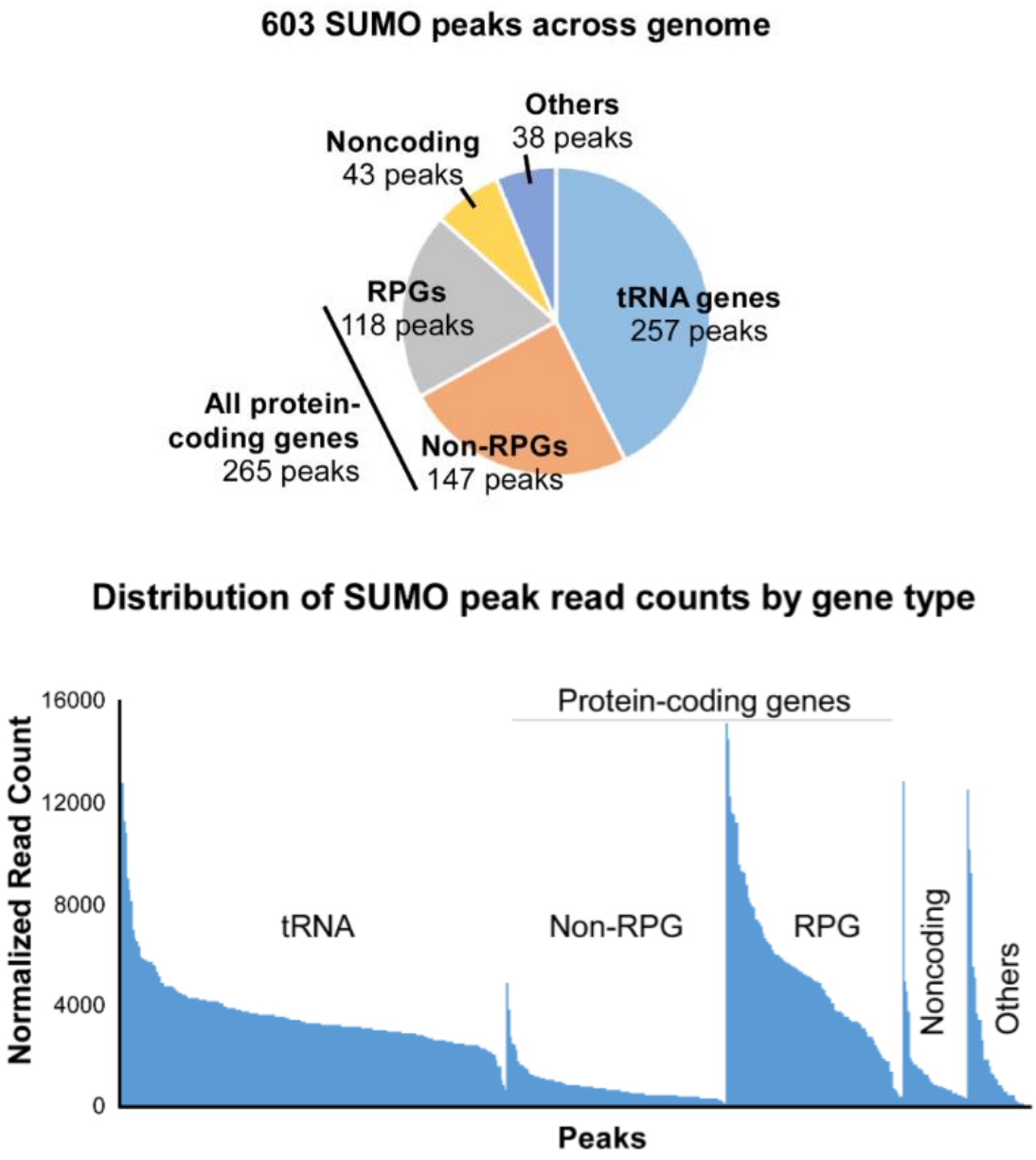


Figure 1: SUMO ChIP-sequencing results presented as a pie chart and as a histogram, showing their distribution and peak intensity. SUMO peaks were classified by gene type based on whether they overlapped gene promoters or other annotated features, and 603 peaks were detected in total. RPG refers to ribosomal protein gene and non-RPG refers to protein-coding gene that is not a ribosomal protein gene.

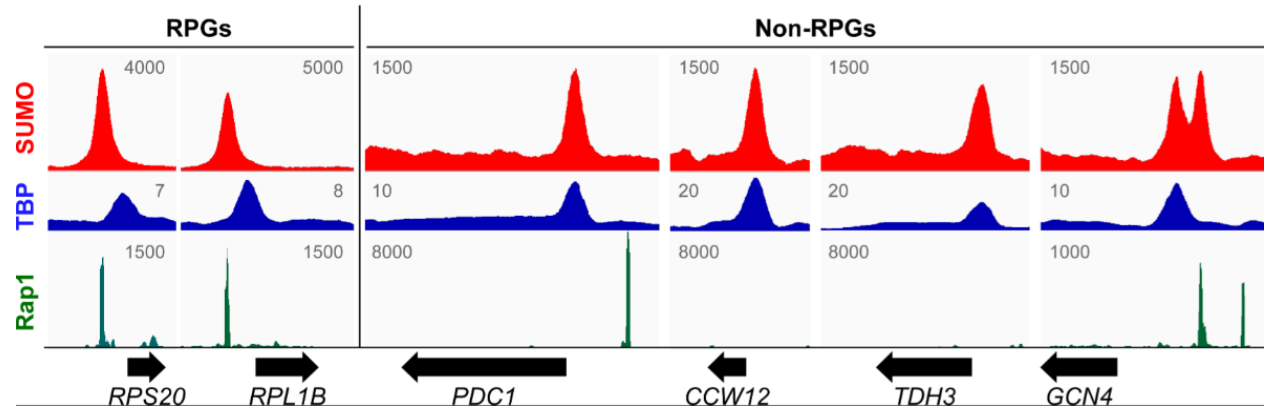
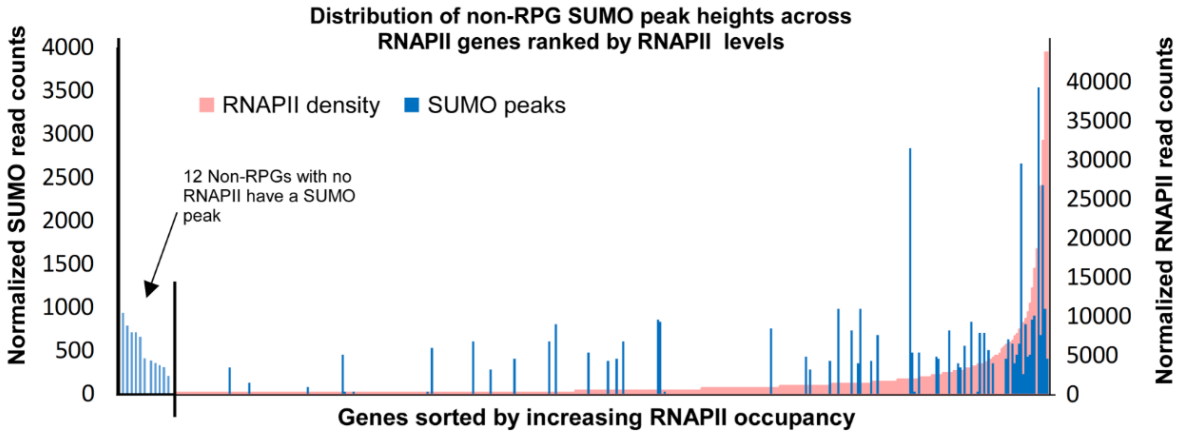


Figure 2: SUMO peaks of sample genes categorized as either ribosomal protein genes (RPGs) or non-RPGs compared to the distribution of TBP and Rap1. TBP is part of the general-transcription factor machinery while Rap1 is gene-specific transcription factor that is typically associated with ribosomal protein gene expression. Alignments of TBP and Rap1 are from published data sets, NCBI GEO database accession numbers GSM2870615 (ChIP-seq) and GSE93662 (ChIP-exo). Peaks were plotted using Integrative Genomics Viewer (IGV). Black arrows indicate open-reading frames of genes only and do not indicate transcription start sites.

A



B

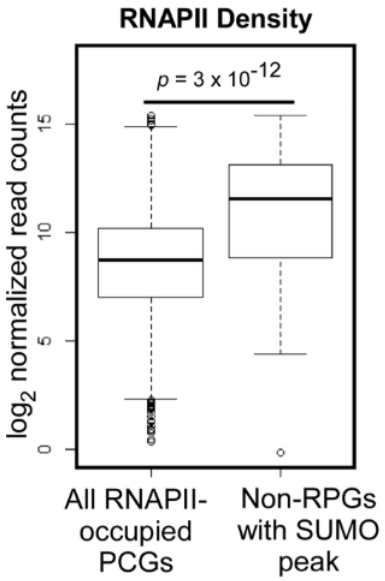


Figure 3: A) Comparing SUMO peaks with RNAPII density on the genome. 111 SUMO peaks are compared with the RNAPII occupation levels of the associated gene. PCG refers to protein coding gene and non-RPGs refers to protein coding genes that are not ribosomal protein genes. B) Box plot representing the RNAPII densities at either all PCGs or just the subset of non-RPGs that also have a SUMO peak detected. P-value is calculated through Student's *t*-test.

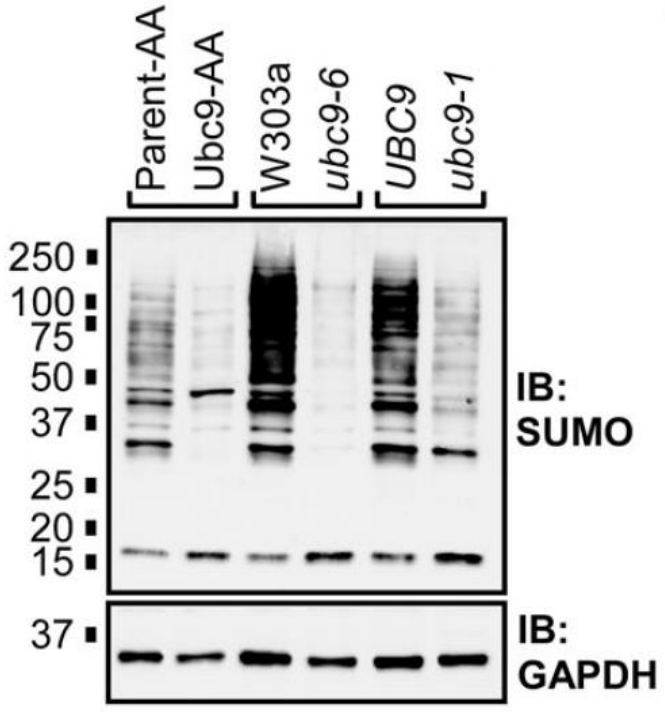


Figure 4: Global sumoylation levels in different Ubc9-defective strains compared to their respective parent strain, at optimal temperature of 30℃. The Ubc9-AA strain has an anchor away tag at the C-terminus of Ubc9 and the tag itself in this strain causes a defect in normal Ubc9 activity. *ubc9-6* and *ubc9-1* both have a point mutation in the same location. *ubc9-6* has the point mutation in normal genomic location and *ubc9-1* has the regular *UBC9* gene deleted and a *UBC9* gene ORF with the point mutation in a different location in the genome.

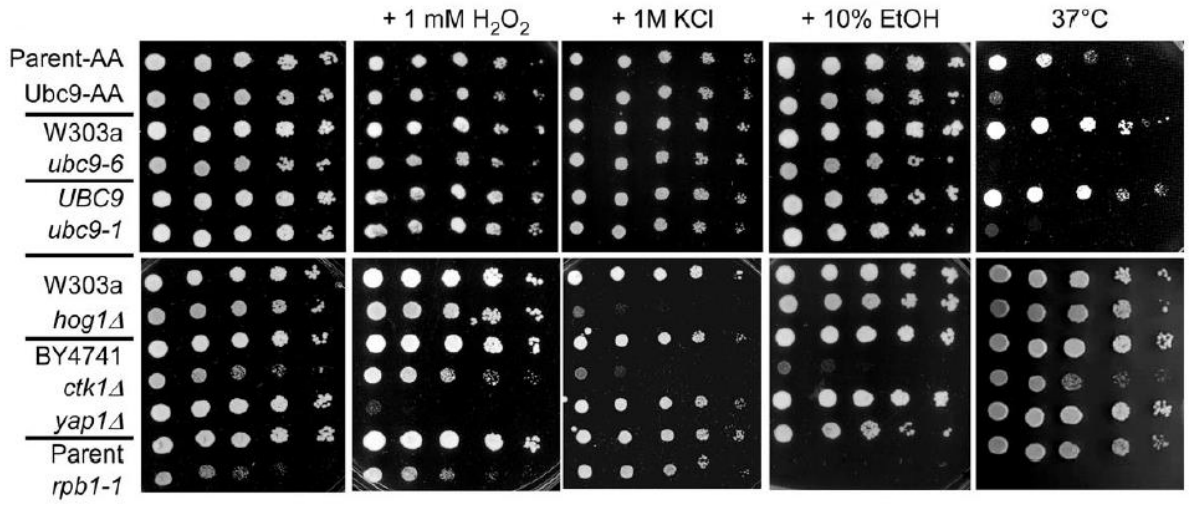


Figure 5: Drop tests to measure growth of different Ubc9 mutants in different stress conditions. The plates on the top panels include the three different Ubc9 mutants and their respective parents. The bottom panel of plates include different positive controls and their parent strains for the different stress conditions. SC refers to minimal media used for all these plates. The following were used as positive controls: *rpb1-1* for heat shock (37°C), *hog1*Δ for osmotic stress (1M KCl), *ctk1*Δ and *rpb1-1* for ethanol stress (10% EtOH), and *yap1*Δ for oxidative stress (1mM H_2O_2).

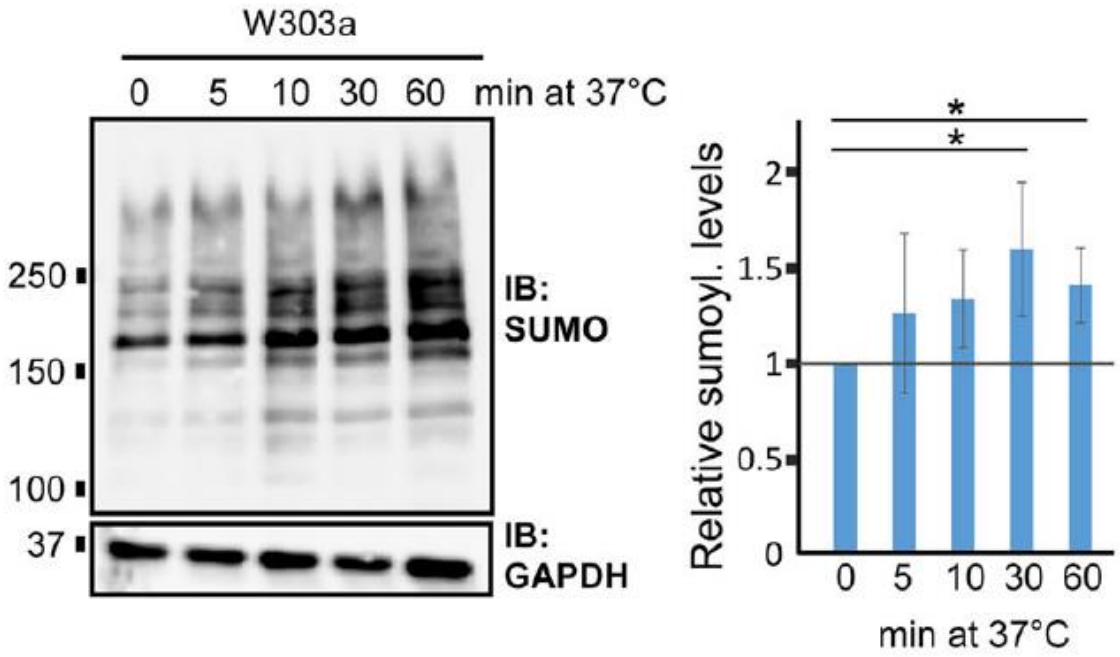


Figure 6: Global sumoylation levels of wild type strain, W303a, undergoing mild heat shock at 37°C at different time intervals. Sumoylation levels were quantified through ImageJ and presented on the right. GAPDH levels were used as a control and used to normalize global sumoylation levels. Asterisks refer to statistically significant differences (p<0.05) between the sample at 0 min of mild heat shock with samples at either 30 or 60 min of mild heat shock.

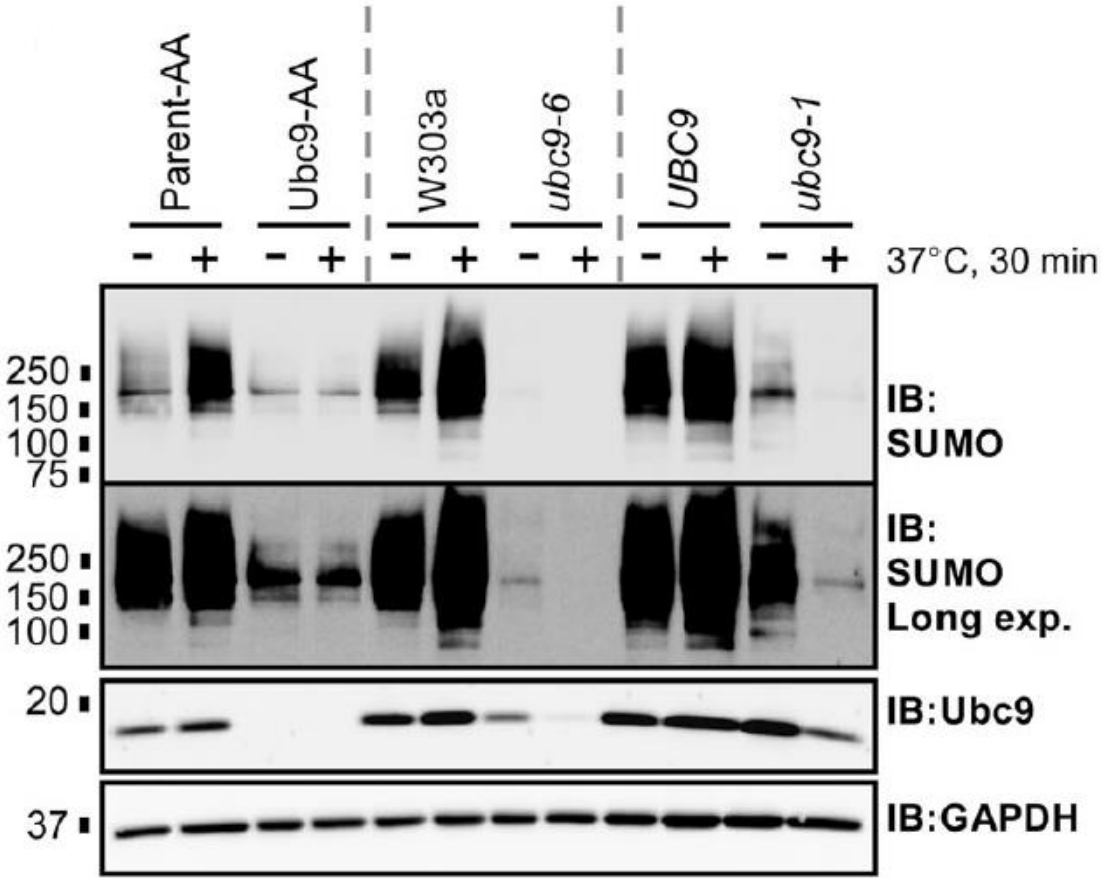


Figure 7: Global sumoylation levels in different Ubc9-defective strains compared to their respective parent strain, with and without heat shock treatment. Heat shock treatment was carried out for 30 min at 37°C, while the regular growing temperature is 30°C.

