REGULATION OF YEAST TRANSCRIPTION FACTORS SKO1 AND CST6 BY SUMOYLATION

VERONI SARATHA SRI THEIVAKADADCHAM

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ABSTRACT

Sumoylation is a post-translational modification that plays an essential role in cellular processes, including transcriptional regulation. Transcription factors represent one of the largest groups of proteins that are modified by the SUMO (Small Ubiquitin-like Modifier) protein. In this study, we focused on finding roles for sumoylation in regulating two gene-specific bZIP transcription factors, Sko1 and Cst6, in *Saccharomyces cerevisiae*. Sko1 plays a unique bifunctional role in regulating transcription: it is a repressor during normal growth, by interacting with co-repressor complexes, and an activator during osmotic stress, via interaction with Hog1 Kinase. We show that Sko1 is poly-sumoylated at Lys 567 but its sumoylation is not regulated by stress. Along with sumoylation, Sko1 also undergoes phosphorylation, by PKA and Hog1, and our experiments show that these two modifications are not interdependent. We find that DNA binding is a requirement for Sko1 sumoylation and genome-wide chromatin immunoprecipitation (ChIP-seq) analysis shows that Sko1 sumoylation controls the occupancy level of Sko1 on target promoters and is involved in preventing Sko1 from binding to non-target promoters. Moreover, blocking sumoylation attenuated the interaction between Sko1 and Hog1 on target promoters.

Cst6, on the other hand, is required for survival during ethanol stress and has roles in the utilization of carbon sources other than glucose. In this study, we show that Cst6 is multi-sumoylated at Lys residues 139, 461 and 547. The level of Cst6 sumoylation increases in ethanol and oxidative stress conditions, but decreases if ethanol is used as the sole carbon source. Unlike Sko1, protein levels of SUMO-deficient Cst6 were moderately reduced compared to the wild-type form, implying that sumoylation promotes Cst6 stability. ChIP experiments suggests that sumoylation is important for the timely recruitment of Cst6 to its target promoters. In addition, we provide evidence that Cst6 sumoylation reduces the expression of some target genes, during non-stress and ethanol stress

conditions. Taken together, our studies suggest that the specific effects of sumoylation in regulating transcription factors are target specific. Nevertheless, SUMO plays a general role by controlling the transcription factor-DNA interaction to maintain proper gene expression.

DEDICATION

I dedicate this dissertation to my mother, **Annette Sri Theivakadadcham**. I pursued with PhD mainly to fulfill my mother's dream. Her constant support and motivation has given me the strength to travel through my PhD journey. Thank you mum, without your love, advice and support, this would not have been possible.

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LIST OF ABBREVIAITONS

Abbreviation	Full name
Aca	ATF/CREB repressor
Acr	ATF/CREB repressor
AP-1	Activator protein-1
ATF	Activating Transcription Factor
ATP	Adenosine triphosphate
bHLH	basic helix-loop-helix
bZIP	basic leucine zipper
CEBP	CCAAT-enhancer binding protein
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation sequencing
COF	Cofactors
CRE	cAMP Response Element
CREB	cAMP Response Element-Binding Protein
Cst6	Chromosome stability 6
DBD	DNA-binding domain
DDR	DNA damage response
EMSA	Electrophoretic mobility shift assay
FDR	False-discovery rate
FE	Fold enrichment
GEO	Gene expression omnibus
GO	Gene ontology
GTFs	General transcription factors
HDAC	Histone deacetylases
IGV	Integrative Genomics Viewer
IP	Immunoprecipitation
Lys	Lysine
NBs	Nuclear bodies
NDSM	Negatively charged amino acid-dependent sumoylation motif
NEM	N-ethylmaleimide
NHR	Nuclear hormone receptor
NLS	Nuclear localization sequence
NMR	Nuclear magnetic resonance