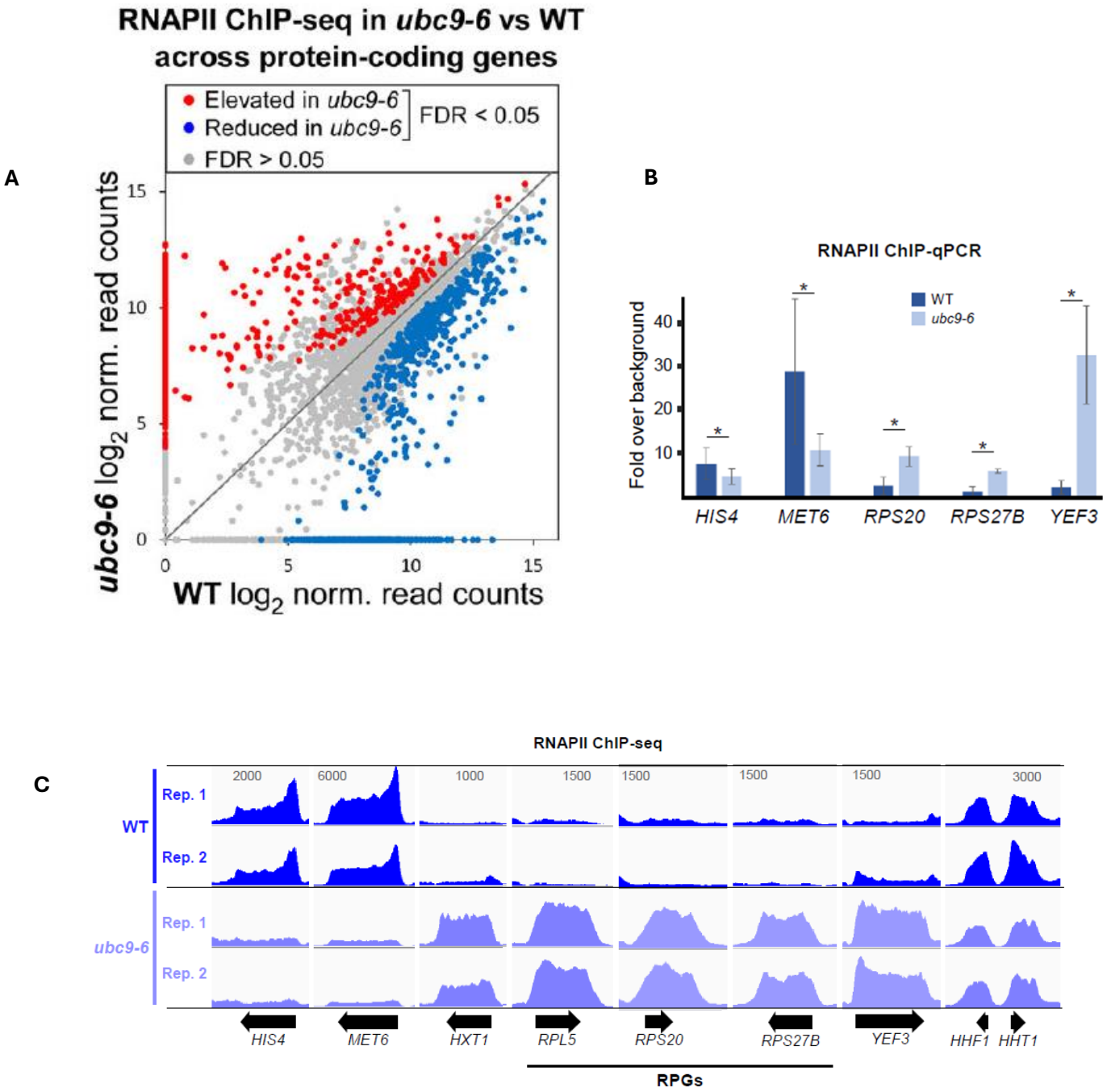


Figure 8: RNAPII ChIP-seq results using *ubc9-6* and its parent strain. A) Scatter plot depicting RNAPII ChIP-sequencing results comparing the *ubc9-6* and its parent. Two biological replicates are presented. B) ChIP-sequencing results are validated with ChIP-qPCR for select genes. *HIS4* and *MET6* represent the metabolic genes that showed a decrease in RNAPII occupation in the ChIP-seq results and *RPS20, RPS27B,* and *YEF3* represent the translation-related genes that showed an increase in RNAPII occupation in the ChIP-seq results. Asterisks mark significant differences of values of $p<0.05$ with a Student's *t*-test. C) RNAPII occupation peaks from ChIP-seq experiment are shown for select genes with either decreased, increased, or same RNAPII occupation levels in the *ubc9-6* strain compared to WT. Peaks plotted using Integrative Genomics Viewer (IGV).

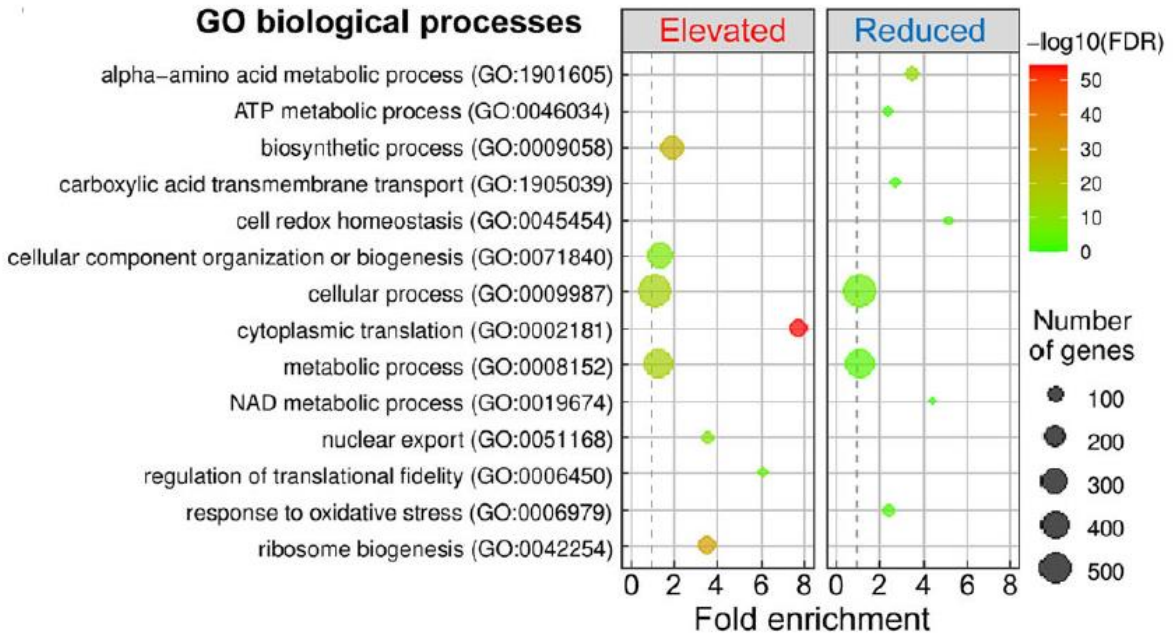


Figure 9: GO-term analysis was performed to group changes detected through the RNAPII ChIP-sequencing experiment. Elevated levels of RNAPII occupation in translation-related genes in the *ubc9-6* strain while reduced levels of RNPAII occupation were found mostly in metabolic processes related genes. The circles represent the number of genes that show either elevated or reduced RNAPII occupation in the *ubc9-6* strain compared to WT and the colour of the circle is the magnitude of difference.

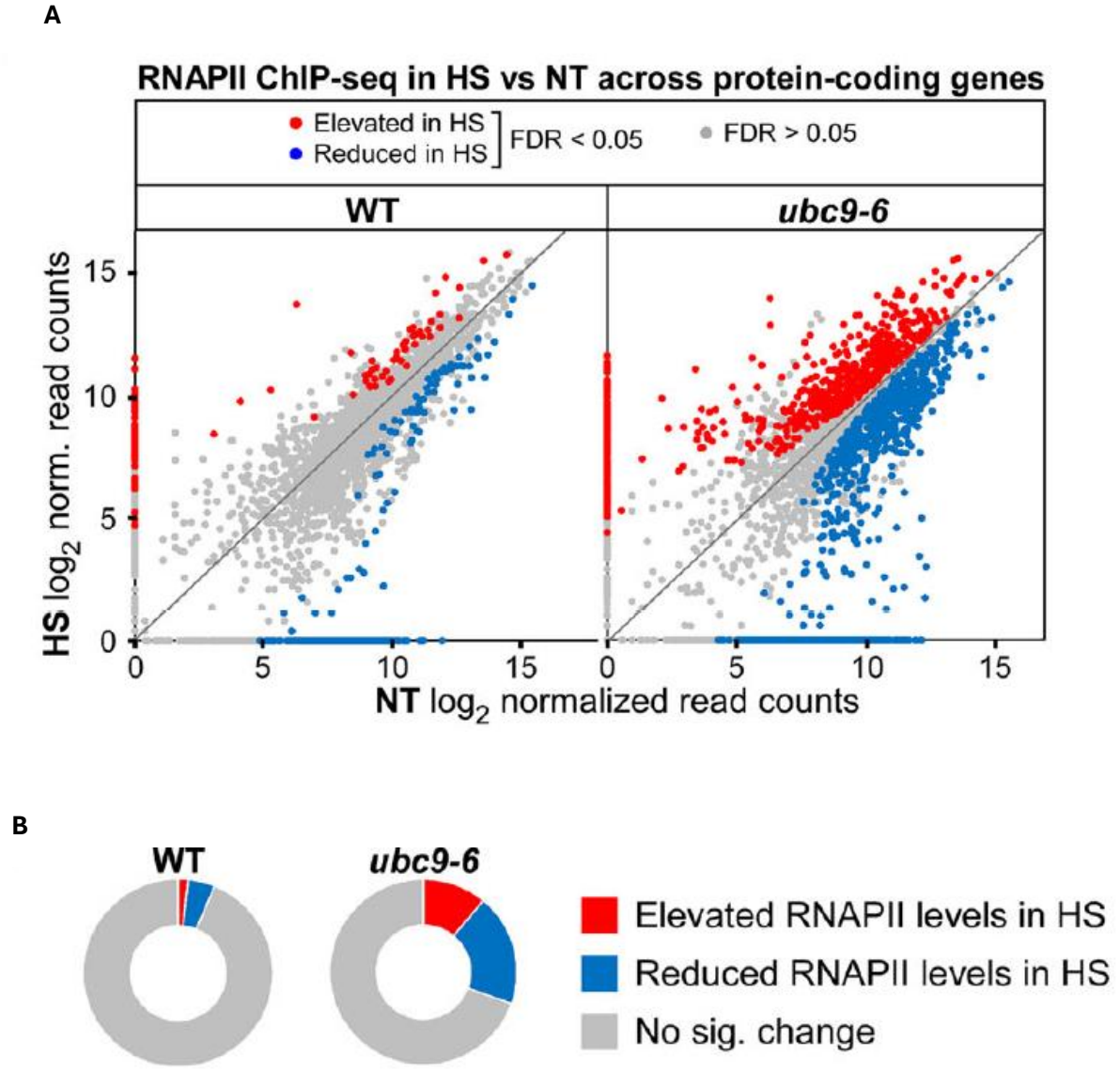


Figure 10: RNAPII ChIP-sequencing results comparing RNAPII occupation genome-wide at optimal temperature for growth at 30°C compared to mild heat shock at 37°C in both wild-type (WT) and *ubc9-6* strains. A) Scatterplot depicting the differences in RNAPII occupation while experiencing mild heat shock in comparison to RNAPII occupation at optimal temperatures in the WT or *ubc9-6* strains. B) Doughnut plots depicting proportion of protein-coding genes that show differences or no significant change in RNAPII occupation during mild heat shock.

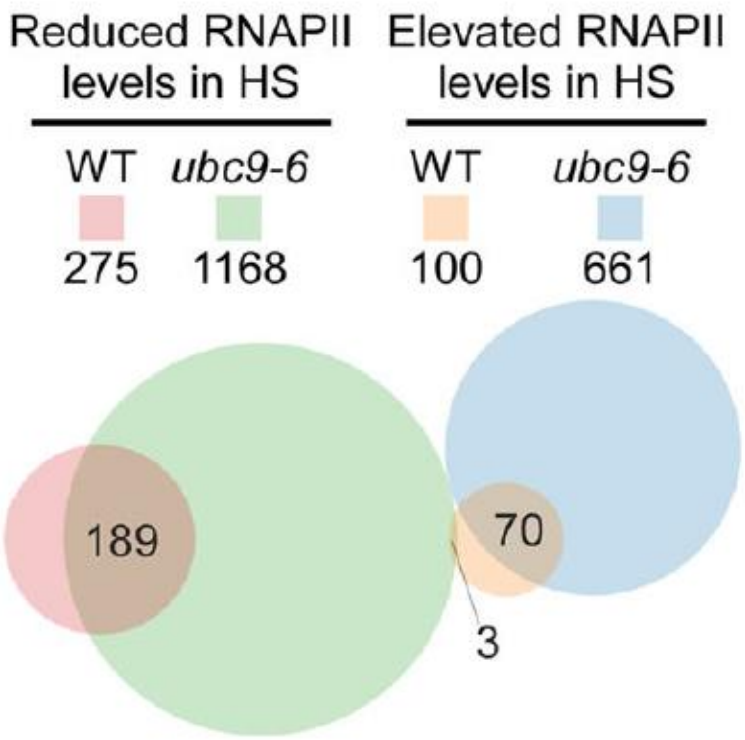
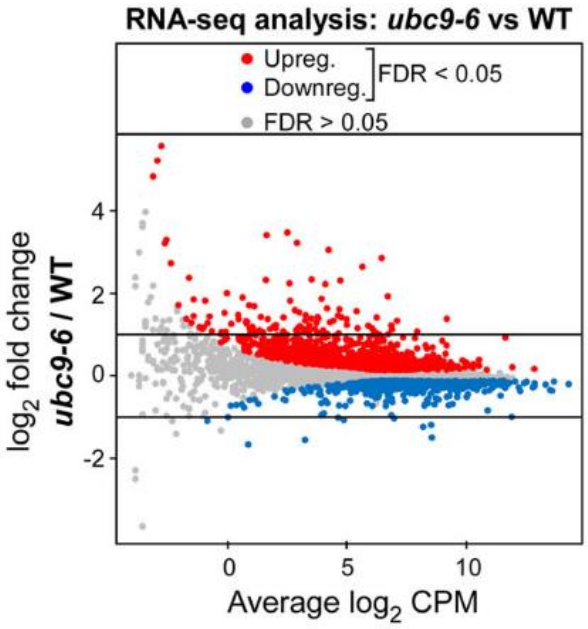


Figure 11: Venn diagram using the RNAPII ChIP-seq results depicting genes that showed RNAPII occupation changes during mild heat shock in the wild-type and *ubc9-6* strain. The numbers in the figure refer to the number of genes in that category.

A



B

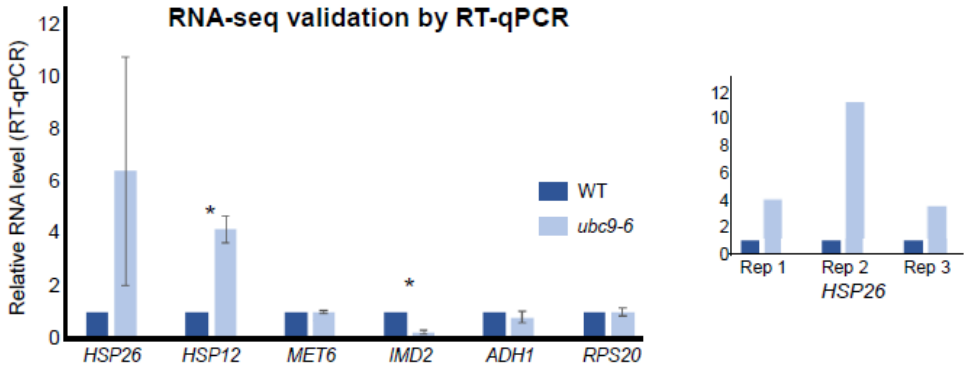


Figure 12: RNA-sequencing results depicting RNA levels in both the wild-type and *ubc9-6* strain. A) Scatterplot depicting RNA-sequencing data where genes that had higher levels of RNA expression in the *ubc9-6* strain are represented by the colour red and genes that are downregulated are represented by blue. Genes that show no significant differences are represented as gray. B) RNA-sequencing results were verified with RT-qPCR for selected genes.

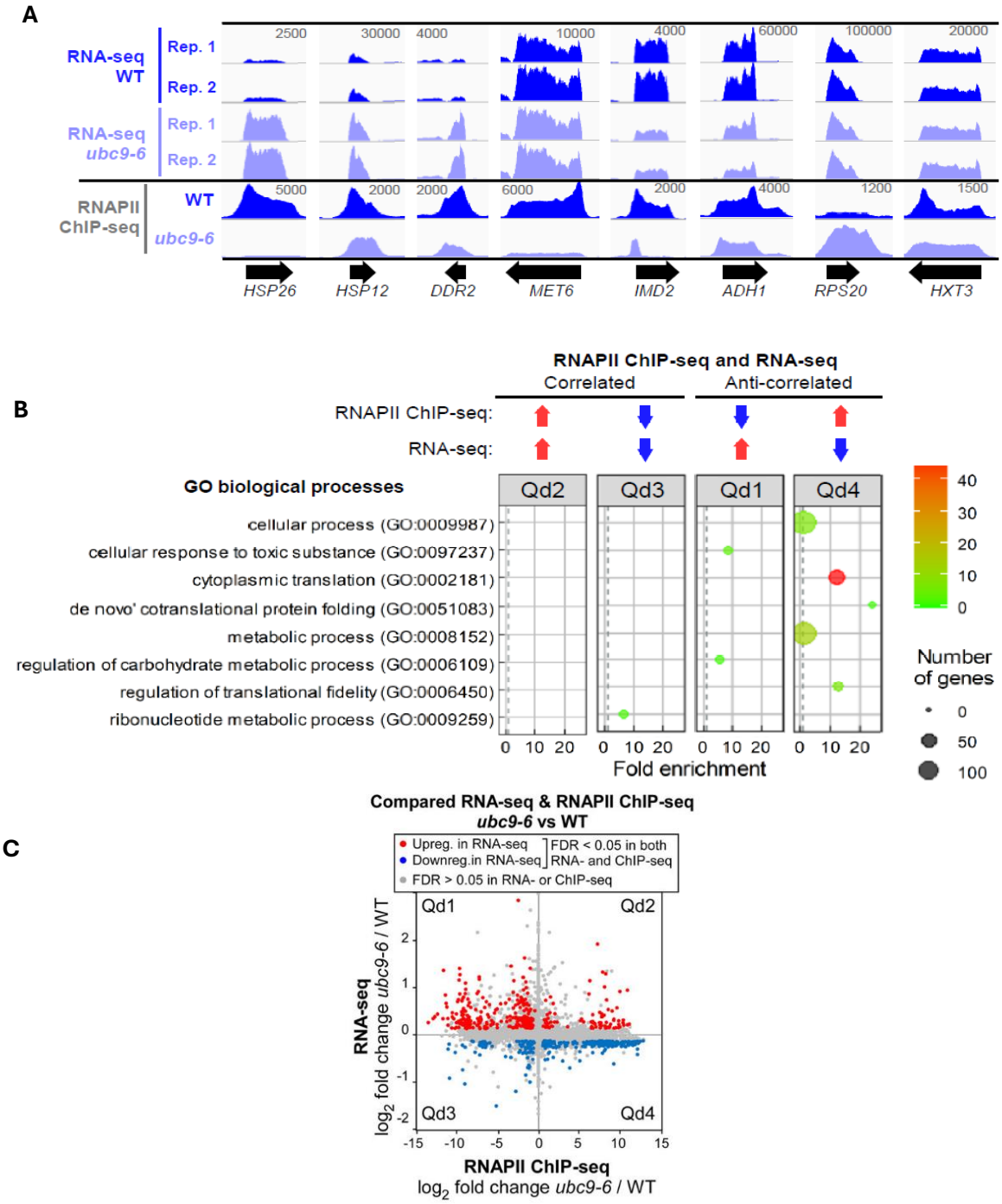


Figure 13: Comparing gene expression levels assessed through ChIP-seq and RNA-seq experiments. A) Sample genes depicting RNA-seq results and comparing them to RNAPII ChIP-seq experiments. Peaks plotted using Integrative Genomics Viewer (IGV). B) GO analysis results compare the RNA-sequencing changes in the *ubc9-6* strain with the RNAPII ChIP-sequencing results to compare transcription and steady-state RNA levels. C) Scatterplot comparing the RNA-sequencing data and ChIP-sequencing data of *ubc9-6* strain changes from the wildtype strain. The scatterplot is divided into quadrants with each one representing a specific pattern of change where correlated and uncorrelated changes are divided and whether the change is in upregulation or downregulation of gene expression.

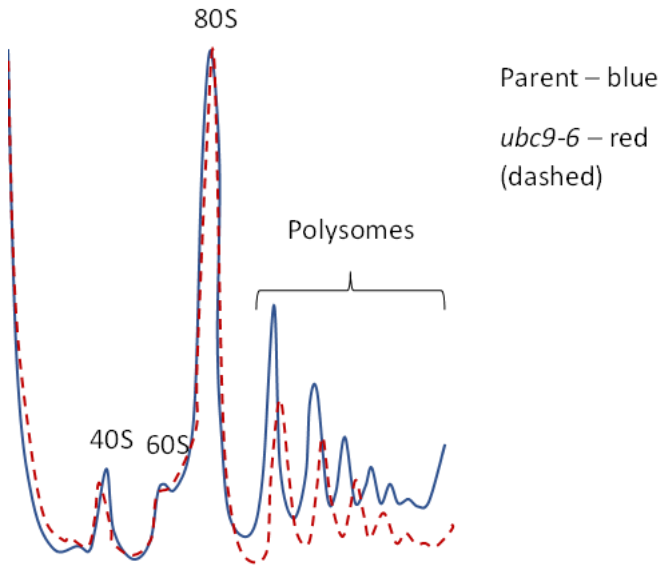


Figure 14: Polysome profiling experiments using parent and *ubc9-6* strains to assess translation. The labels above the absorbance peaks show what the peaks represent, either 40S or 60S free ribosome subunits, 80S subunits or polysomes, with each peak from left to right representing one more ribosome on mRNA. The blue line represents the parent strain while the red dashed line represents the *ubc9-6* strain peaks. Three biological trials show a decrease in polysomes in the *ubc9-6* strain, even though the levels of subpolysomes and monosomes are the same.

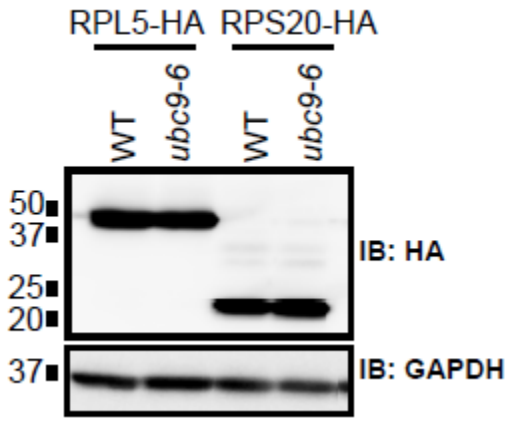


Figure 15: Comparing ribosomal protein levels by western blot. Strains with tagged RPL5 and RPS20 with 3xHA were used for this experiment. GAPDH was used for control. Levels of ribosomal proteins do not appear to be different in the two strains, parent or *ubc9-6.*

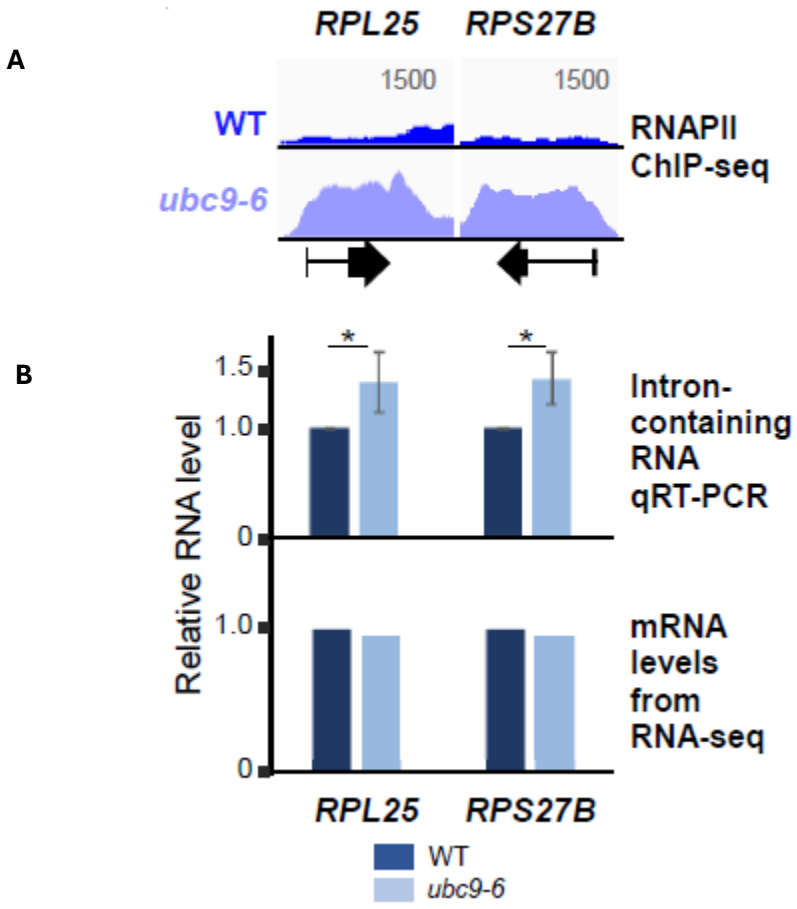


Figure 16: Comparing nascent transcription of RPGs in parent and *ubc9-6* strains. A) RNAPII occupation at the coding region of RPL25 and RPS7B based on RNAPII ChIP-seq data with wild type and *ubc9-6* strains. Peaks plotted using Integrative Genomics Viewer (IGV). B) RT-qPCR results comparing intron-containing pre-mRNA with processed mRNA in both the wild-type and *ubc9-6* strain. Asterisks refer to significant difference with p-value <0.05 when Student's t-test was conducted. mRNA levels shown on the bottom panel for comparison based on the RNA-seq data representing the steady-state mRNA levels.

2.7 Supplementary Data

This table is presented as an Excel Sheet and can be found under supplemental material of the published paper at PLOS Genetics

Table S1 (original manuscript Table S9): SUMO peaks from the SUMO ChIP-sequencing experiment using WT and *ubc9-6* strain listed and organized into different groups of promoters of genes associated with peak. https://doi.org/10.1371/journal.pgen.1009828.s013

All of these tables are presented as Excel Sheets and can be found under supplemental material of the published paper at Molecular and Cellular Biology at https://doi.org/10.1080/10985549.2023.2166320

Table S2 (original manuscript Table S1): RNAPII ChIP-sequencing analysis of two independent biological replicates of RNAPII occupation genome-wide in the *ubc9-6* strain and its parent strain (wild-type strain) in both optimal temperatures and after heat shock at 37°C for 12 min. All peak concentrations listed along with fold changes in peaks listed and corresponding False Discovery Rate (FDR).

Table S3 (original manuscript Table S2): GO Term analysis of either the RNAPII ChIP-sequencing data, RNA-sequencing data shown in groups of upregulated genes or downregulated genes. GO Term analysis also done comparing both sets of sequencing data separated into four different categories depending on correlation between the two data sets and upregulation or downregulation of gene expression.

Table S4 (original manuscript Table S3): RNA-sequencing analysis of *ubc9-6* strain and wild-type strain of two independent biological replicates. Data is shown for all counts and differential gene expression between the two strains along with significant differences in gene expression.

Table S5 (original manuscript Table S4): Comparing RNAPII ChIP-sequencing and RNA-sequencing data to compare the gene expression for both upregulated and downregulated genes and whether there is any correlation between the two data sets. LogFC (Logarithmic Fold Change) and corresponding FDR were calculated and presented.

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Chapter III: Dynamic sumoylation is essential for suppressing stress-induced gene expression in non-stress conditions

This Chapter consists of an entire manuscript that was submitted for publication:

Dynamic sumoylation is essential for suppressing stress-induced gene expression in non-stress conditions

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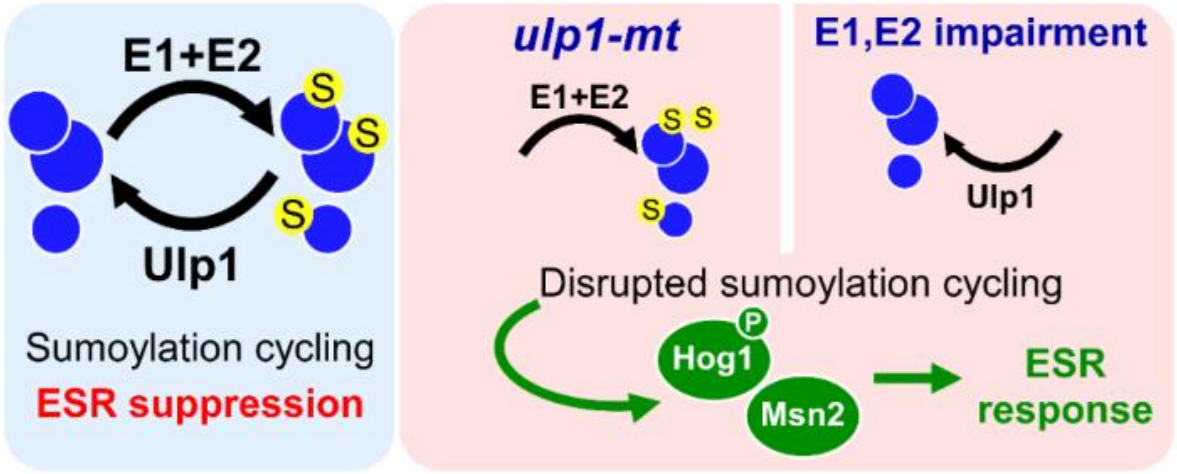
Author contributions:

Marjan Moallem conducted RNA-seq experiments, assisted in SUMO ChIP-seq experiments, conducted the western blots and RT-qPCR experiments using the drug BCP, conducted all western blots analyzing phosphorylation status of Hsf1 and Hog1, and conducted all SUMO IP experiments. J. Bryan McNeil transformed yeast strains and performed qRT-PCR, western blots, and drop test experiments using those strains (Figures 2, 3 A-C, 4D, 4E, 5A-C). Bejamin Bergey assisted in SUMO-ChIP seq experiments and conducted ChIP-qPCR experiments using *ULP2* and *ulp2*Δ strains. Dr. Emanuel Rosonina analyzed sequencing data.

3.1 Graphical Abstract



3. 2 Abstract

Environmental stresses such as heat shock trigger widespread changes in gene expression, collectively known as the environmental stress response (ESR), along with a global increase in SUMO conjugation. However, whether elevated sumoylation contributes to establishing the ESR gene expression program remains unclear. Here, we show that mutant yeast with constitutively high sumoylation levels due to a defect in the SUMO protease Ulp1, *ulp1-mt*, display stress-like gene expression changes, including strong upregulation of heat shock-induced genes, in the absence of external stress. The ESR regulators Hog1 and Msn2 are constitutively active in this mutant and drive the observed transcriptional changes. Surprisingly, both heat shock and Ulp1 impairment lead to a reduction in chromatin-associated SUMO across all gene classes, suggesting that increased sumoylation of chromatin-bound factors does not underlie stress-like transcriptional changes. Intriguingly, mutant yeast with reduced overall sumoylation due to impairment of sumoylation enzymes also show ESR-like transcription effects, including induction of heat shock genes. These findings indicate that disruption of normal sumoylation/desumoylation cycling, rather than absolute SUMO conjugation levels, triggers an ESR-like transcriptional program. This work reveals a new layer of regulation in stress-responsive gene expression and implicates SUMO homeostasis as a key modulator of transcriptional stress signaling.

3.3 Introduction

In eukaryotes, hundreds of proteins are subject to sumoylation, a post-translational modification that can impact activity, stability, interactions, and localization of its targets (1–7). Fractionation experiments reveal that most SUMO-conjugated proteins are chromatin-associated, consistent with the frequent identification of SUMO targets involved in DNA metabolism, RNA synthesis, and RNA processing (8). Among these are histones, multiple components of the general RNA polymerase II (RNAPII) transcription machinery, including TFIIF and RNAPII itself, as well as the majority of sequence-specific transcription factors (SSTFs) (8–11), indicating that sumoylation of gene-bound factors may regulate the expression of numerous protein-coding genes. However, sumoylation can also regulate gene expression by modifying cytoplasmic signaling proteins or by controlling the stability or nuclear access of transcription factors (12–16), indicating that its effects are not limited to gene-bound targets. Indeed, extensive evidence supports the idea that SUMO regulates gene expression through multiple mechanisms, contributing to both activation and repression of transcription in a gene- and context-specific manner (11, 17, 18).

Sumoylation involves the covalent and reversible attachment of the 12-kDa SUMO peptide to target proteins through a three-enzyme cascade that is analogous to ubiquitination. The dimeric E1 enzyme, whose subunits are called Aos1 and Uba2 in budding yeast, activates free SUMO peptides and transfers them to the E2 conjugating enzyme (19). In all eukaryotes, Ubc9 is the sole E2 enzyme which is responsible for the attachment of SUMO to specific lysine residues within target proteins, a process which is facilitated in vivo by E3 ligases (20). Desumoylation is accomplished by SUMO proteases, including Ulp1 and Ulp2 in yeast, which have distinct targets and roles (19, 21). Whereas Ulp2 functions in regulating levels of polysumoylation of its targets,

Ulp1, which is situated on the inner surface of the nuclear pore complex, processes the SUMO precursor peptide to its mature form and maintains low levels of conjugation for most SUMO targets under normal (i.e., non-stress) growth conditions (22–25). Indeed, most SUMO conjugations in yeast last mere seconds as a consequence of the continuous activity of Ulp1, an observation that is consistent with sumoylation being a highly transient modification (24, 26, 27).

While cellular levels of sumoylation are generally low under optimal growth conditions, exposure to heat, osmotic, oxidative, and ethanol stresses leads to a rapid elevation of SUMO conjugation (23, 28–32). This surge in sumoylation, often referred to as the SUMO stress response, occurs largely through a widespread reduction in SUMO deconjugation that results from stress-mediated regulation of SUMO proteases. During ethanol stress, for example, Ulp1 is re-localized to the nucleolus where it cannot access its targets, while heat shock triggers degradation of Ulp1 in yeast and inactivation of several human SUMO proteases (23, 33, 34). Yeast mutants that are deficient in SUMO conjugation, and human cells lacking SUMO isoforms SUMO2 and SUMO3, cannot survive heat shock, suggesting that the stress-mediated surge in SUMO conjugation serves an evolutionarily conserved protective role during heat stress (23, 35). Although the precise role for elevated sumoylation during heat and other stresses remains unclear, some have postulated that elevated sumoylation of chromatin associated factors functions to control gene expression as part of the environmental stress response (ESR) (31, 32, 36).

The ESR is a cellular program that enables yeast cells to adapt to diverse environmental stressors by modulating gene expression through multiple mechanisms (37–40). Although each stress condition elicits a unique transcriptional response, many stresses induce overlapping sets

of genes (38, 39). Central to this response are master regulatory transcription factors that coordinate the expression changes required for survival. Hsf1 is activated in response to heat shock, glucose starvation, and misfolded proteins, regulating hundreds of downstream genes (41, 42). Msn2 and Msn4 are similarly activated under a range of stress conditions, including heat shock, osmotic stress, and oxidative stress, and function redundantly to control stress-responsive gene expression (43). Another key regulator is Hog1, a MAP kinase best known for its role in the osmotic stress response, but also involved in other stress responses, including heat shock (44, 45). Unlike Hsf1, Msn2, and Msn4, Hog1 is activated during heat shock through an indirect mechanism involving phosphorylation via the cell wall integrity pathway, one of the five MAPK signaling pathways in yeast (46). Once activated, Hog1 cooperates with other transcription factors, including Msn2 and Msn4, to drive the expression of stress-responsive genes.

Here, we explore whether elevated global sumoylation levels play a role in establishing the transcriptional response to stress. We find that non-stressed mutant yeast that harbour constitutively high SUMO conjugation levels show gene expression changes that resemble those that result from heat shock, including highly elevated expression of some ESR genes. Unexpectedly, strains with constitutively low SUMO conjugation levels also show ESR-like gene expression pattern changes, suggesting that it is the disruption of dynamic sumoylation, not simply elevated or reduced SUMO conjugation levels per se, that triggers an ESR-like transcription response. Finally, we implicate activation of Hog1 and, in particular, Msn2, as key for mediating the ESR-like response in non-stress conditions when sumoylation and desumoylation are disrupted. Together, our findings demonstrate that maintaining cycles of SUMO conjugation and deconjugation is important for suppressing the transcriptional stress response in the absence of stress.

3.4 Materials and Methods

Yeast growth

Yeast strains used in this study are listed in Table S1. Cultures were grown in rich medium (YPD), or synthetic complete medium (SC) where specified, shaking at 30°C until they reached optical densities (ODs) between 0.5 to 0.8 at an absorbance of 595 nm. For heat shock, cultures were transferred to an incubator set to 42°C (or 37°C where indicated) for the indicated times. For treatment with bathocuproine (BCP; Sigma; cat. 140910), a stock solution was prepared by dissolving 70 mg in 100 mL of ethanol, then samples were treated with the indicated final concentrations of BCP or with an equal volume of ethanol for controls. To compare growth of different strains, spot assays were performed as follows. Strains were grown overnight in YPD then diluted to an optical density (OD at 595 nm) of 0.2. Samples were then spotted side-by-side in five-fold serial dilutions on YPD solid-media plates. Plates were incubated at either 30°C or 37°C and images were taken after 1 – 3 d.

Generation of new yeast strains

Previously unreported Kl TRP1 and URA3 knockout strains were generated by transformation with PCR-amplified DNA fragments that were incorporated at specific genomic loci through homologous recombination (47). The *MSN2-6HA::Kl TRP1* tagged strains were generated by insertion of the 6HA::Kl *TRP1* PCR amplicon cassette at the *MSN2* genomic loci in the MH1006 and MH1018 parent strains through homologous recombination as previously described (47). Proteins were C-terminal tagged with a shortened IAA17 degron (mAID) with sequences available upon request (48). PCR was used to assemble and amplify the 6HA-Kl *TRP1*/mini degron tag. PCR products included approximately 50 bp of gene-specific sequences for homologous recombination.

Protein lysates and immunoprecipitation (IP)

Cultures of 10 mL were harvested through centrifugation at 3000 g for 5 min, then pellets were washed with 10 mL of IP buffer, which consists of 50 mM Tris-HCl, pH8; 150 mM NaCl; 0.1% nonidet P-40 (NP40); supplemented with a protease inhibitor cocktail (Sigma; cat. P8215), 1 mM phenylmethylsulphonyl fluoride (PMSF), and 2.5 mg/mL N-ethylmaleimide (NEM; Sigma; cat. E3876). Samples were then resuspended in 500 µL of IP buffer. Lysis and IP were performed as described previously (8, 23). For IPs, 15 µL of pre-conjugated HA agarose beads (Sigma; cat. A2095) were used. For phosphatase treated samples, IP buffer without EDTA and NEM was used, and lysates were treated by adding 600 units/µL of λ phosphatase (NEB; cat. P0753S) in 1X NEBuffer for PMP (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij-35, and 1 mM MnCl2, at pH of 7.5), then incubated for 30 min at 30°C. Samples were then boiled in 2X SDS sample buffer for 5 min at 95°C.