NP40	Nonidet P-40
NTP	Nucleoside triphosphates
OD	Optical density
PDSM	Phosphorylation-dependent sumoylation motifs
PIAS	Protein inhibitor of STAT
PIC	Pre-initiation complex
PMB	Protein binding microarrays
PMSF	Phenylmethylsulfonyl fluoride
PTM	Post-translational modifications
PTP1B	Protein-tyrosine phosphatase-1B
PXR	Pregnane X receptor
RanGAP1	Ran GTPase-activating protein 1
RNAP	RNA polymerase
RT	Reverse transcription
SAE	SUMO-activating enzyme
SENP	Sentrin-specific protease
SC	Synthetic complete
SIM	SUMO Interacting Motifs
Sko1	Suppressor of Kinase Overexpression 1
SM	Sulfometuron methyl
SSR	SUMO stress response
STUbL	SUMO-targeted ubiquitin ligases
SUMO	Small Ubiquitin-like Modifier
TAF	TBP-associated factor
TBP	TATA box-binding protein
TCA	Trichloroacetic acid
TCAG	The Centre for Applied Genomics
TDG	Thymine-DNA glycosylase
TSS	Transcription start site
TF	Transcription factor
UAS	Upstream activation sequences
ZF	Zinc finger

CHAPTER 1
LITERATURE REVIEW

1.1 Post-translational modification

Proteins play vital roles in many cellular functions such as catalysis, transport, structural building, defence, hormones, and many more. While the human genome consists of about 25,000 protein coding genes, there are more than 1 million proteins found in cells that are functionally different [1]. Much of the diversity among proteins is due to alternative splicing and post-translational modifications (PTMs). Alternative splicing takes place before an mRNA is translated to a protein by ribosomes, whereas, PTMs occur after protein synthesis. Many proteins are not functionally active immediately after the synthesis process; they must first undergo certain chemical modifications to become fully functional and these modifications are known as PTMs [1]. PTMs are most often driven by enzymes that either add a functional group or peptides, or perform proteolytic cleavage to orchestrate protein activity [2]. PTMs can regulate a protein throughout its lifespan starting from its intracellular localization, DNA-binding, protein-protein interactions, and all the way to its degradation [2].

To date, more than 200 PTMs have been identified and some common PTMs include phosphorylation, acetylation, glycosylation, methylation, ubiquitination, and sumoylation [1-3]. Other PTMs, such as oxidation, neddylation, formylation, propionylation, and butyrylation have also gained attention in recent years. The effects of the modifications vary, but can include altering the charge state, hydrophobicity, conformation and stability of the target proteins [3]. In addition, most of these PTMs are reversible and can have different functions in different organelles. For example, phosphorylation controls nuclear localization of some proteins by either assisting (e.g.

inflammatory response transcription factor RelA) or preventing (e.g. Bach2, a regulator of the immune system) their nuclear import [4, 5]. Whereas in the nucleus, phosphorylation can regulate gene expression by influencing the DNA binding activity of transcriptional regulators. For example, ZEB1, an essential transcription factor in the development of the eye and neurons, is subjected to phosphorylation at Thr 867 which promotes ZEB1-DNA binding [6]. In contrast to this, YY1 phosphorylation at Tyr 383 interferes with its DNA binding thereby supressing transcription of genes involved in embryogenesis [7].

In many cases, proteins can be modified by multiple PTMs that are dependent or independent of each other [1-3]. These modifications can work together to alter the functional outcomes of a target protein. For example, pregnane X receptor (PXR) is a ligand-activated transcription factor that has a pivotal role in transcription regulation of drug metabolism and drug transport pathways in the liver and intestine [8-10]. PXR is regulated by acetylation, sumoylation and ubiquitination, and recent studies have shown an interconnected role for these modifications. Both the ligand binding and recruitment of p300 induces a conformational change in PXR that promotes p300-mediated histone acetylation which facilitates transcriptional activation. Sustained transcription induces p300 mediated acetylation of PXR at Lys109 [8]. PXR acetylation induces further conformational changes that reduces its affinity to its DNA binding partner RXRα, resulting in the destabilization of transcriptional complex and transcriptional repression.

Another study indicated that this acetylation of PXR is important in mediating PXR sumoylation which is involved in transcriptional repression of target genes [9]. PXR is sumoylated with SUMO-2/3 at Lys108 and 128/129 by the PIASy SUMO E3 ligase. This sumoylation, in turn, stimulates K48-linked poly-ubiquitination at Lys170 to promote proteasome-mediated degradation of PXR

[10]. Interestingly, increased acetylation on ligand-activated PXR correlated with an increase in poly-ubiquitination at Lys170 and 160 [9]. These results together show the interplay between acetylation, sumoylation, and ubiquitination in the regulation of PXR. Importantly, deregulation of PTMs are found in human diseases such as cancer, neurodegenerative diseases, and diabetes [2].

1.2 Sumoylation

Since the discovery of sumoylation in 1996 by Günter Blobel and his team, this PTM was shown to be involved in a wide range of cellular processes such as DNA damage response, nuclear/cytoplasmic transport, cell cycle progression, signalling, cellular stress response and transcriptional regulation [11-14]. Sumoylation involves the attachment of a SUMO (Small Ubiquitin-like Modifier) peptide to lysine residues of substrate proteins [12-14]. SUMO is a small protein that consists around 95-115 amino acids and has a calculated molecular mass of ~12 kDa. The first SUMO target was identified in mammals, the Ran GTPase-activating protein 1 (RanGAP1), a protein that functions in regulating nuclear import and export systems [11].

Three-dimensional structures of both SUMO and ubiquitin are very similar and these proteins contain a tightly packed ββαββαβ fold (Figure 1.1) [3]. SUMO shares about an 18% amino acid sequence homology with ubiquitin and these two proteins contain a double glycine at their C-termini (Figure 1.2A). In contrast, SUMO has a long N-terminal domain that is absent in ubiquitin and SUMO and ubiquitin have different surface charge distributions [15]. These observations suggest that they interact with distinct substrates to exert different functions.

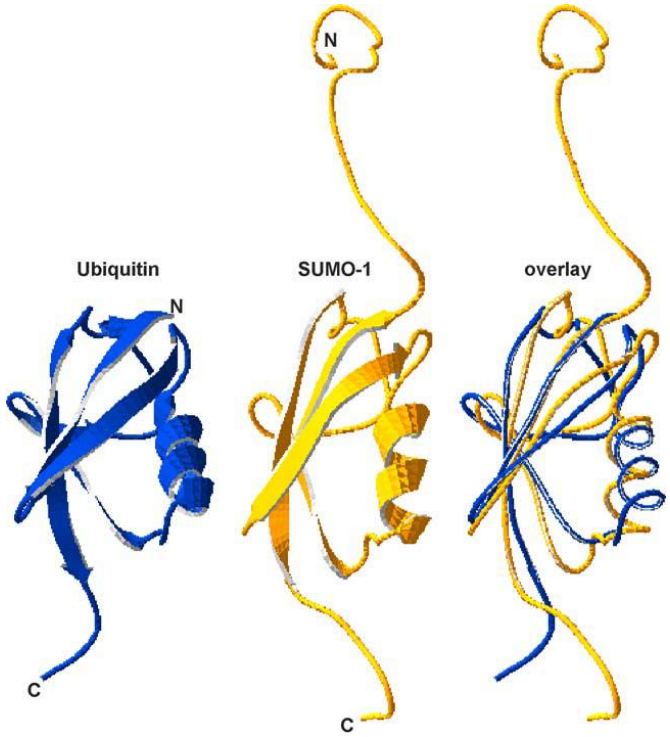

Figure 1.1: Structural comparison between Ubiquitin and the human SUMO1 isoform. Both proteins share structural similarities with the tightly packed ββαββαβ fold. Contrastingly, SUMO1 has a long N- terminus which is absent in ubiquitin. The structure of ubiquitin was determined by X-ray crystallography whereas the structure of SUMO was discovered by NMR [15].

SUMO is conserved among eukaryotes and since its discovery, at least one SUMO isoform has been identified in all eukaryotes. *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila* express only a single SUMO protein. Contrastingly, mammals express five SUMO isoforms: SUMO1, SUMO2, SUMO3, SUMO4 and SUMO5 [13]. *S. cerevisiae* SUMO (also known as Smt3), encoded by the *SMT3* gene, shares similarities to the amino acid sequences of the mammalian SUMO isoforms (Figure 1.2A) [12]. SUMO1 shares only about 47% sequence similarity to SUMO2 and SUMO3. On the other hand, since SUMO2 and SUMO3 share 97% sequence identity, they are often referred to as SUMO2/3.

SUMO4 was identified through DNA sequence analysis as it shares about 87% similarity with the SUMO2/3 sequence [16, 17]. However, differences between SUMO4 and the other SUMO isoforms have led to questions about whether it is expressed or functions like other SUMO peptides. Unlike other SUMOs, the *SUMO4* gene lacks introns, thus making it possible that it is actually a pseudogene. In addition, it has a proline instead of glutamine in position 90, which questions whether it can be processed by known SUMO proteases to a mature form (Figure 1.2A) [16, 17].