Western blots

Prior to western blotting, protein lysates were analysed by SDS-polyacrylamide gel electrophoresis (PAGE). For Hsf1 blots, 6% Phos-tag gels containing 0.05 mmol/L Phos-tag acrylamide solution (NARD Institute; cat. AAL-107) and 0.1 mmol/L MnCl2 were used and washed with 1 mmol/L EDTA for 10 min prior to transfer. Wet transfers were performed at 100 V for 1 h using a buffer containing 10% methanol, 50 mM Tris-HCl, and 380 mM glycine. Nitrocellulose membranes were used in all cases except for analysis with Hsf1, in which case PVDF was used. Primary antibody incubation was performed overnight using the following dilutions: SUMO (Santa Cruz; cat. sc-28649) 1:1000, GAPDH (Sigma; cat. G9545) 1:3000, HA (Sigma; cat. H6908) 1:1000, Hog1 (Santa Cruz; cat. sc-165978) 1:1000, Hog1p (NEB; 92155) 1:1000, Hsf1 (49) 1:3000. Chemiluminescence imaging was performed with a MicroChemi

Imager (DNR).

RNA analysis

For isolating RNA, 15-mL cultures were harvested through centrifugation at 3000 g for 4 min, then RNA was extracted using a phenol-based freeze-heat method that was previously described (8), with the addition of an extra chloroform wash before ethanol precipitation. RT-qPCR was performed as previously described using primer pairs listed in Table S2, and a CFX Opus real-time PCR detection system (Bio-Rad) (8, 50). The averages of three biological trials were presented, and standard deviations are plotted as error bars, with statistical analysis performed as described below. For RNA-seq, samples were further purified using the RNeasy kit (Qiagen) and eluted with 100 µL of nuclease free water. Further details of the RNA-seq procedure and bioinformatics analysis are indicated in Table S3, and analysis results are shown in Table S4. For heat shock and *ubc9-1* comparisons (Figs. 1E, 1F, and 1G), previously published datasets were used with database accessions GSE135568 (GEO database) and PRJEB7579 (ENA database), respectively (51, 52). Details of the analysis of these datasets is indicated in Table S4.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously using 50-mL culture volumes and 10 µL of the SUMO antibody for IP (50). Analysis of isolated chromatin by qPCR was performed as previously described using primers listed in Table S2 and the CFX Opus real-time PCR detection system (Bio-Rad) (50). Gene-specific signals are shown relative to the signal at a non-transcribed region of Chromosome V (Fig. 6C) or to input signals (Fig. S2A). The averages of three biological trials were presented, and standard deviations are plotted as error bars, with statistical analysis performed as described below. ChIP-seq was performed as previously described, with

details of the analysis indicated in Tables S5 and S7, and analysis results shown in Tables S6 and S8 (50). SUMO and RNAPII ChIP-seq analyses in the W303a strain, with and without heat shock, were previously described and corresponding datasets were previously deposited to the GEO database with accession GSE167428 (8). ChIP-seq and RNA-seq genome alignment views were generated using the Integrative Genomics Viewer (IGV; desktop version 2.16.2) and the sacCer3 yeast genome assembly.

Statistical analyses

For the statistical analyses shown in Figs. 2A, 3B, 4E, and 5C, GraphPad Prism (version 10.3.1) was used for ANOVA analyses. In each case, ordinary one-way ANOVA was performed with a Fisher's least significant difference (LSD) test. Sample pairs showing no significant difference are indicated with ns, whereas asterisks indicate significant difference between indicated pairs as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. For the statistical analyses shown in Figs. 3F, 3G, 5A, 6C, and S2A, two-tailed Student's *t*-tests were applied, with *p*-values < 0.05 indicated with an asterisk. Output for all statistical analyses is presented in Table S9. Data for all graphical figures are presented in Table S10.

3.5 Results

3.5.1 Gene expression patterns in *ulp1-mt* and *ubc9-6* cells resemble stress-induced changes

Previously, we demonstrated that a mild heat shock of 37°C results in the degradation of Ulp1 and a concomitant increase in SUMO conjugation levels in yeast cells (23). To determine whether this effect is also observed after a more severe heat shock, cultures growing at the optimal temperature of 30°C were transferred to a 42°C incubator for a range of durations, then lysates were prepared and analyzed by immunoblot. As shown in the SUMO immunoblot in Fig. 1A, overall sumoylation levels increased rapidly during the severe heat shock to a maximal level around 30 min before dropping somewhat by 60 min. In a strain expressing an HA epitope-tagged version of Ulp1 from its natural locus, HA immunoblot showed that a severe heat shock also results in the loss of Ulp1, with virtually none being detected by 30 min (Fig. 1B). The increase in SUMO conjugation levels after heat shock is reminiscent of increased sumoylation observed in a strain harbouring a partially defective and temperature-sensitive mutant form of Ulp1, *ulp1-mt* (also known as *ulp1-I615N*; (53)). To compare these directly, cultures of the *ulp1-mt* and its wild-type parent strain, *ULP1*, were grown continuously at 30°C or treated with a 12-min 42°C heat shock. Side-by-side SUMO immunoblot analysis showed that, indeed, both untreated *ulp1-mt* and heat-shocked wild-type cells show a similar level of elevated sumoylation (Fig. 1C). These data strongly support a role for the loss of Ulp1 activity in elevating cellular levels of sumoylation during both mild and severe heat shocks.

Considering the strong connection between sumoylation and transcriptional regulation, one possibility is that elevated sumoylation during heat shock can itself facilitate the establishment, at least in part, of the environmental stress response (ESR) gene expression pattern. To investigate this, we sought to determine whether there are similarities in the

transcriptomes of untreated *ulp1-mt* cells, which show constitutively high sumoylation, and heat-shocked wild-type cells, which show transiently increased sumoylation. The transcriptome of the *ulp1-mt* strain has not been reported, so we isolated RNA from *ULP1* and *ulp1-mt* cultures grown at 30°C and performed RNA-seq (Table S3). As shown in Fig. 1D and Table S4, approximately half of all genes express at significantly different levels in the *ulp1-mt* and *ULP1* strains, with 322 showing at least twofold higher levels, and 43 showing at least twofold lower levels, in the *ulp1-mt* strain. Intriguingly, the gene with the most significant and dramatic difference in expression is the heat-induced gene *HSP12*, which is upregulated 37-fold in *ulp1-mt* cells (Table S4 and see Fig. S1A).

Next, we compared the *ulp1-mt* transcriptome with that of heat-stressed cells obtained from a previously published study in which a wild-type yeast strain grown at 30°C was transferred to 39°C for 20 min (51). Specifically, we compared gene expression changes in *ulp1-mt* cells and in heat-shocked wild-type cells, relative to their respective controls (*ULP1* and untreated wild-type cells). As shown in Fig. 1E, S1A, and Tables S4 and S10, gene expression changes in the two strains show a moderate level of similarity, with a Pearson coefficient, *r*, of 0.47. When considering only genes that show significant changes compared to their respective controls, however, there is a strong correlation ($r = 0.78$; and see heat map in Fig. 1E). Intriguingly, when considering only the direction but not the magnitude of change, 71% of genes that are significantly upregulated in heat-shocked cells are also significantly upregulated in *ulp1-mt*, while 66% of heat shock-downregulated genes are also downregulated in *ulp1-mt*. These data indicate that heat shock and impaired Ulp1 activity, both of which result in elevated levels of SUMO conjugation, have similar effects on the transcriptome.

We reasoned that gene expression changes that result from elevated sumoylation in the

ulp1-mt strain may be reversed in a strain harbouring constitutively low levels of sumoylation. To test this, we compared our *ulp1-mt* data with a published RNA-seq analysis performed in a strain expressing a partially defective form of Ubc9, *ubc9-1*, and its parent strain (52). Surprisingly, as shown in Fig. 1F and Tables S4 and S10, changes in gene expression due to Ubc9 impairment largely resemble those that result from impairment of Ulp1. Indeed, most genes showing upregulation in the *ubc9-1* strain are also upregulated in *ulp1-mt*, and most downregulated genes in *ubc9-1* are also downregulated in *ulp1-mt* (Fig. 1F heatmap). Consistent with this, gene expression changes in *ubc9-1* and *ulp1-mt* show a comparable degree of similarity to the heat-shock data (compare Figs. 1E and 1G). Observing that elevating and reducing global sumoylation levels have similar effects on the transcriptome is unexpected. However, this suggests that modulating SUMO conjugation and deconjugation cycles, which we refer to hereafter as "sumoylation cycling", rather than the presence or absence of the SUMO modification itself, is key to controlling gene expression patterns. As such, when dynamic sumoylation is disrupted by inhibiting either sumoylation (e.g., in *ubc9-1*) or desumoylation (e.g., in *ulp1-mt*), similar effects are seen, and intriguingly, these effects resemble those triggered by heat shock.

3.5.2 Disrupting sumoylation or desumoylation results in expression of stress-induced genes

To validate the RNA-seq comparisons described above and to explore the idea that disrupted sumoylation cycles can lead to an ESR-like gene expression pattern, we examined expression levels of stress-induced genes in strains that display constitutively altered SUMO conjugation levels (Fig. 2A). These include the *ulp1-mt* and *ubc9-1* strains mentioned above and four strains that display varying levels of reduced sumoylation. Like *ubc9-1*, the *ubc9-6* strain harbours a partially inactivating point mutation in Ubc9, while in the UBC9-AA strain, fusion to

the rapamycin-induced cytoplasmic-targeting Anchor Away tag itself reduces Ubc9 activity, even in the absence of rapamycin (Fig. 2B; (23)). Similarly, fusion of either of the SUMO E1 enzyme subunits, Aos1 or Uba2, to the mini auxin-induced degron tag, mAID (54), leads to reduced sumoylation, even in the absence of auxin analogs (strains AOS1.mAID and UBA2.mAID; Fig. 2B). RNA was isolated from each of the six sumoylation-altered strains and their respective wild-type or parent strains, and RT-qPCR was performed on heat shock-inducible genes (*HSP12*, *HSP26*, *DDR2*, and *HSP82*), the ribosomal protein gene *RPS22B*, and the constitutively expressed gene *PM*A1, either with or without a 5-min heat shock at 42°C.

Consistent with the RNA-seq analyses, *HSP12* is highly expressed in unstressed *ulp1-mt* and *ubc9-1* strains, strikingly, at levels that are even higher than when their respective wild-type parent strains were heat shocked (Fig. 2A). Heat shock did not result in a large increase in *HSP12* expression in these strains, suggesting that it is already near-maximal. A similar effect was observed in the *ubc9-6* and AOS1.mAID strains, whereas the UBC9.AA strain showed a more modest increase and the UBA2.mAID strain showed no significant increase in *HSP12* levels in unstressed conditions. Notably, of all the sumoylation-deficient strains tested, UBC9.AA and UBA2.mAID have the smallest defect in sumoylation (i.e., smallest reduction compared to their parent strains; Fig. 2 B). The level of *HSP12* expression, therefore, appears to be related to the severity of the sumoylation/desumoylation defect in these strains (compare Figs. 2A and 2B). Analysis of *HSP26* and *DDR2* also revealed high levels of expression in a manner dependent on the severity of the sumoylation defect, but expression levels of *HSP82*, *RPS22B*, and *PMA1* were unaffected or barely affected by the defects in these strains (Fig. 2A). These results indicate that a subset of ESR genes is highly expressed in non-stress conditions when sumoylation cycling is disrupted, implying that sumoylation and desumoylation of one or more

factors is needed to suppress their expression.

Next, we examined whether disrupted sumoylation cycling and constitutive expression of stress-response genes in these strains impacts cell growth in normal and heat-shock conditions. A temperature of 37°C was used to assess temperature sensitivity in this case since many yeast strains are unable to sustain growth at temperatures greater than 37°C (23). As previously reported, strains *ulp1-mt* and *ubc9-6* show modest growth defects at 30°C, and like *ubc9-1*, they are unable to grow at 37°C (Fig. 2C) (23, 53, 55). Similarly, AOS1.mAID, which shows high expression levels of *HSP12*, *HSP26*, and *DDR2*, is also temperature sensitive. UBC9.AA, which shows a modest increase in expression of these genes, is partially temperature sensitive, while UBA2.mAID, in which expression of these genes is unaffected, shows no growth defect at the elevated temperature (Fig. 2C). Taken together, the data in Figs. 2A, 2B, and 2C reveal a remarkable correlation between the severity of sumoylation cycling defects, the expression of a subset of stress-induced genes, and temperature sensitivity. Paradoxically, constitutively elevated expression of some stress-response genes appears to relate to increased sensitivity to heat stress.

To continue to explore the relationship between sumoylation cycling defects and expression of stress response genes, we generated a strain that harbours both *ubc9-6* and *ulp1-mt* mutations. Compared to the individual mutations, the *ubc9-6 ulp1-mt* strain shows an intermediate level of SUMO conjugation that is somewhat reduced compared to wild-type yeast (Fig. 3A). Nonetheless, RT-qPCR analysis showed significantly elevated expression for *HSP12*, *HSP26*, and *DDR2* without heat shock, as observed in the individual *ubc9-6* and *ulp1-mt* strains (compare Fig. 2A with Fig. 3B). Furthermore, partially restoring sumoylation levels by combining the mutations did not reverse the temperature-sensitivity defect (Fig. 3C). This supports the idea that it is defects in sumoylation cycling that impacts expression of stress-

response genes and viability at elevated temperatures, and not the level of SUMO conjugation itself. Previously, we demonstrated that treatment of yeast cells with the compound 1,10-phenanthroline results in a dramatic and rapid reduction in SUMO conjugation levels (24). While investigating whether analogs of this drug also impact sumoylation, we found that exposure of yeast cultures to bathocuproine (BCP) leads to an increase in global sumoylation that peaks around 10 min before returning to normal levels (Figs. 3D, E, F). We examined whether this transient elevation of sumoylation impacts expression of stress-induced genes. Indeed, as shown in Fig. 3G, treatment with BCP for 10 min led to a sharp increase in expression of *HSP12*, *HSP26*, *DDR2*, and *HSP82*. Although we cannot rule out that BCP is able to trigger a stress response by unknown mechanisms, these data are consistent with a strong connection between sumoylation and expression of stress-response genes, where any disruption of SUMO conjugation-deconjugation homeostasis leads to their induction.

3.5.3 Altered sumoylation cycling activates Hog1, resulting in induction of some stress-response genes

To explore mechanisms by which disrupting sumoylation cycling can lead to elevated expression of ESR genes, we examined the status of key regulators of the transcriptional response to environmental stresses. Even under normal growth conditions, Hsf1 is associated with up to ~370 genes, including *HSP12*, *HSP26*, and *HSP82*, but not *DDR2*, and heat shock triggers its phosphorylation which coincides with activation of its target genes (40, 56–58). To determine whether disrupted sumoylation leads to constitutive activation of Hsf1, lysates were prepared from untreated or heat-shocked cultures, and Hsf1 immunoblots were performed. As shown in Fig. 4A, heat shock resulted in a notable upward shift in migration for Hsf1 that was not apparent when samples were treated with λ phosphatase, confirming that the shift is indeed

due to heat-induced phosphorylation. Migration of Hsf1 in the analysis of untreated ulp1-mt and *ubc9-6* cells, however, corresponds to the unphosphorylated form, implying that Hsf1 is not constitutively active, and is therefore not likely responsible for the expression of the subset of ESR genes, in these strains (Fig. 4A, B). Interestingly, treatment of yeast with BCP did result in a migration shift for Hsf1, suggesting that, although the drug impacts sumoylation levels, it may induce expression of ESR response genes through an Hsf1-mediated pathway, unlike in untreated *ulp1-mt* and *ubc9-6* (Fig. 4A, B). This is supported by the observation that BCP robustly induces *HSP82*, an Hsf1-target gene, whereas this gene is barely impacted in strains with constitutive sumoylation defects (see Figs. 3G and 2A).

Next, we investigated whether the mitogen-activated kinase Hog1 could be involved. Hog1 has been largely studied for its key role in osmoregulation, but its signaling affects ~70% of ESR genes, likely through its regulation of multiple transcription factors, including Msn2 and Msn4 (59–62). Furthermore, phosphorylation activates Hog1 in response to high osmolarity, but also upon exposure to other stresses including heat (59, 63). Using a phosphorylation-dependent Hog1 antibody, we examined lysates derived from untreated or heat shocked cultures by immunoblot. As shown in Figs. 4A and B, phosphorylated Hog1 indeed appeared after heat shock, supporting previous findings. Intriguingly, however, this analysis shows that Hog1 is substantially phosphorylated in untreated *ulp1-mt*, *ubc9-6,* and BCP-treated cells. In untreated *ulp1-mt* cells, the level of Hog1 phosphorylation appears to be somewhat greater than in heat shocked wild-type cells, but heat shock in *ulp1-mt* further elevates this. In untreated *ubc9-6*, however, the level of Hog1 phosphorylation is dramatically higher than in heat shocked wild-type cells, and heat shock does not lead to a further increase (Figs. 4A and B). These data indicate that disrupting sumoylation cycling leads to activation of Hog1, which might at least

partly explain the ESR-like gene expression pattern in these conditions.

We wished to further explore the relationship between Hog1 and sumoylation. First, we tested whether Hog1 is needed for the increase in sumoylation that takes place during heat shock or for elevated SUMO conjugation in the *ulp1-mt* strain. However, deletion of *HOG1* did not impact either of these, as shown in the immunoblots in Fig. 4C and D. Because disrupted sumoylation cycling triggers Hog1 phosphorylation, but Hog1 is not needed for modulating sumoylation, we presume that activation of Hog1 takes place downstream of altered sumoylation cycling. This raises the intriguing possibility that Hog1 activation during heat stress is a consequence, at least in part, of the heat-triggered drop in SUMO deconjugation levels. To determine whether Hog1 signaling is needed for mediating the gene expression effects of disrupted sumoylation cycling, RT-qPCR was performed using RNA isolated from the *ULP1*, *ulp1-mt*, *ULP1 hog1Δ*, and *ulp1-mt hog1Δ* strains. As shown in Fig. 4E, in the *ulp1-mt* background, the high level of expression of *HSP12* and *DDR2*, but not *HSP26*, was dramatically reduced in the absence of Hog1. These data strongly suggest that disrupted sumoylation cycling in the *ulp1-mt* strain leads to constitutive Hog1 activation, which consequently results in elevated expression of some ESR genes. Whereas Hog1 appears to be largely responsible for induction of *HSP12* and *DDR2*, another pathway must be involved in the induction of *HSP26* in the *ulp1-mt* strain.

3.5.4 Constitutive expression of stress response genes in *ulp1-mt* depends on Msn2

The transcriptional activators Msn2 and Msn4 function through an Hsf1-independent pathway to induce expression of ESR genes in response to a variety of stressors, including heat (38, 40, 64, 65). Although they have overlapping functions, Msn2 is constitutively expressed while Msn4 expression is stress-induced and dependent on Msn2 activation (38). To determine

whether Msn2 is activated and if *MSN4* is induced in conditions of disrupted sumoylation cycling, we examined the expression levels of *MSN4* and *PGM2*, both of which depend on active Msn2 for their activation (38, 66). RNA was isolated from *ulp1-mt*, *ubc9-1*, *ubc9-6* and their respective parental wild-type strains, and from a wild-type strain treated with BCP, and RT-qPCR was performed with primers specific for *MSN2*, *MSN4*, and *PGM2* (Fig. 5A). *MSN2* expression is not affected by changes to sumoylation cycling, consistent with it being constitutively expressed under a variety of tested conditions (38). However, *MSN4* expression is elevated in the *ulp1-mt*, *ubc9-1*, and *ubc9-6* strains, while *PGM2* expression was increased in all conditions in which sumoylation cycling was altered. Since these data suggest that Msn2 activity is sensitive to the status of sumoylation in the cell, we examined whether Msn2, and/or Msn4, could be responsible for the expression of ESR genes in the *ulp1-mt* strain. Deletion of *MSN2*, *MSN4*, or both in the *ulp1-mt* and its parental wild-type strains did not impact sumoylation levels (Fig. 5B). However, RT-qPCR analysis showed that the high level of expression of *HSP12*, *HSP26*, and *DDR2* in *ulp1-mt* is strongly dependent on Msn2, but not Msn4 (Fig. 5C).