Lastly, SUMO5 was the latest identified SUMO isoform to be identified, and it shares a high sequence similarity with SUMO1 (Figure 1.2B). Although these five isoforms share some sequence similarities, their expression profiles are different [12]. SUMO1 and SUMO2/3 are ubiquitously expressed while SUMO4 and SUMO5 are tissue specific. Although the SUMO4 mRNA has been found in specific organs such as spleen, lymph nodes, and kidneys, the endogenous SUMO4 protein has not been detected [12, 16, 17], whereas SUMO5 is translated in the lungs and spleen [18].

```
Ubiquitin -------------------------MQIFVKTLTGKTITLEVESSDTIDNVKAKIQDKEG 35
Smt3      MSDSEVNQEAKPEVKP--EVKPETHINLKVS-DGSSEIFFKIKKTTPLRRLMEAFAKRQG 57
SUMO1     MSD----QEAKPSTEDLGDKKEGEYIKLKVIGQDSSEIHFKVKMTTHLKKLKESYCQRQG 56
SUMO2     MAD----E--KPKE--GVKTENNDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQG 52
SUMO3     MSE----E--KPKE--GVKTE-NDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQG 51
SUMO4     MAN----E--KPTE--EVKTENNNHINLKVAGQDGSVVQFKIKRQTPLSKLMKAYCEPRG 52
SUMO5     MSD----LEAKPSTEHLGDKIKDEDIKLRVIGQDSSEIHFKVKMTTPLKKLKKSYCQRQG 56

Ubiquitin IPPDQQRLIFAGKQLEDGRTLADYNIQKESTLHLVLRLRGG   76
Smt3      KEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGG   98
SUMO1     VPMNSLRFLFEGQRIADNHTPKELGMEEEDVIEVYQEQTGG   97
SUMO2     LSMRQIRFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG   93
SUMO3     LSMRQIRFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG   92
SUMO4     LSMKQIRFRFGGQPISGTDKPAQLEMEDEDTIDVFQQPTGG   93
SUMO5     VPVNSLRFLFEGQRIADNHTPEELGMEEEDVIEVYQEQIGG   97
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Figure 1.2: Sequence alignment between Ubiquitin and SUMO family proteins. Mammalian SUMO isoforms and yeast SUMO (Smt3) share some sequence similarity with ubiquitin. Dark blue background shows the identical residues in all sequences, including the di-glycine motif. Light blue background represents conserved positions among ubiquitin and SUMO. Positions that are identical in at least three of the SUMO proteins, but not in Ub, are shaded with a purple background. Sequence alignment of SUMO isoforms and ubiquitin was made using Clustal Omega.

1.2.1 Sumoylation Pathway

SUMO proteins are synthesized as immature precursors and must undergo a C-terminal cleavage mediated by SUMO-specific proteases to become mature proteins [11, 14]. This cleavage exposes a di-glycine motif ('GG') that is required for SUMO conjugation to occur on lysine residues in substrate proteins. Similar to ubiquitination, sumoylation is a dynamic process which requires catalysis by three classes of enzymes: E1 activating enzymes, E2 conjugating enzyme, and E3 ligases (Figure 1.3). The mammalian and yeast proteins involved in the sumoylation pathway are listed in Table 1.1.