Our data indicate that disrupted sumoylation cycling leads to activation of Hog1 and Msn2, and that these factors are required for stress-independent expression of ESR genes in the *ulp1-mt* strain. Whereas Msn2 is required for elevated expression of all tested ESR genes in this strain, expression of *HSP26* relies on Msn2 but not Hog1, suggesting that activation of Msn2 in particular is involved. To test this, we compared our *ulp1-mt* RNA-seq data with data from a previously published study in which gene expression patterns were determined for a strain that conditionally expresses an active mutant form of Msn2, Msn2A6, by microarray (67). In that study, 194 genes were induced, and 338 repressed, following induced expression of Msn2A6.As shown in Fig. 5D, these gene expression changes showed strong similarity to changes due to

impairment of Ulp1 function (r = 0.71). In particular, most genes that are upregulated due to activation of Msn2 are also activated in the *ulp1-mt* strain (heatmap in Fig. 5D). In another study, ChIP-seq was used to identify 193 genes whose promoter regions are bound by Msn2 prior to or after nutritional stress and determined whether binding of Msn2 led to induction, repression, or had no significant effect ("neutral") on expression of these genes during the stress (68). Fig. 5E shows the effects of Ulp1 impairment on expression of each of these Msn2-targeted genes, as determined by our *ulp1-mt* RNA-seq analysis. Strikingly, genes that were previously shown to be bound and induced by Msn2 mostly show elevated expression in the *ulp1-mt* strain, whereas genes previously associated with a neutral or repressive effect of Msn2 showed a reduced or more muted level of expression in *ulp1-mt*. Together, these data strongly support that much of the gene expression pattern that is specific to the *ulp1-mt* strain is due to activation of Msn2.

3.5.5 Chromatin associated sumoylation is reduced during heat shock and in the *ulp1-mt* strain

Next, we explored whether changes to the sumoylation status of chromatin-associated proteins specifically could be responsible for the establishment of a stress gene expression pattern. In a previous study, through SUMO ChIP-seq, we showed that sumoylated proteins are found at most tRNA and ribosomal protein genes, as well as at the promoters of ~150 non-ribosomal protein-coding genes ("non-RPGs") (Table S5) (8). As shown in Figs. 6A, S1B, S1C, and S1D heat shock results in a widespread decrease in chromatin-associated sumoylation, across all gene types (Table S6). Since heat shock leads to an overall increase in SUMO conjugation (e.g., Fig. 1A), the decrease in chromatin-associated SUMO levels is an unexpected result. We then examined how constitutively elevated sumoylation, through impaired Ulp1 activity, impacts the genomic SUMO landscape. SUMO ChIP-seq was performed in the *ulp1-mt*

and *ULP1* parent strains, and strikingly, as in heat shock, chromatin-associated SUMO was significantly reduced across the genome, at all gene classes (Tables S7, S8; Fig. 6B). Similarly, deletion of the gene encoding the SUMO protease Ulp2 results in reduced levels of SUMO associated with non-RPGs (Fig. 6C). Intriguingly, these findings suggest that the global increase in SUMO conjugation seen during heat shock and in desumoylation mutants occurs at the expense of chromatin-associated sumoylation, suggesting a redistribution of SUMO away from chromatin during stress and impaired SUMO protease function.

Previously, we performed an RNAPII ChIP-seq analysis in cultures that were untreated or heat shocked in the same conditions as the heat-shock SUMO ChIP-seq experiment described above (23). On comparing the analyses, we found that the drop in sumoylation at non-RPGs occurred regardless of whether RNAPII levels also dropped during heat shock, implying that the reduction in sumoylation is not necessarily transcription-dependent (Fig. 6D). Intriguingly, however, only five genes showed a heat shock-dependent increase in SUMO occupancy, all of which are heat stress-induced (Figs. 6A, 6D, S2A; Table S8). The pattern of sumoylation across the genes includes a promoter-associated peak and a more diffuse signal across the gene body (Fig. 6D; see *HSP82*, *SSA2*, *HSP26*, and *KAR2*). Promoter-associated SUMO peaks at non-RPGs can be largely attributed to TFIIF sumoylation, while RNAPII is also a known SUMO target suggesting that it may be the source of the signal detected at gene bodies (8, 9). However, mutations that impair sumoylation of Tfg1 or Rpb1, which are the SUMO-targeted subunits of TFIIF and RNAPII, respectively, did not impact the induction of heat-shock genes, indicating that sumoylation of these chromatin-associated factors is not involved in the transcriptional response to heat shock (Fig. S2B). Furthermore, SUMO occupancy is not elevated at these heat shock genes in *ulp1-mt* even though their expression is induced in that strain (Fig. 6E). Taken

together, these data suggest that expression of ESR genes in the *ulp1-mt* strain results from disrupted sumoylation cycling of one or more factors that are not necessarily associated with chromatin.

3.6 Discussion

In response to environmental stress, global sumoylation levels increase rapidly while transcription patterns genome-wide are adjusted to establish a stress-adaptive gene expression profile. Although previous studies have shown connections between these two observations, it is not known whether they are directly linked (23, 29, 69). Our findings show that disrupting sumoylation cycling, by decreasing levels of SUMO conjugation or deconjugation, leads to the induction of ESR genes and an overall ESR-like gene expression pattern, even under optimal, non-stress conditions. This suggests that dynamic sumoylation equilibrium is required to suppress the ESR transcriptional response in the absence of stress. The idea that sumoylation cycling, rather than merely the presence or absence of the SUMO modification itself, serves cellular functions is not new. An analysis of the role of sumoylation of the RNAPII general transcription factor TFIIF showed that both impairing its sumoylation and expressing a "permanently sumoylated" form, through fusion of the targeted subunit with SUMO, leads to reduced binding of RNAPII at active gene promoters, suggesting that dynamic sumoylation of TFIIF is somehow involved in facilitating transcription (8). In another study, mutant yeast strains expressing partially defective or null forms of Ubc9, Ulp1, or Ulp2, that consequently lead to correspondingly reduced or elevated SUMO conjugation levels, all show significantly reduced lifespans, implicating sumoylation cycling of one or more factors as important in promoting longevity (70). These studies, and the transient nature of SUMO modifications, support the importance of sumoylation cycling, but uncovering the specific molecular mechanisms through which this dynamic process contributes to cellular functions remains an important goal.

Towards understanding the mechanisms by which disrupted sumoylation causes an ESR-like gene expression profile, our work implicates Hog1 and Msn2, but not Hsf1. We provide

evidence that both Hog1 and Msn2 are activated in *ulp1* and *ubc9* mutant strains grown in non-stress conditions, and both contribute significantly to the anomalous expression of *HSP12* and *DDR2* in the *ulp-1mt* background. The activated Hog1 and Msn2 pathways appear, then, to function coordinately in the expression a subset of ESR genes when sumoylation cycling is disrupted. This is supported by work that showed that Hog1 and Msn2 interact with each other to regulate gene expression and are not entirely independent (59, 62, 71). Nonetheless, our data suggest that Msn2 plays a more important role in establishing gene expression patterns in the *ulp1-mt* strain, considering that *HSP26* expression in that strain is dependent on Msn2 but not Hog1, and that there are strong similarities between the *ulp1-mt* regulon and the previously reported Msn2 regulons (Fig. 5) (67, 68). In any case, we find that neither Hog1 nor Msn2 is required for establishing elevated sumoylation in the *ulp1-mt* strain, which aligns with the observations that Hog1 is not required for the surges in sumoylation seen after heat shock or osmotic stress (Fig. 4C) (69). Together, these findings support a model in which Msn2 and Hog1 mediate downstream transcriptional responses to disrupted sumoylation cycling, but do not contribute to the underlying elevation in sumoylation levels.

By this model, then, we conclude that Hog1 and Msn2 activation may be a consequence of disrupted sumoylation cycling of one or more factors that lie upstream of Hog1 and Msn2 signaling. One possibility is that changes in global sumoylation impact the protein kinase A (PKA) pathway, which functions upstream of Msn2. Amongst its roles, the PKA pathway is important for responding to environmental changes including through downstream regulation of gene expression (72, 73). Environmental stress downregulates PKA activity which reduces phosphorylation of its downstream targets, including Msn2 (67). Msn2 dephosphorylation allows for its entry into the nucleus where it then binds to promoter regions of its target genes. Although

there is evidence of crosstalk between the PKA pathway and sumoylation in other species, it is not clear how sumoylation levels impact the PKA pathway in yeast and whether modulated global sumoylation levels occurs prior to or after PKA inhibition when cells are exposed to environmental changes (74–76).

Two unexpected observations were made in this study. First, we found that strains that constitutively express a subset of ESR-induced genes show sensitivity to heat stress in a manner that correlates with the expression level of these genes. Whereas one might expect that expression of stress-specific gene products in non-stress conditions could prime cells for a more robust adaptation to elevated temperatures, this was not observed. Although paradoxical, our results are consistent with the finding that activation of heat shock genes is not always necessary for survival at high temperatures (77, 78). Future work can examine whether only transient expression of ESR genes after exposure to stress, as opposed to continual expression, is critical for stress adaptation. The second unexpected observation is that elevated SUMO conjugation, whether triggered by elevated temperatures or reduced SUMO protease activity, leads to a significant reduction of chromatin associated sumoylation levels. This suggest that conditions that increase SUMO conjugation levels lead to widespread dissociation of sumoylated proteins from chromatin, or it somehow promotes desumoylation of chromatin-bound targets. The former possibility is strongly supported by previous work that demonstrated that sumoylation of some sequence-specific transcription factors in yeast and human cells stimulates their dissociation from DNA (50, 79, 80). Determining whether specific DNA-bound, sumoylated proteins are actively desumoylated or dissociate from chromatin during heat shock will help distinguish between these possibilities.

Our findings shed light on how altered sumoylation dynamics can regulate the transcriptional response to stress. Based on our data, we propose that exposure to stress leads to a surge in global sumoylation, triggered by Ulp1 degradation. This, in turn, activates the stress-responsive transcription factors Hog1 and Msn2, leading to changes in transcription that establish an ESR gene expression pattern. Identifying the specific factor or factors whose activity is modulated by disrupted sumoylation cycling will be critical to validating this model. Notably, our SUMO ChIP-seq analysis revealed that many genes with altered expression in the *ulp1-mt* strain do not exhibit significant changes in SUMO occupancy, and that impairing sumoylation of RNAPII or TFIIF does not affect ESR gene expression in this context. Additionally, we were unable to detect Msn2 sumoylation under non-stress, heat shock, or *ulp1-mt* strain conditions (Fig. S2C). Although sumoylated Hog1 was observed, its sumoylation level is unaffected by heat shock (Fig. S2D). These observations suggest that sumoylation of chromatin-associated proteins, Msn2, or Hog1 is not likely to be the regulatory node. Future work can examine whether sumoylation or desumoylation of components of the PKA pathway can trigger the activation of Hog1 and Msn2. This would help elucidate how sumoylation cycling homeostasis functions to supress expression of ESR genes in non-stress conditions, and how modulated sumoylation can serve as an early regulatory signal that coordinates the transcriptional changes needed to survive stress conditions.

3.7 Figures

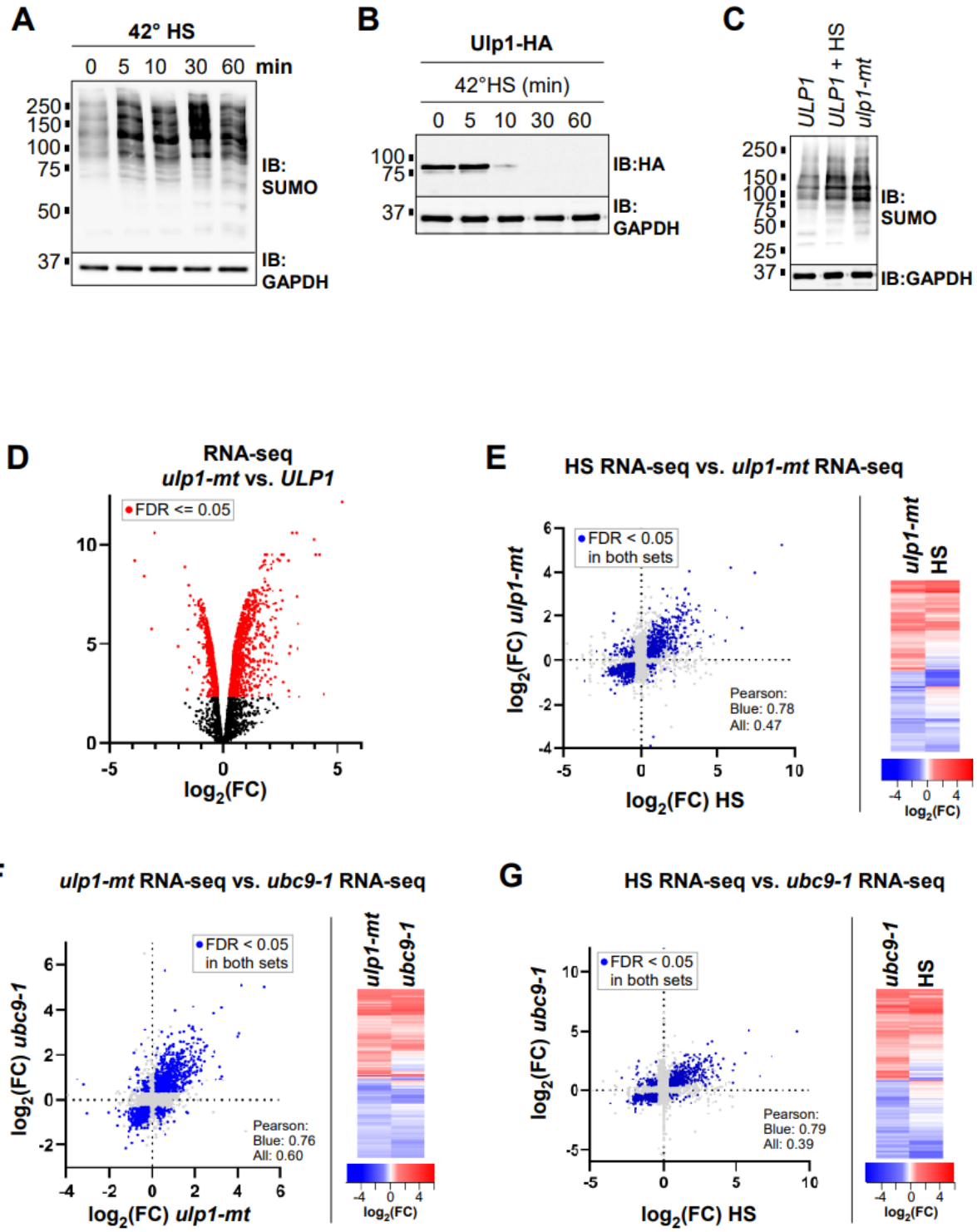


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Figure 1. Constitutively reduced Ulp1 activity leads to stress-like changes in gene expression. (**A**) Cultures of the W303a strain were grown at 30°C then transferred to 42°C (i.e., heat-shocked) for the indicated times. Lysates were prepared and analyzed by SUMO immunoblot (IB) to detect levels of SUMO conjugation, and a GAPDH loading control immunoblot was performed in parallel. (**B**) Cultures of the Ulp1-HA strain were treated as in *A*, the analyzed by HA and GAPDH immunoblots. (**C**) Lysates were prepared from cultures of the ulp1-mt strain grown at 30°C, its isogenic parental strain, ULP1, grown at 30°C, and the *ULP1* strain heat-shocked at 42°C for 12-min. Lysates were analyzed by SUMO and GAPDH immunoblot. (**D**) RNA-seq was performed using RNA isolated from the *ULP1* and *ulp1-mt* strains, both maintained at 30°C. A volcano plot displaying differential gene expression between the two strains is shown, with $\log_2$ fold-change values ($\log_2$(FC)) plotted against -$\log_{10}$ of the false discovery rate (FDR). Expression changes showing an FDR of less than 0.05 are highlighted in red. Values for this and all graphs are shown in Table S10. (**E**) Scatterplot comparing gene expression changes ($\log_2$(FC)) due to mutation of Ulp1 (from the *ULP1* and *ulp1-mt* RNA-seq analysis) and due to heat shock (HS; from a previously published RNA-seq analysis using wild-type yeast; (51)). Pearson correlation analysis was performed with all genes from which data was available for both analyses ("All") and for the subset of genes that show significant changes in both data sets ("Blue"; FDR < 0.05). At right, a heatmap depicts gene expression changes in the two analyses for the subset of genes that show significant changes in both data sets. (**F**) Comparison, as in *E*, of gene expression changes from the *ulp1-mt* RNA-seq analysis and from a previously published RNA-seq analysis performed using the *ubc9-1* strain and its wild-type parental strain (52). (**G**) Comparison, as in *E*, of gene expression changes detected in the HS and *ubc9-1* RNA-seq analyses.

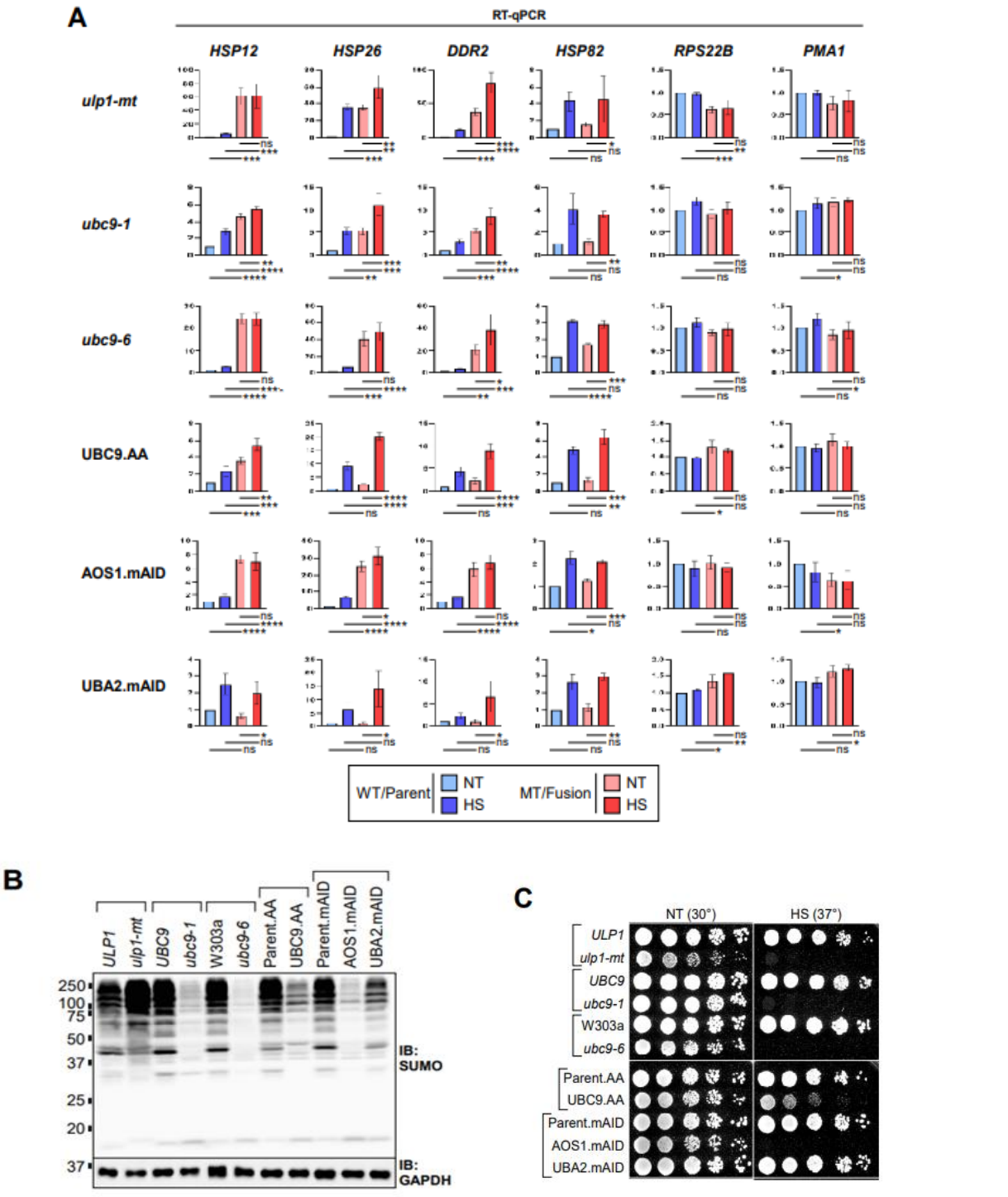

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Figure 2. Disrupted sumoylation or desumoylation leads to expression of some stress-response genes in non-stress conditions. (**A**) RNA was isolated from cultures of strains expressing partially-inactivating mutations in Ulp1 or Ubc9 (*ulp1-mt*, *ubc9-1*, and *ubc9-6*) and their wild-type isogenic parent strains, or from strains expressing C-terminal fusions of the Anchor Away (AA) or mini auxin-inducible degron (mAID) tags to components of the sumoylation machinery (UBC9.AA, AOS1.mAID, and UBA2.mAID), and their untagged parental strains, in the absence of any treatments or after a 5-min 42°C heat shock. RT-qPCR was performed using primers specific to the genes indicated, and average values are shown from three independent experiments, relative to the respective untreated parental condition. Error bars represent standard deviation amongst the replicates. For statistical analysis, an ordinary one-way ANOVA was performed with a Fisher's least significant difference (LSD) test. Sample pairs showing no significant difference are indicated with ns, whereas asterisk indicate significant difference between indicated pairs as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (**B**) Comparison of SUMO conjugation levels in the strains described in *A* (paired with their isogenic parent strains) is shown. Lysates were prepared from cultures of the strains that were grown at 30°C, then analyzed by SUMO and GAPDH immunoblot. (**C**) Yeast growth was compared by spot assay for each of the strains described in *A* and their parental strains when grown at 30°C ("NT") or at 37°C (HS).

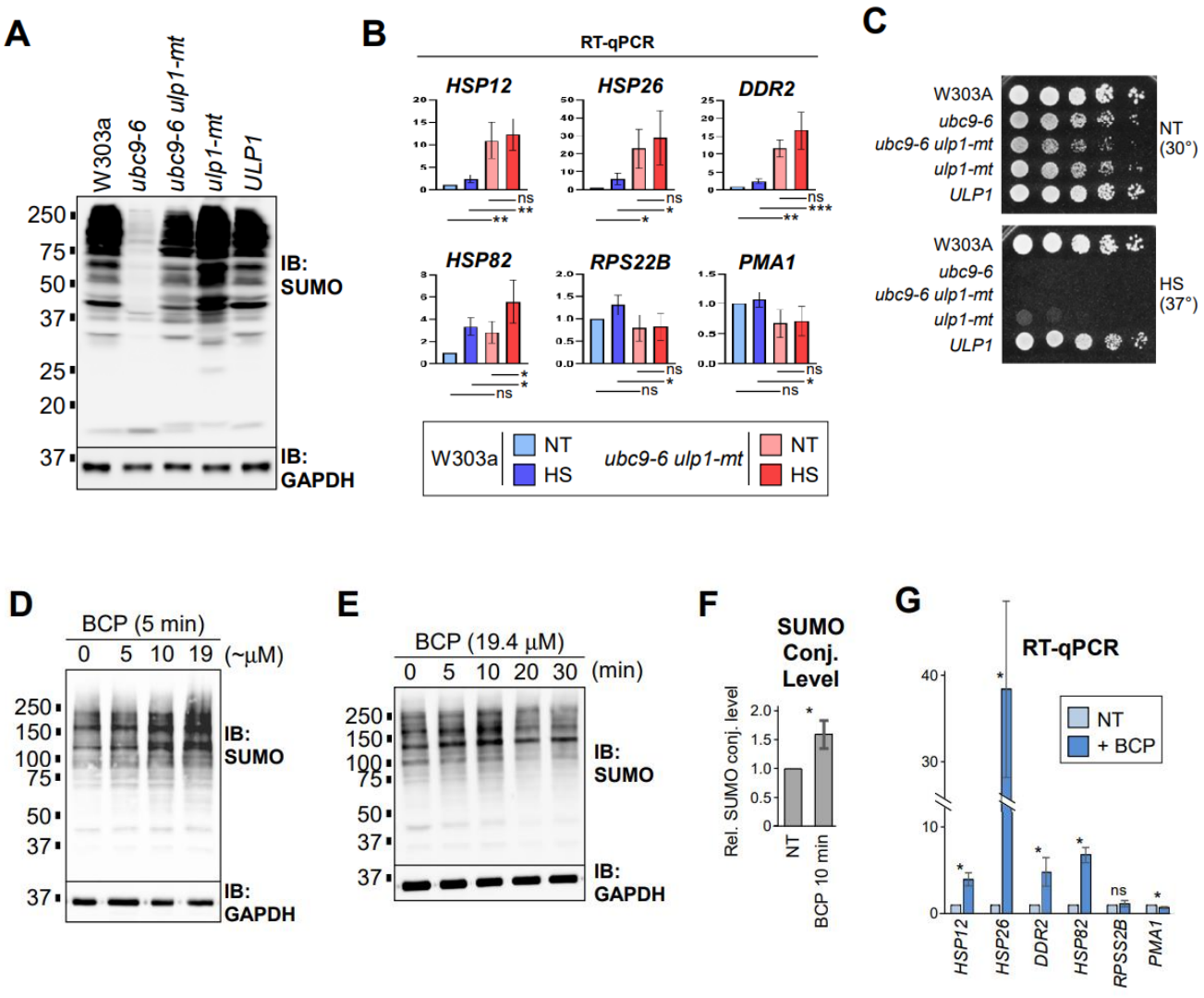

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Figure 3. Combining opposing defects in Ubc9 and Ulp1 or transiently elevating SUMO conjugation levels leads to induction of stress response genes. (**A**) Cultures of the indicated strains, including the *ubc9-6 ulp1-mt* strain which partially restores SUMO conjugation levels compared to strains harbouring the individual mutations, were grown at 30°C, and lysates were prepared and analyzed by SUMO and GAPDH immunoblot. (**B**) RT-qPCR was performed using RNA from the *ubc9-6 ulp1-mt* strain, or its wild-type parent, W303a, untreated or after a 5-min 42°C heat shock. Analysis was performed as in Fig. 2A. (**C**) Spot assay comparing growth of the strains indicated in A at 30°C (NT) or 37°C (HS). (**D**) Cultures of a wild-type yeast strain, W303a, were treated with the indicated concentrations of bathocuproine (BCP) for 5 min, then lysates were prepared and analyzed by SUMO and GAPDH immunoblot. (**E**) Lysates were prepared from cultures of W303a that were treated with 19.4 μM of BCP for the indicated durations and analyzed by SUMO and GAPDH immunoblot. (**F**) Densitometry quantification of SUMO conjugation levels in lysates from W303a cultures either untreated (NT) or after 10-min of treatment with 19.4 μM of BCP. Relative quantification is shown from three independent experiments, and a Student's *t*-test was used to demonstrate that BCP results in significantly elevated sumoylation levels (asterisk, $p < 0.05$). (**G**) RT-qPCR was performed using RNA from untreated W303a cultures, or after treatment with 19.4 μM of BCP for 10-min, using primers specific for the indicated genes. Relative quantification is shown from three independent experiments, with Student's *t*-test analysis showing significantly elevated expression levels of stress response genes (asterisks, $p < 0.05$).

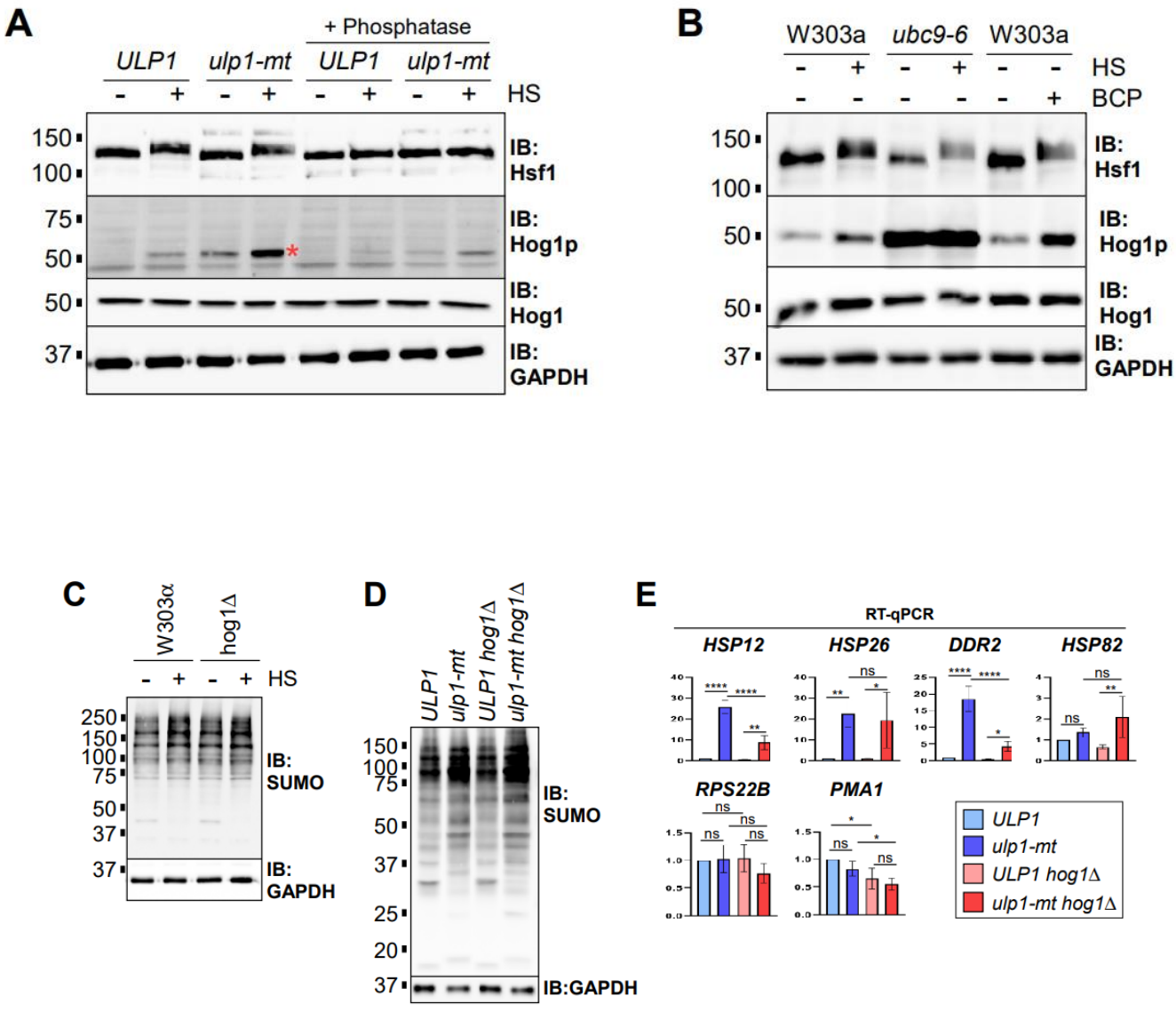

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Figure 4. Hog1 is activated when sumoylation or desumoylation are disrupted. (**A**) Lysates were prepared from cultures of the *ULP1* and *ulp1-mt* strains that were untreated (-) or after a 5-min 42°C heat shock (+ HS) and analyzed by immunoblot with antibodies that recognize Hsf1, phosphorylated Hog1 (Hog1p), total Hog1, or GAPDH. A portion of each the lysates was treated with λ phosphatase prior to immunoblot analysis (+ Phosphatase). (**B**) Lysates were prepared from W303a and *ubc9-6* strains that were untreated or heat-shocked for 5 min, and from the W303a strain treated with 19.4 μM of BCP for 10 min and analyzed by Hsf1, Hog1p, Hog1, and GAPDH immunoblot. (**C**) Lysates were prepared from cultures of the *hog1Δ* strain and its isogenic wild-type parental strain, W303α, that were untreated or after a 30-min 42°C heat shock, then analyzed by SUMO and GAPDH immunoblot. (**D**) SUMO and GAPDH immunoblot analysis was performed from lysates generated from the indicated strains. (**E**) RT-qPCR analysis was performed from RNA isolated from the indicated strains, as in Fig. 2A.

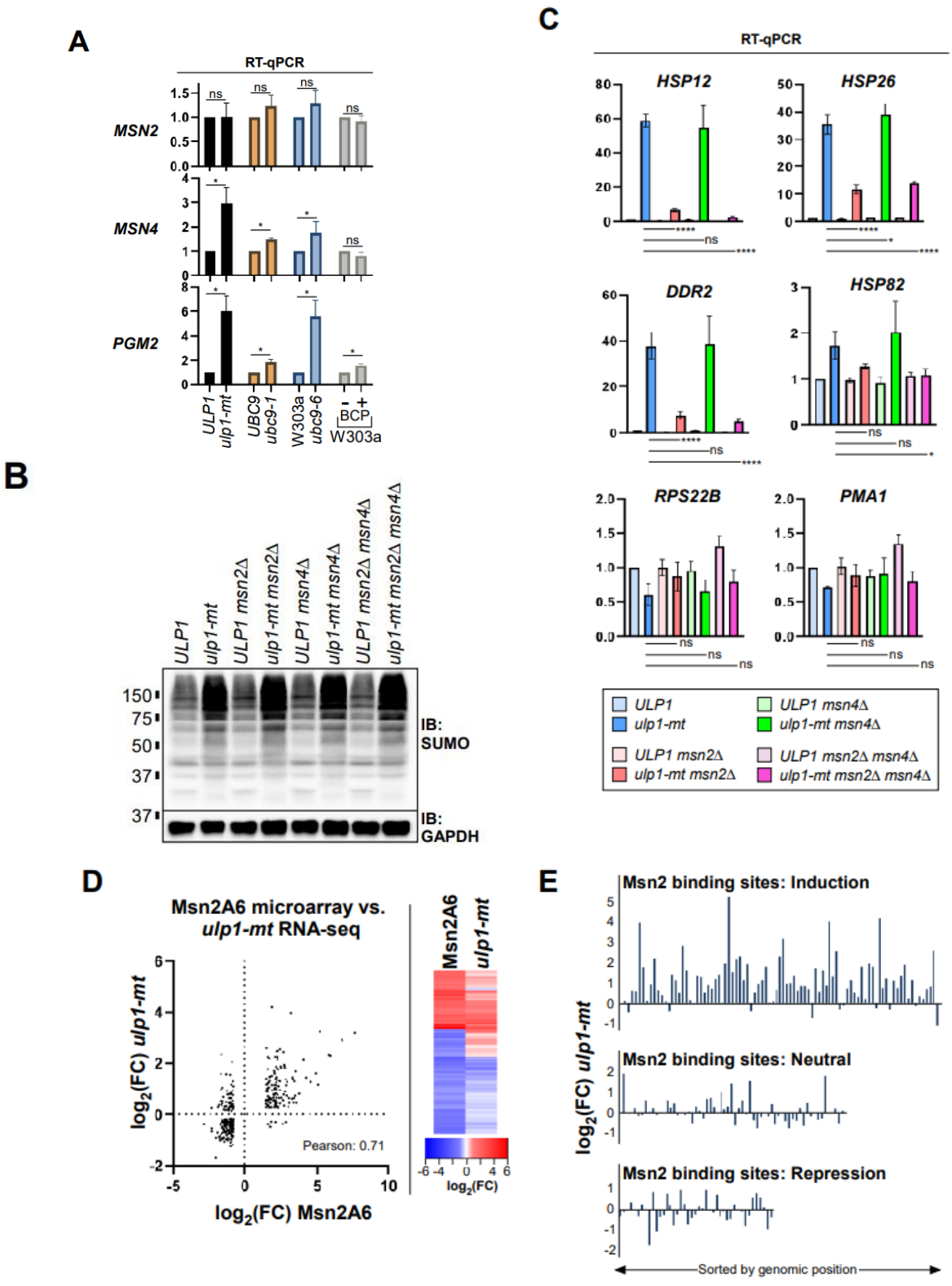

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Figure 5. Msn2 is largely responsible for the elevated expression of stress response genes in the *ulp1-mt* strain. (**A**) RT-qPCR analysis was performed using RNA from the indicated strains, including the W303a strain treated with 19.4 μM of BCP for 10 min. Relative expression levels of the *MSN2*, *MSN4* and *PGM2* genes is shown from three independent experiments, and Student's *t*-tests were performed to determine whether the respective gene modifications or treatment with BCP significantly affects expression of the genes (asterisks, $p < 0.05$). (**B**) Lysates from the indicated strains were analyzed by SUMO and GAPDH immunoblot. (**C**) RT-qPCR analysis was performed using RNA isolated from the indicated strains, as in Fig. 2A. (**D**) Gene expression changes were compared in the *ulp1-mt* RNA-seq analysis and with those provided in the analysis of a previously published expression microarray analysis performed in a strain that expresses an inducible active form of Msn2, Msn2A6 (67). Only genes that were reported as differentially expressed in that study are shown, and analysis was performed as in Fig. 1E. (**E**) Gene expression changes in the *ulp1-mt* strain as determined by RNA-seq analysis are shown for the 193 genes that were found to bind Msn2 at their promoters in a previously published ChIP-seq analysis (68). That study classified the genes as likely to be induced (Induction) or repressed (Repression) by Msn2, or not affected by its binding (Neutral). Here, genes are sorted by their genomic position in each of the three categories.