Once the mature form of SUMO is formed, it undergoes an activation step by the E1 activation enzyme in an ATP dependent manner. The mammalian SUMO E1 enzyme is composed of two subunits, SUMO-activating enzyme subunit 1 (SAE1; known as Aos1 in yeast) and SUMO activating enzyme subunit 2 (SAE2; known as Uba2 in yeast) [11-14]. The activation of SUMO is a two-step process: (i) SUMO E1 complex adenylates the exposed 'GG' of the SUMO protein; (ii) SAE2 forms a thioester bond between its active site cysteine and the carboxyl terminus (at the 'GG') of SUMO [12]. Formation of the thioester bond activates SUMO, therefore enabling its interaction with the SUMO E2 enzyme, Ubc9. Activated SUMO is then transferred to a cysteine in the active site of Ubc9, maintaining the thioester bond by a trans-thioesterification reaction [11-14]. In contrast to ubiquitination, in which E3 ubiquitin ligases are the primary determinant of substrate specificity, Ubc9 can directly specify its targets via the consensus motif ΨKxD/E, where Ψ is a hydrophobic residue, K is the lysine (Lys) to be modified and x is any amino acid (Figure 3: Step 4A).

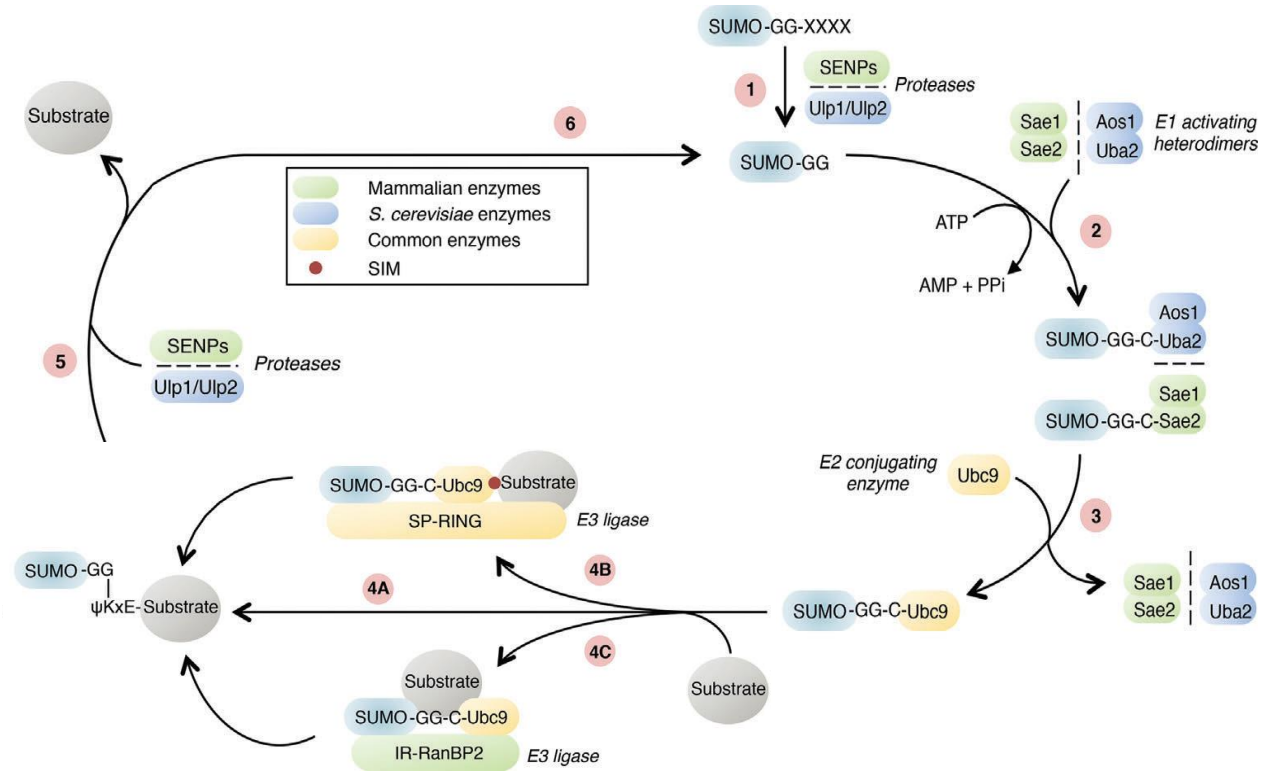

Figure 1.3: The sumoylation pathway in mammals and *S. cerevisiae*. **(1)** SUMO precursors are cleaved at the C-terminus by SUMO-specific protease to reveal the GG motif. **(2)** Mature SUMO is activated by the E1 activating enzyme in an ATP-dependent manner. **(3)** Activated SUMO is then transferred to the E2 conjugating enzyme, Ubc9. Substrate is sumoylated by one of the three methods: **(4A)** Ubc9 can trigger the conjugation of SUMO to a lysine residue within a consensus modification motif (ΨKxE) of a given substrate; or **(4B)** SUMO E3 ligase (e.g. SP-RING) interacts with both the Ubc9-SUMO complex and the substrate to facilitate substrate sumoylation; or **(4C)** SUMO E3 ligase (e.g. IR-RanBP2) interacts only with the Ubc9-SUMO complex during sumoylation. **(5)** Finally, de-sumoylation is carried out by SUMO-specific protease that cleaves the SUMO peptide from the substrate proteins, and **(6)** allowing the recycling of free SUMO. SIM: SUMO interacting motif. Figure adapted from [13].

Although Ubc9 can transfer the SUMO protein to a substrate protein, there are a number of SUMO E3 ligases discovered, that enhance SUMO conjugation and substrate specificity. The identified SUMO E3 ligases are classified into three groups: Siz/PIAS-RING (SP-RING), Internal repeats (IR)-RanBP2, and the others. Similar to Ubc9, SUMO E3 executes the transfer of SUMO by forming an isopeptide bond between the lysine of a target protein and the C-terminal glycine of SUMO [11-14] (Figure 1.3: Steps 4B and 4C).

1.2.2 De-sumoylation

Sumoylation is a highly dynamic process, and the removal of SUMO from substrates is referred to as de-sumoylation. This step is mediated by the same enzymes that cleave the SUMO precursors before the activation step (Figure 1.3; compare Step 1 and Step 5). There are several SUMO proteases identified to date, including Ulp1 and Ulp2 in yeast, and the sentrin-specific protease (SENP) family in humans [11-14] (Table 1.1). SUMO proteases contain shallow clefts lined with conserved amino acids, such as tryptophans, that clamp the di-glycine motif leading to the cleavage of the SUMO C-terminus [19]. These proteases show substrate specificity and have been discovered to be localized to different subcellular compartments in cells. For example, Ulp1, SENP1 and SENP2 localize to the nuclear envelope to function in cleaving SUMO precursors and de-sumoylating substrates. In Contrast, Ulp2, SENP6 and SENP7 localize in the nucleoplasm in order to remove poly-SUMO chains [19].

Table 1-1: Enzymes mediating the sumoylation pathway in *S. cerevisiae* and *H. sapiens*.

Enzymes	*S. cerevisiae*	*H. sapiens*
SUMO	Smt3	SUMO1, SUMO2, SUMO3, SUMO4, SUMO5
E1 activating enzyme	Aos1, Uba2	Sae1, Sae2
E2 conjugating enzyme	Ubc9	Ubc9
E3 ligase	Siz1, Siz2, Cst9, Mms21	PIAS1, PIAS2, PIAS3, PIAS4, ZMIZ1, NSE2, RanBP2, Pc2, MUL1, TOPORS, HDAC4, HDAC7, TRAF7, FUS, RSUME
SUMO protease	Ulp1, Ulp2	SENP1, SENP2, SENP3, SENP4, SENP5, SENP6, SENP7, DESI1, DESI2, USPL1

Adapted from [13]

1.2.3 SUMO acceptor sites

Approximately 75% of SUMO substrates are modified within the consensus motif (ΨKxD/E). However, sumoylation can also occur at a lysine residue within at least two other types of SUMO acceptor sites: phosphorylation-dependent sumoylation motifs (PDSM) and negatively charged amino acid-dependent sumoylation motif (NDSM) [20-22].

The PDSM is composed of a SUMO consensus motif and a proline-directed phosphorylation site (ΨKxExxSP) [11, 21]. Binding of SUMO within PDSM is dependent on prior phosphorylation. The first evidence of PDSM-dependent sumoylation was observed in heat-shock factor 1 and heat-shock factor 4 [21]. Subsequent bioinformatics analyses showed the presence of PDSM in multiple transcriptional regulators, including erythroid transcription factor GATA-1, Smad nuclear interacting protein-1, and myocyte enhancer factor 2 [21]. On the other hand, NDSM (ΨKxExxxxxxxxxx) contains negatively charged residues within a 10-amino-acid region downstream of the SUMO consensus motif [19, 22]. NDSM was first discovered in Elk-1 and later in PML, Sin3A and NF1.