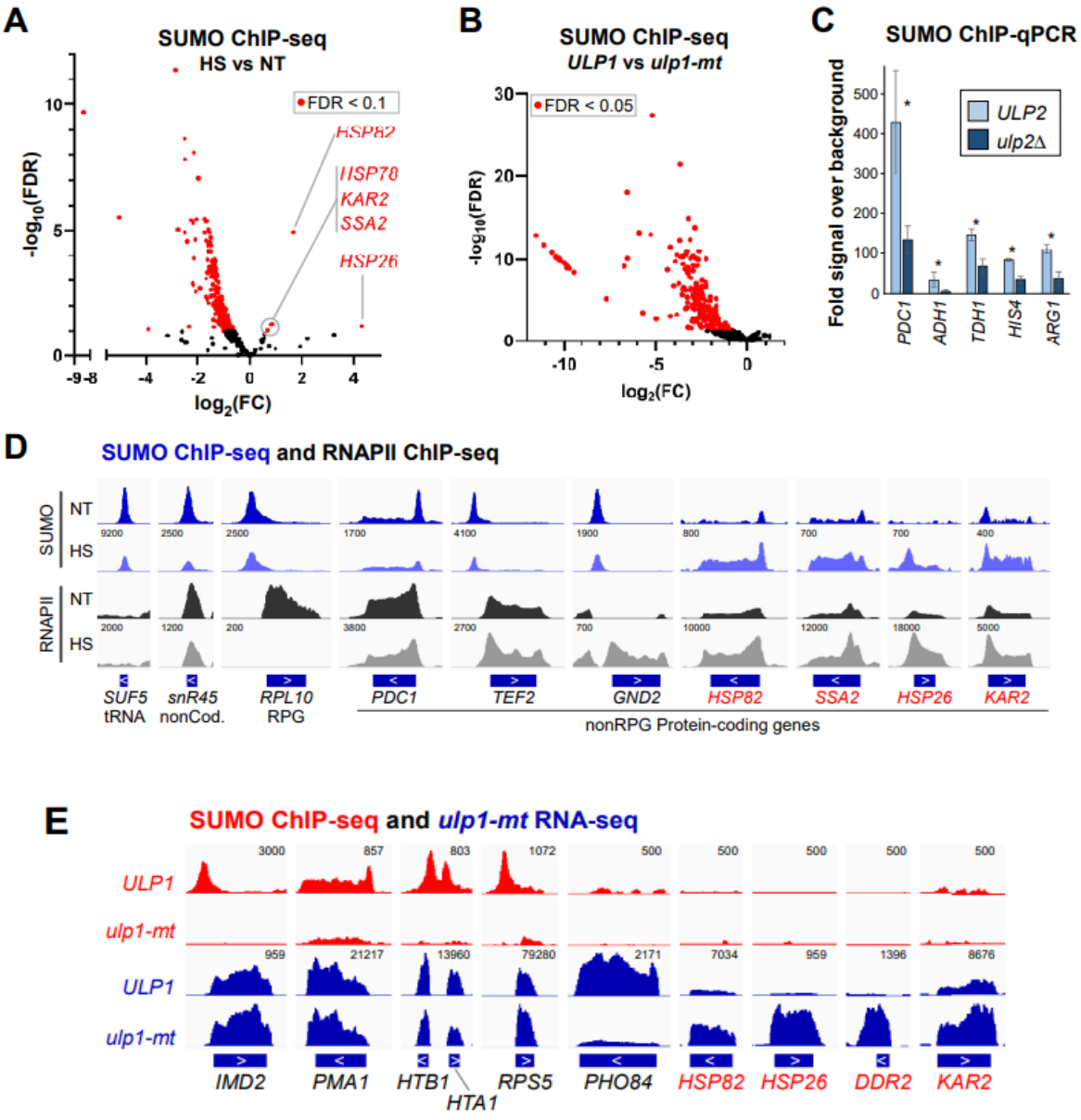

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Figure 6. Heat shock or Ulp1 mutation leads to reduced chromatin-associated sumoylation. (**A**) Independent duplicate SUMO ChIP-seq analyses were performed in the W303a strain that was untreated (NT) or after a 12-min 37°C heat shock (HS). A volcano plot is shown indicating the change in the level of SUMO at 608 loci after heat shock, with those changes having an FDR of less than 0.1 indicated in red. The names of the only genes that show significantly elevated SUMO levels are indicated on the graph. (**B**) Independent duplicate SUMO ChIP-seq analyses were performed in the *ULP1* and *ulp1-mt* strains and a volcano plot is shown indicating the change in SUMO levels at 598 loci due to the *ulp1-mt* mutation with those changes having an FDR < 0.05 indicated in red. No loci showed significantly elevated sumoylation in the *ulp1-mt* strain. (**C**) SUMO ChIP was performed in the *ulp2Δ* strain and its isogenic wild-type parent strain, *ULP2*. qPCR was then performed using primers specific for promoter-proximal regions of the indicated genes. The average of three experiments is shown with standard deviations indicated as error bars. Student's *t*-tests were performed to demonstrate statistically significant reductions in SUMO levels at these sites in the *ulp2Δ* strain (asterisks, $p < 0.05$). (**D**) ChIP-seq genomic alignments are shown at selected gene regions for the SUMO heat-shock ChIP-seq experiment described here, and for an RNAPII ChIP-seq that we previously described ((8, 23); GEO accession: GSE167424), with both sets examining untreated and heat shocked (37°C for 15 min) W303a cultures. Regions shown include genes for a tRNA and a noncoding RNA (nonCod.), an RPG, and non-RPGs, including four heat-induced genes labeled in red. Values refer to maximum data range (read numbers) for the view shown. Alignments are normalized, averaged, and background (input)-subtracted, and displayed using IGV. (**E**) Genomic alignments of the SUMO ChIP-seq and RNA-seq experiments performed in *ULP1* and *ulp1-mt* strains are shown at selected gene regions. Values refer to maximum data range (read numbers) for the view shown. Alignments, displayed using IGV, are normalized and averaged, and ChIP-seq alignments are background (input)-subtracted.

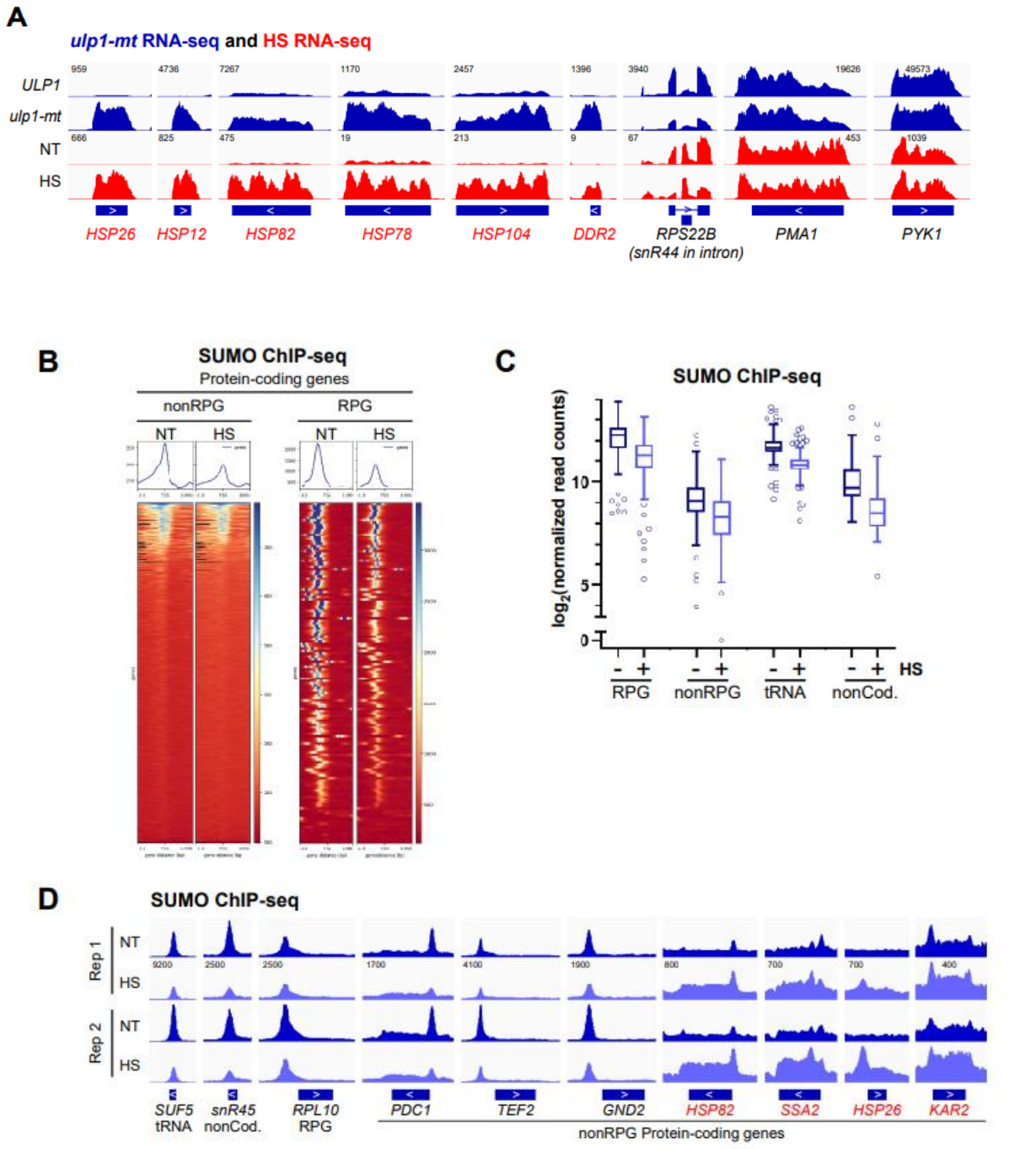

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Figure S1. (**A**) Many heat shock induced genes are expressed in non-stress conditions when Ulp1 activity is impaired. Genomic alignments are shown for RNA-seq experiments from this study (with *ULP1* and the *ulp1-mt* strains) and from a previously published analysis of wild-type yeast, untreated (NT) or after a 20-min 39°C heat shock (HS; (51)). Selected gene regions are shown including for six heat-induced genes (names indicated in red). Values refer to maximum data range (read numbers) for the view shown. Alignments, displayed using IGV, are normalized and averaged. (**B**) Heat shock results in reduced SUMO occupancy at genomic sites. Heat maps and composite profiles are shown for regions surrounding the transcriptional start sites (TSS) of nonRPG and RPG gene subsets from the SUMO ChIP-seq analysis in untreated (NT) and heat-shocked (HS) samples. Plots were generated using the computeMatrix tool from deepTools using custom gene lists. (**C**) Heat shock reduces sumoylation at all gene types. Box plots comparing SUMO ChIP-seq signals ($\log_2$ of the normalized read counts, as determined by DiffBind), for peaks associated with the four classes of genes indicated, from samples that were untreated or after heat shock. (**D**) Genomic alignments from the SUMO heat-shock ChIP-seq analysis are shown at selected gene regions, with each replicate shown separately (compare with Fig. 6D). Heat shock-induced genes are indicated with red names. Values refer to maximum data range (read numbers) for the view shown. Raw alignments (i.e., unnormalized and without background subtraction) were generated using IGV.

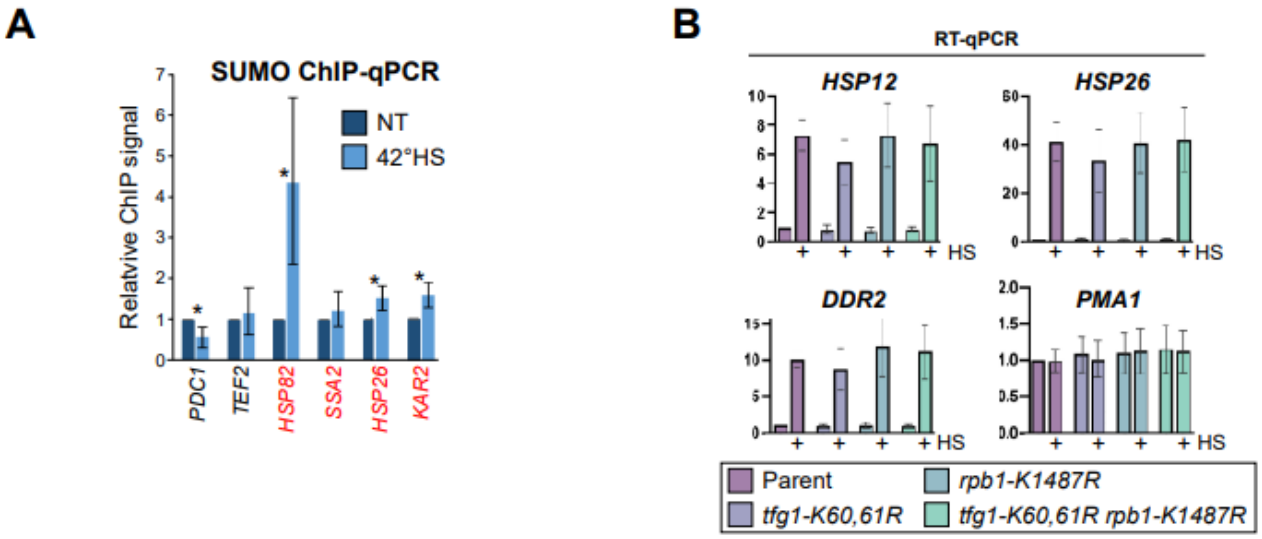

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Figure S2. (**A**) Sumoylation at some heat shock-induced genes increases during heat shock. Cultures of the W303a strain were grown untreated or heat shocked at 42°C for 12 min, then SUMO ChIP was performed followed by qPCR using primers specific for the promoter region of the indicated genes, with heat shock-induced genes indicated in red. Whereas the level of SUMO at *PDC1* is reduced after heat shock, SUMO levels increased at the four heat shock genes tested. This strongly parallels the data obtained from the SUMO heat shock ChIP-seq analysis. (**B**) Impaired sumoylation of the largest subunits of RNAPII (Rpb1) and TFIIF (Tfg1) does not impact the induction of heat shock genes. Cultures of strains harbouring sumoylation-diminishing point mutations on Rpb1 and/or Tfg1 were grown and either left untreated or heat shocked for 5 min at 42°C. RNA was then isolated, and qRT-PCR was performed using primers specific for the indicated genes. The mutations did not impact the induction of the heat shock genes. (**C**) Sumoylated Msn2 is not detected. Strains were generated in the *ULP1* and *ulp1-mt* background that express the Msn2 gene with a C-terminal 6x HA tag from its natural locus. The strains were then grown and left untreated, or heat shocked for 5 min at 42°C, then lysates were prepared in a buffer containing *N*-ethylmaleimide (see Methods) and immunoprecipitated (IPed) with an HA antibody. Inputs and IPed Msn2-HA was then analyzed by SUMO and HA immunoblot. (**D**) Hog1 is sumoylated, but its sumoylation level is not impacted by heat shock. Cultures of a strain that express Hog1 with a C-terminal 6x HA tag from its natural locus were grown and left untreated or heat shocked for 5 min at 42°C. As in *C*, lysates were prepared, IPed, and analyzed by SUMO and HA immunoblot.

3.8 List of supplementary tables

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Table S1: Yeast strains used in this study

Name	Code	Background/Parent Strain	Genotype	Source
W303a			*MAT a ura3-52 trp1Δ2 leu2-3_112 his3-11 ade2-1 can1-100*	Dharmacon
W303α			*MAT α ura3-52 trp1Δ2 leu2-3_112 his3-11 ade2-1 can1-100*	Dharmacon
ULP1	MH1006		*leu2-3,112 ura3-52 trp1-289 ade2Δ ade3Δ lys1::kanMX4*	Martine Heude (Soustelle et al., 2004)
ulp1-mt	MH1018	MH1006	*ulp1-I615N*	Martine Heude (Soustelle et al., 2004)
UBC9	DF5		*his3-Δ200, leu2-3,2-112, lys2-801, trp1-1(am), ura3-52*	Stefan Jentsch; Sufert et al., 1995
ubc9-1	YWO102 alpha	DF5	*ubc9Δ::TRP1, leu2::ubc9Pro-Ser::LEU2*	Stefan Jentsch; Sufert et al., 1995
ubc9-6	D370	W303a	*ubc9-1:Tadh1:TRP1:ubc9-1*	Baig et al., 2021
Parent.AA	HHY221	W303a	*MATa tor1-1 fpr1::loxP-LEU2-loxP RPL13A-2 × FKBP12::loxP*	Euroscarf
UBC9.AA	YJBa001A	W303a	*UBC9-FRB::kanMX tor1-1 fpr1::loxP-LEU2-loxP RPL13A-2 × FKBP12::loxP*	Moallem et al., 2023
Parent.mAID	YMK728	W303-1a	*ura3-1::ADH1-OsTIR1(pMK200, URA3)*	Masato Kanemaki/ National BioResource Project - Yeast

AOS1.mAID	YJBM118	YMK728	*AOS1-mAID (mini IAA17) 6HA::Kl TRP1*	This study
UBA2.mAID	YJBM119	YMK728	*UBA2-mAID (mini IAA17) 6HA::Kl TRP1*	This study
ubc9-6 ulp1-mt	YJBM111	W303a	*ulp1-I615N:URA3 ubc9-1:Tadh1:TRP1:ubc9-1*	This study
hog1Δ	YVS015a	W303a	*hog1Δ::kanMX Matα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]*	Moallem et al., 2023
ULP1 hog1Δ	YJBM130	MH1006	*hog1Δ::Kl TRP1*	This study
ulp1-mt hog1Δ	YJBM131	MH1018	*hog1Δ::Kl TRP1*	This study
ULP1 msn2Δ	YJBM141	MH1006	*msn2Δ::Kl TRP1*	This study
ulp1-mt msn2Δ	YJBM142	MH1018	*msn2Δ::Kl TRP1*	This study
ULP1 msn4Δ	YJBM143	MH1006	*msn4Δ::URA3*	This study
ulp1-mt msn4Δ	YJBM144	MH1018	*msn4Δ::URA3*	This study
ULP1 msn2Δ msn4Δ	YJBM145	YJBM143	*msn2Δ::Kl TRP1 msn4Δ::URA3*	This study
ulp1-mt msn2Δ msn4Δ	YJBM146	YJBM142	*msn2Δ::Kl TRP1 msn4Δ::URA3*	This study
ULP2	BY4741		*Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
ulp2Δ	ERYM579B	BY4741	*ulp2Δ::URA3MX*	McNeil et al., 2024
TFGI-HA	YJB002A	W303a	*TFG1-6XHA::Kl TRP1*	Baig et al., 2021
tfg1-K60,61R-HA	YRB013A	W303a	*tfg1-K60/61R-6HA::Kl TRP1*	Baig et al., 2021
TFG1-HA rpb1-K1487R	YBB063	YJB002A	*rpb1-K1487R::KanMX*	This study
tfg1-K60,61R-HA rpb1-K1487R	YBB061	YRB013A	*rpb1-K1487R::KanMX*	This study
ULP1 MSN2-HA	YJBM149	MH1006	*MSN2-6HA::Kl TRP1*	This study
ulp1-mt MSN2-HA	YJBM150	MH1018	*MSN2-6HA::Kl TRP1*	This study
HOG1-HA	ERYM334E	YPH499	*ura3-52 lys2-801_amber ade2-101_ochre trp1Δ63 his3Δ200 leu2Δ1 HOG1-6HA::Kl TRP1*	This study

Table S2. Oligonucleotide primer sequences

Gene	Forward Primer	Reverse Primer
Oligonucleotide primers for RT-qPCR		
HSP12	CTCTGCCGAAAAAGGCAAGG	GACGGCATCGTTCAACTTGG
HSP26	TACTGGCAAGGAAGTTGCTAG	TCATCTAAGGTTTCATCTCTGG
DDR2	TTCATTTCTGCCATCTCTGTC	GTTACTCGTGGTGTTGGATG
HSP82	GGCTAGTGAAACTTTTGAATTTCAAG	CCAACGCATCCGAGGCATTAG
RPS22B	GATCTGGTAAGATTGTCGTCCAAC	GTCTGGCTGGCAATAAGTTGGCG
PMA1	CTGGTCCATTCTGGTCTTCTATC	TCAGACCACCAACCGAATAAG
MSN2	CATCCTCCACGTCTCTTGCAAC	GGCACCGTACCCATCAAAGGCAC
MSN4	CAACATGCAACTTCTCCCAAGAC	GTCGTTCAATCCTGTTGTACTG
PGM2	GTCTCTTAAGGTCACCGATTGTGG	CAATCTTAGAACGAATCTTGCACC
25S	TCTAGCATTCAAGGTCCCATTC	CCCTTAGGACATCTGCGTTATC
Oligonucleotide primers for ChIP-qPCR		
PDC1	CTCCTTGCAATCAGATTTGG	TTGCGTGAGGTTATGAGTAG
ADH1	GTTTGCTGTCTTGCTATCAA	GAAAAAGAAACAAGGAAGAA
TDH1	TGACCAAAACTGGAGTCTCG	TGCAAGAGAGAGAATAGAACTG
HIS4	TCACCTCCGATGTGTGTTGT	ACAGCGCAGTTGTGCTATGA
ARG1	GACGGCTCTCCAGTCATTTAT	TTCCATACGGCACCGTTAAT
TEF2	CCGCCAATCAGCAAACTACC	ACGTTCTCTTGTACGACGCC
HSP82	ACCTCCTCATTTCTTCCCGC	TCTTTGCGTGTTTGTTTGTGC
SSA2	GATCGATGTTGACGGTAAGC	GTAAGCTGGGACAGTGACG
HSP26	ACTCCGTGTGTACCCCTAACTC	TTTGGACGCATAAGGGGGA
KAR2	CAACAGACTAAGCGCTGGC	CGAAAAGGGCGTACAGGACC
Chr V	CATTATCCGTAACGCCACTTT	CGATCTTAGTTCCAATGGTGAAA