Both PDSM and NDSM provide negatively charged residues downstream of the Lys residue [20, 21]. These negatively charged residues are used to interact with the basic patches on Ubc9, thereby stabilizing the interaction between Ubc9 and the SUMO target to facilitate the transfer of SUMO [19-21]. The main difference between these two motifs is that PDSM provides the acidic nature by phosphorylation of a nearby serine residue, whereas the negative charge in the NDSM is a property of the primary sequence of the protein [17].

1.2.4 SUMO chain formation

Through the sumoylation cycle, a substrate can undergo different types of sumoylation: mono-sumoylation (sumoylated at a single Lys); multi-sumoylation (sumoylated at multiple Lys); or poly-sumoylation (formation of SUMO chains on one or more Lys) (Figure 4A) [12-14]. SUMO 2/3 contain a SUMO consensus motif in their N-terminal region, centered on K11 [12, 23] (highlighted on Figure 1.2B), whereas yeast SUMO (Smt3) contains three SUMO consensus motifs around K11, K15, and K19 [23, 24]. These Lys residues with these three SUMO forms serve as a platform to be modified by SUMO chains. There are non-consensus Lys residues in SUMO1 (K7, K17, K25), SUMO2 (K32, K34), and SUMO3 (K33, K35) that accept the formation of SUMO chains. Interestingly, in humans, SUMO chains can be formed with the combination of different SUMO isoforms. For example, SUMO1 conjugates to K11 of SUMO2/3 to terminate the chain formation in an *in vitro* reaction [25]. This termination is due to the absence of K11 on SUMO1 hindering the ability for further chain elongation (Figure 1.4A) [25, 26].

De-sumoylation of SUMO chains is mainly executed by Ulp2 and SENP6 SUMO proteases, in yeast and mammalian cells respectively, as seen by the heavy accumulation of poly-sumoylated proteins in the absence of these two proteases [23]. Multiple SUMO targets have been identified to be poly-sumoylated and the SUMO chain has many biological functions. The well-studied primary function of poly-sumoylation is that it targets the substrate for ubiquitin-mediated proteasomal degradation (Figure 1.4B) [27, 28]. SUMO-targeted ubiquitin ligases (STUbLs) bind poly-sumoylated targets to form poly-ubiquitin chains. Subsequently, the 26S proteasome is recruited to degrade the target protein. In addition, poly-sumoylation has been found to increase sporulation efficiency in yeast, and it plays a role in kinetochore function in humans [29, 30].