Table S5. Analysis details for SUMO ChIP-seq in HS vs. NT

SUMO ChIP-seq in HS vs. NT	
Samples and conditions	Two independent replicates were prepared from cultures grown in SC medium at 30°C. Inputs and SUMO IPs were sequenced. 1 – WT (W303a): untreated 2 – WT (W303a): HS (37°C for 15 min)
Library synthesis	NEBNext Ultra II DNA library prep kit (New England Biolabs)
Sequencing	Illumina HiSeq 2500; Paired-end reads; 2x 126 nt; 10 million reads/sample
Quality control	FastQC (0.11.5)
Trimming	Trim Galore (0.4.4_dev) Cutadapt (2.3) Quality Phred score cutoff: 25, Adapter sequence: 'AGATCGGAAGAGC', Minimum required adapter overlap (stringency): 5 bp, Minimum required sequence length for both reads before a sequence pair gets removed: 40 bp, All sequences trimmed by 6 bp from their 5' end.
Genome alignment	Bowtie2 (2.3.5) with sacCer3 reference genome
Peak calling	MACS (2.1.1.20160309) ***Parameters***: paired-end; input as control; narrow peaks; effective genome size: 1.2e7; *q*-value cut-off: 0.05
Differential binding analysis	DiffBind (2.10.0) ***Parameters***: Defaults with minMembers=2 for dba.contrast; see notes below
Peak analysis and annotation	ChIPpeakAnno (3.18.2) from Bioconductor ***Parameters***: TxDb.Scerevisiae.UCSC.sacCer3.sgdGene genome annotation package was used, but each annotation was confirmed visually using IGV.
Notes	• Each peak was visually inspected on IGV to validate and classify by associated gene type and confirm annotations attributed by ChIPpeakAnno. • See Table S9 in Baig et al., PLOS Genetics, 2021 (PMID 34587155).
Generation of coverage tracks (bigWig) for alignment visualization	bamCompare (deepTools 3.3.2) was used to generate average bigWig files from replicate BAM sequencing alignment files. ***Parameters***: bin size: 10, operation: mean
Heatmaps	computeMatrix (deepTools 3.3.2) ***Parameters***: reference-point, custom regions lists

Table S7. Analysis details for SUMO ChIP-seq in *ULP1* vs. *ulp1-mt*

SUMO ChIP-seq in *ULP1* vs. *ulp1-mt*	
Samples and conditions	Two independent replicates were prepared from cultures grown in YPD medium at 30°C. Inputs and SUMO IPs were sequenced. 1 – *ULP1* 2 – *ulp1-mt*
Library synthesis	NEBNext Ultra II DNA library prep kit (New England Biolabs)
Sequencing	Illumina NovaSeq SP flowcell; Paired-end reads; 2x 150 nt; 10 million reads/sample
Quality control	FastQC (0.11.9)
Genome alignment	Bowtie2 (2.3.5.1) with sacCer3 reference genome
Peak calling	MACS3 (3.0.0) ***Parameters***: paired-end; input as control; effective genome size: 1.2e7; *q*-value cut-off: 0.05
Differential binding analysis	DiffBind (3.12.0) ***Parameters***: Defaults with minOverlap=2 for dba.count and minMembers=2 for dba.contrast.
Peak analysis and annotation	ChIPpeakAnno (3.18.2) from Bioconductor ***Parameters***: TxDb.Scerevisiae.UCSC.sacCer3.sgdGene genome annotation package was used, but each annotation was confirmed visually using IGV.
Generation of coverage tracks (bigWig) for alignment visualization	bamCompare (deepTools 3.3.2) was used to generate average bigWig files from replicate BAM sequencing alignment files, normalized to inputs. ***Parameters***: bin size: 10 bigwigCompare (deepTools 3.3.2) was used to generate average bigwig files for replicates. ***Parameters***: operation: mean; bin size 10

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Chapter IV: Conclusions and Future Directions

4.1 Summary

Although previous studies have focused on the impacts of sumoylation on individual target proteins, not much is known about how coordinated changes to global sumoylation levels impact gene expression. Considering that a large fraction of SUMO targets are nuclear and involved in gene expression and considering that diverse eukaryotic organisms display a surge in SUMO conjugation levels upon exposure to stress, this work aimed to explore how modulated global sumoylation impacts gene expression at multiple levels in both optimal growth conditions and under stress, specifically heat shock (Seeler and Dejean 2003; Zhou et al. 2004; Makhnevych et al. 2009; Wohlschlegel et al. 2004; Enserink 2015). Disrupting the activities of enzymes of the sumoylation pathway, which leads to either an increase or decrease in global sumoylation, leads to the activation of heat shock related gene expression, changes in transcription for a large subset of genes, and an inability to tolerate heat shock. Altogether, the work detailed in this dissertation highlights the importance of normal sumoylation cycling in regulating gene expression and cell adaptability to stress.

4.1.1 Impairment of sumoylation or desumoylation leads to temperature sensitivity

Our analysis of strains expressing different defective versions of Ubc9 demonstrated that the vast majority of SUMO conjugations that occur in non-stress conditions are dispensable for normal growth. Intriguingly, the strains also displayed normal resistance to osmotic, oxidative, and ethanol stresses, even though these stresses are associated with a surge in sumoylation (Lewicki et al. 2015; Zhou et al. 2004). However, the Ubc9-defective strains all showed strong growth impairment when incubated at the elevated temperature of 37°C. This initially led us to conclude that a minimal threshold level of SUMO conjugation during non-stress conditions is

required pre-emptively to prepare cells for adaptation to heat shock, should it occur, but not for other stress types.

Subsequently, we tested for temperature sensitivity in strains expressing partially defective forms of the E1 activating enzyme Aos1, which displays reduced global sumoylation, and the SUMO protease Ulp1, which shows elevated sumoylation. As previously shown for the *ulp1-mt* strain, both of these strains exhibited temperature sensitivity and could not survive mild heat shock (Soustelle et al. 2004). Furthermore, a strain simultaneously expressing impaired versions of both Ulp1 and Ubc9 also exhibited sensitivity to mild heat shock, even though the global levels of sumoylation were somewhat restored to wild type levels. These results suggest to us that it is not the absolute level of SUMO conjugation under non-stress conditions that determines adaptability to heat stress, but rather the cell's ability to carry out normal cycles of sumoylation and desumoylation, maintaining SUMO as a transient, dynamic modification, that enables survival at elevated temperatures. Similar experiments need to be done in other species to determine whether disrupting sumoylation cycling also specifically impacts heat shock survival in other eukaryotes.

4.1.2 Normal sumoylation cycling is needed for suppressing expression of heat shock related genes

We observed that both abnormally reduced or elevated global sumoylation results in a high level of expression of heat shock related genes such as *HSP12*, *HSP26*, *HSP82*, and *DDR2*. This points to the requirement for normal sumoylation-desumoylation cycling in supressing their expression in non-stress conditions. Unexpectedly, however, strains with defective sumoylation or desumoylation, which constitutively express these heat shock genes, are unable to survive mild heat shock. Paradoxically, this suggests that persistent expression of heat shock genes

during normal growth conditions compromises, rather than enhances, thermotolerance. Previous studies have shown that pre-exposure to mild heat shock increases survival upon subsequent heat stress (Piper 1993; Yamamoto et al. 2008). At first glance, this appears to contradict our findings, where pre-induction of heat shock genes does not confer heat shock resistance. However, in those prior studies, the normal cycling of sumoylation likely remained intact, allowing cells to adjust global sumoylation levels and fine-tune transcriptional responses during subsequent stress. In contrast, in our strains with deregulated sumoylation cycling, cells may be unable to properly reprogram transcription in response to heat shock, even though heat shock genes are already expressed. Supporting this idea, another study revealed that the expression of heat shock proteins alone is insufficient for heat shock survival, and it was suggested that dynamic changes in transcription of additional genes is essential for gaining thermotolerance (Smith and Yaffe 1991).

4.1.3 Msn2 and Hog1 are activated when normal sumoylation cycling is disrupted

The common gene expression changes observed in the strains where sumoylation cycling was disrupted led to the investigation of whether there is a specific transcription factor that mediates these effects. In all strains that we tested with altered global sumoylation, both Msn2 and Hog1 were found to be active, even in non-stress, optimal conditions. Hog1 activity was confirmed by detecting its phosphorylated form, while Msn2 activity was inferred by the elevated expression of its known target genes. To assess their contribution to the induction of heat shock genes in the *ulp1-mt* strain, we tested whether deletion of either *HOG1* or *MSN2* reversed heat shock gene expression. Indeed, these experiments demonstrated that both Hog1 and Msn2 play significant roles in driving heat shock gene activation when sumoylation cycling is disrupted. Although it is considered a key regulator of the heat shock gene expression program (Solís et al. 2016; Amorós and Estruch 2001; Verghese et al. 2012), Hsf1 activation was notably

absent in strains with disrupted sumoylation cycling and occurred only during heat shock, similar to wild type cells. This indicates that Hsf1 does not mediate the heat shock gene induction observed under non-stress conditions. Interestingly, although Hsf1 was activated normally during heat shock in the sumoylation-defective strains, this activation was insufficient to confer thermotolerance. These findings support the idea that, even when Hsf1 is activated, the gene expression program required for heat shock survival cannot proceed properly when normal sumoylation cycling is disrupted.

Overall, our work supports a model in which exposure to elevated temperature disrupts the normal balance of sumoylation-desumoylation cycling by triggering the proteasomal degradation of the Ulp1 SUMO protease. This imbalance in sumoylation dynamics, through a yet-unknown mechanism, contributes to the activation of the key stress responders Msn2 and Hog1 and subsequent induction of their target genes, including heat shock genes. Consistent with this model, we find that Hog1 activation is not required for the surge in sumoylation that is observed during heat shock, indicating that increased sumoylation occurs upstream of Hog1 activation. The mechanism by which disrupted sumoylation cycling leads to the activation of Hog1 and Msn2 is unclear. However, it is unlikely to involve altered sumoylation of these proteins directly. Although Hog1 is sumoylated in non-stress conditions, its level does not change upon heat shock, and we did not detect a sumoylated form of Msn2 in either non-stress or heat shock conditions. Therefore, altered sumoylation likely affects other regulatory factors that, in turn, trigger downstream activation of Hog1 and Msn2.

Although *S. cerevisiae* is a well-established model studying for the heat shock response, the distinct contributions of Msn2 and its paralog Msn4 relative to Hsf1 remain to be fully defined (Amorós and Estruch 2001; Gasch et al. 2000). Further work is also needed to determine

how modulated sumoylation influences activation of the heat shock gene expression response and whether key components of these pathways are regulated by sumoylation-desumoylation cycling, or whether altered sumoylation cycling exerts its effects indirectly. Recent studies have shown that components of the sumoylation machinery, such as Ubc9 and SUMO peptides, play critical roles in assembling and disassembling stress granules in response to heat shock (Marmor-Kollet et al. 2020b). Also, Ulp1, which localizes to the nuclear pore complex and contributes to mRNA transport (Ptak et al. 2025), is degraded upon heat shock, as shown in our experiments. Integrating these observations could provide a more comprehensive understanding of how global changes in sumoylation impact diverse cellular processes to promote adaptation to heat shock.

4.1.4 Chromatin-associated sumoylation decreases when sumoylation cycling is disrupted

Multiple proteomics analyses identified many sumoylation targets as proteins that are known to interact with chromatin, including transcription factors and chromatin remodelling factors (Hendriks and Vertegaal 2016; Albuquerque et al. 2015; Makhnevych et al. 2009; Esteras et al. 2017) .Consistent with this, in 2015, a yeast SUMO ChIP-seq analysis was reported that determined that sumoylated proteins were largely situated genes transcribed by RNAPIII, particularly tRNAs genes, and at RNAPII-transcribed ribosomal protein coding genes (Chymkowitch, Nguéa P, Aanes, et al. 2015). Subsequently, we reported our own SUMO ChIP-seq study that identified sumoylated proteins additionally at promoters of highly transcribed non-ribosomal protein coding genes (Baig et al. 2021). These SUMO peaks were found to align specifically where TBP binds, indicating that one or more components of the RNAPII pre-initiation complex, composed of RNAPII and the general transcription factors, are modified by SUMO during active transcription.

SUMO ChIP-seq experiments were also performed in the *ubc9-6* and *ulp1-mt* strains to determine how modifying global levels of SUMO conjugation impacts the sumoylation landscape genome-wide. As expected, since the *ubc9-6* strain has impaired sumoylation, SUMO ChIP-seq revealed reduced SUMO peak intensities genome-wide. Unexpectedly, however, SUMO ChIP-seq analysis in the *ulp1-mt* strain also showed reduced SUMO peak intensities on chromatin, even though there are elevated SUMO conjugation levels in that strain. One possible explanation is that elevated SUMO conjugation of soluble (non-chromatin-bound) proteins comes at the expense of reduced sumoylation of chromatin-bound factors in *ulp1-mt*, or that, for some reason, SUMO-targeted chromatin factors bind less to chromatin in that strain. Further analysis will be needed to distinguish between these and other possibilities. Nonetheless, these observations support the finding that, although the *ubc9-6* and *ulp1-mt* strains have opposing effects on steady state SUMO conjugation levels, they display similar phenotypes with respect to heat shock sensitivity, heat shock gene expression in non-stress conditions, Msn2/Hog1 activation, and reduced chromatin-associated sumoylation. Consequently, we propose that normal cycling of sumoylation (i.e., balanced sumoylation and desumoylation) during non-stress conditions is required for proper gene expression and for cell survival during subsequent heat shock, and that it is not simply elevated global sumoylation levels that confer protection.

4.2 Future directions

To explore the mechanisms by which disrupted sumoylation cycling triggers an Msn2-mediated gene expression pattern, we will examine regulators of Msn2. Msn2 is activated when it is translocated to the nucleus upon downregulation of PKA activity (Gorner et al. 1998), suggesting that the PKA pathway could act as an upstream regulator of Msn2 activation when sumoylation cycling is impaired. In *S. cerevisiae*, PKA consists of four subunits that assemble by

combining two of its three possible regulatory subunits, Tpk1-3, with a dimer of its catalytic subunit, Bcy1 (Broach 1991). Future work will determine how disrupted sumoylation cycling activates Msn2, for instance by assessing whether these PKA subunits are being sumoylated or whether altered global sumoylation levels affect PKA phosphorylation and lead to its downregulation.

This can be done by first assessing whether the PKA subunits are sumoylated in wild-type strains or strains with modified global sumoylation levels through immunoprecipitation (IP) of the PKA subunits and western blots to visualize whether there any sumoylated forms of those proteins. Even if the PKA subunits are not being directly sumoylated, disruptions in sumoylation cycling globally in the cell can still be impacting their function. Therefore, identifying the phosphorylation status of the PKA regulatory or catalytic subunits can determine its activity. This can be performed by western blotting PKA subunits after electrophoresis with Phos-Tag gels, which resolve phosphorylated proteins from their unphosphorylated forms.

Although strains with either reduced or elevated global sumoylation showed similar expression levels of heat shock-related genes and activation of both Msn2 and Hog1, we still observed gene expression differences between strains such as *ubc9-6* and *ulp1-mt*. In the *ubc9-6* strain, Hog1 exhibited higher phosphorylation and activation levels than in *ulp1-mt*. Therefore, it would be informative to compare genome-wide gene expression between these strains and assess expression of Hog1 target genes to determine whether differential Hog1 activation drives these gene expression differences. For example, comparing RNA-seq results from wild-type, *ulp1-mt*, and *ubc9-6* strains could identify which Hog1 target genes are being upregulated in the mutant strains and the magnitude of RNA level differences between *ulp1-mt* and *ubc9-6* strains could be determined to correlate Hog1 phosphorylation levels with the expression levels of Hog1 target

genes. Additionally, upstream components of Hog1 activation, such as the plasma membrane pressure sensor Sho1, could also be investigated through co-immunoprecipitation experiments to test its association with other proteins needed for activation (Winkler et al. 2002).

Hog1 is also known to be activated during heat shock through the cell wall integrity pathway, and previous studies have shown interactions between Hog1 and Msn2 (Dunayevich et al. 2018). However, further work is needed to clarify the relationship between Hog1 and Msn2 activation when global sumoylation levels are altered. Our experiments show that both Hog1 and Msn2 contribute to heat shock gene expression when sumoylation cycling is disrupted. To test whether these two proteins interact and one is activated before the other, we can track their localization during heat shock through subcellular fractionation or immunofluorescence analysis through a heat shock time-course. Both Msn2 and Hog1 translocate from the cytoplasm to the nucleus in response to stress conditions once they are activated (Alepuz et al. 2001; Gorner et al. 1998). Monitoring their nuclear localization at different time points after heat shock could reveal which protein is activated first.

A notable observation in our studies was that the chromatin association of sumoylated proteins in the *ulp1-mt* strain was reduced compared to wild type, despite the elevated sumoylation levels in the mutant strain. One possible explanation is that sumoylated proteins are unable to bind chromatin and instead remain soluble in the nucleus. To further determine the correlation between transcription factor sumoylation status and its binding to chromatin, sumoylation levels of gene-specific transcription factors that are known to be sumoylated in wild-type strains, such as Rap1 or Sko1, could be first assessed through IPs followed by SUMO western blots. This can be done in the wild-type, *ulp1-mt* and *ubc9-6* strains, as each strain has a different global sumoylation levels. To then assess the binding of the transcription factor to

promoter regions of target genes, ChIP experiments can be done to relate chromatin binding levels with sumoylation levels of the tested transcription factors. These experiments would allow for us to confirm that there is an inverse correlation between sumoylation levels of transcription factors and their chromatin binding. They could also provide further evidence that dynamic sumoylation cycling, rather than the absolute level of sumoylation, is critical for chromatin binding. If so, the chromatin association pattern might remain similar whether a transcription factor is sumoylated or not sumoylated in the *ubc9-6* and *ulp1-mt* background strains.

A recent study by Ptak *et al.* (2025) revealed that Ulp1 associates with the nuclear pore complex and plays a role in maintaining global sumoylation levels. Under normal conditions, Ulp1 interacts with nucleoporins such as Nup60. Since the strains used in our study exhibit altered global sumoylation, future work could examine whether Ulp1 remains associated with nucleoporins in these strains and whether the structure of the nuclear pore complex is affected. Nup60 contributes to nuclear pore stability, so its localization and interactions could be assessed, along with potential changes in Ulp1-nucleoporin interactions. Experiments such as co-immunoprecipitation and immunofluorescence microscopy could be used to determine whether Nup60 interacts with other nucleoporins and Ulp1. Understanding whether the nuclear pore structure is altered in strains with disrupted sumoylation could provide insight into the mechanisms underlying gene expression changes and explain the downstream effects of Ulp1 degradation during heat shock in wild-type cells.

In our study, gene expression was examined using genome-wide ChIP-seq and transcriptome-wide RNA-seq analyses. Translation in the *ubc9-6* strain was also assessed using polysome profiling, and protein levels of select factors were examined in strains with altered sumoylation. However, proteomic studies would help quantify the abundance of each protein in

each strain and identify the specific sumoylated proteins present similar to previous sumoylation-specific proteomic studies (Zhou et al. 2004; Albuquerque et al. 2015). Comparing proteomics data from strains with disrupted sumoylation to data from heat-shocked cells could reveal which proteins remain sumoylated under both conditions, indicating essential SUMO targets required for survival. Although the *ulp1-mt* strain showed a strong correlation with the gene expression profile of heat-shocked cells, some differences remained. Comparing the SUMO-modified proteome of *ulp1-mt* cells and heat-shocked wild-type cells could clarify whether differences in SUMO conjugation explain these gene expression differences.

Altogether, the proposed future will enhance our understanding of sumoylation's role in coordinating the cellular heat shock response and regulating gene expression under normal conditions. Using *S. cerevisiae* as a model organism, insights into global sumoylation dynamics will inform studies of human diseases involving abnormal sumoylation, as observed in certain cancers and neurodegenerative diseases. Moreover, understanding how sumoylation enables adaptation to environmental stress could have practical applications in the agricultural industry where there is a great need for stress-resistant plants.

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Page or Page Range of Portion	115-158	**Publication Date of Portion**	2012-06-01