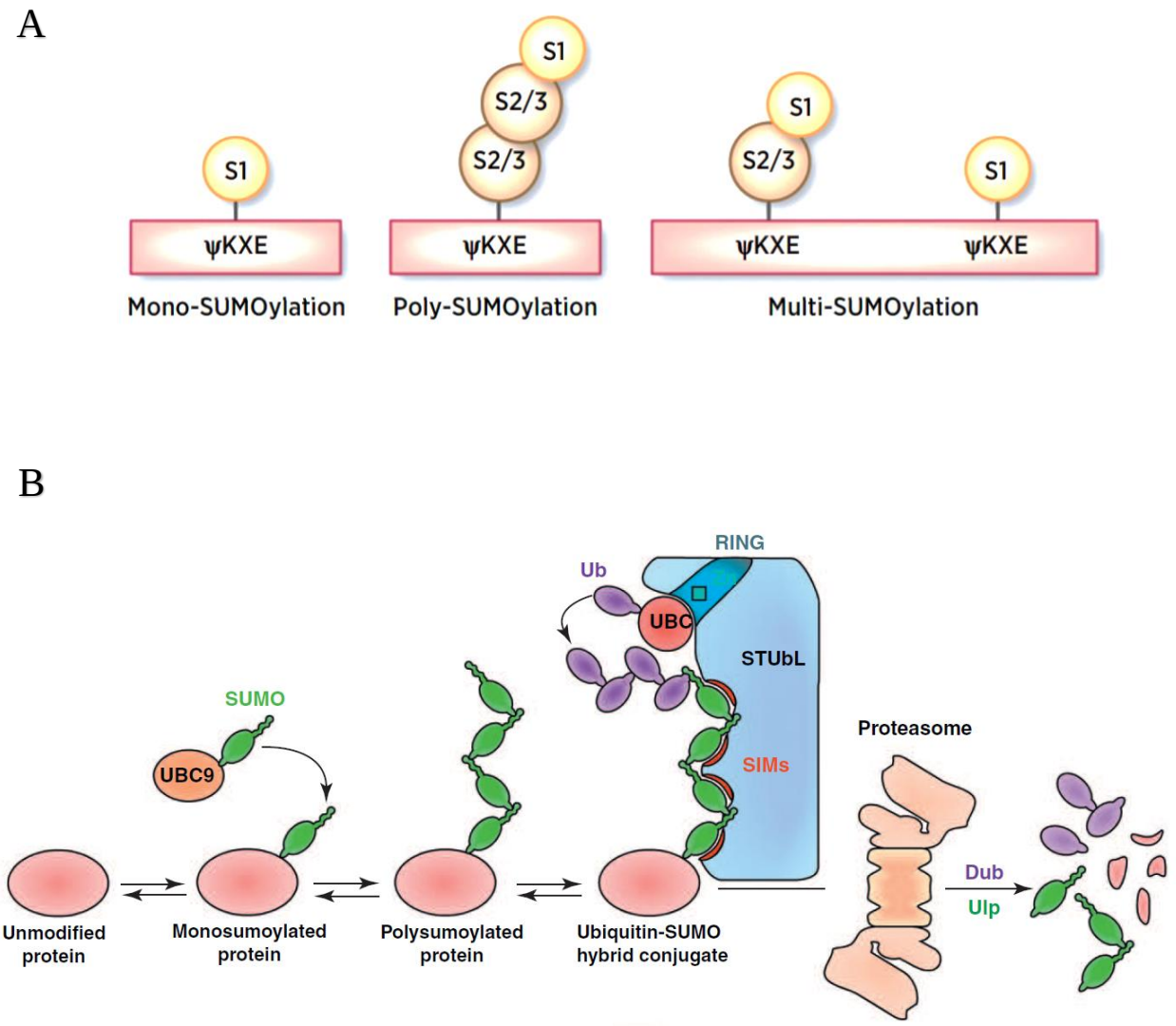

Figure 1.4: Types of sumoylation and the function of poly-sumoylation in ubiquitin-mediated proteasomal degradation. (A) SUMO targets can be either mono-sumoylated, poly-sumoylated or multi-sumoylated. Combinations of SUMO2/3 (S2/3) and SUMO1 (S1) can form poly-sumoylation and SUMO1 often terminates the chain elongation. **(B)** Schematic representation to explain how poly-sumoylation on target proteins leads to ubiquitin-mediated proteasome degradation. Poly-sumoylated protein is recognized by SUMO-targeted ubiquitin ligases (STUbLs), RING finger type of ubiquitin ligase. STUbL adds poly-ubiquitin chains that are recognized by the 26S proteasome, which then facilitates protein degradation. Finally, ubiquitin and SUMO are recycled by de-ubiquitination (DUB) and de-sumoylation (ULP), respectively. SIM: SUMO interacting motif. Figures are adapted from [26] and [28], respectively.

1.2.5 Molecular consequences of sumoylation

Sumoylation can affect protein localization, enzymatic activity, protein stability or influence interactions with other proteins [20, 31]. For example, sumoylation of RanGAP1, a GTPase activating protein, increases its interaction with RanBP2, a component of the nuclear pore complex, to facilitate RanGAP1 nuclear import [32]. In addition, the sumoylation of p53 promotes its interaction with the nuclear export receptor CRM1 to allow nuclear export of p53 [33].

SUMO also influences protein stability by either enhancing stability directly, or by assisting in ubiquitin-mediated degradation. For example, in the absence of viral infection, cyclic GMP-AMP synthase (cGAS) is modified by SUMO1, which prevents poly-ubiquitination and the subsequent degradation of cGAS, thereby enhancing its stability [34]. On the other hand, RNF4, a ubiquitin E3 ligase, preferentially binds to the poly-sumoylated promyelocytic leukaemia (PML) protein to induce poly-ubiquitination of PML and the subsequent degradation by the ubiquitin-proteasome pathway [35].

All these general functions of SUMO are mediated by altering the surface of the target protein, thereby influencing interactions with other proteins. There are multiple ways that this can be achieved: (i) target conformation changes after the modification by SUMO (Figure 1.5A); (ii) the presence of SUMO can mask the binding sites that are needed for other proteins to interact (Figure 1.5B); or (iii) SUMO provides the binding sites/surface for other proteins to bind (Figure 1.5C) [13, 20, 31].

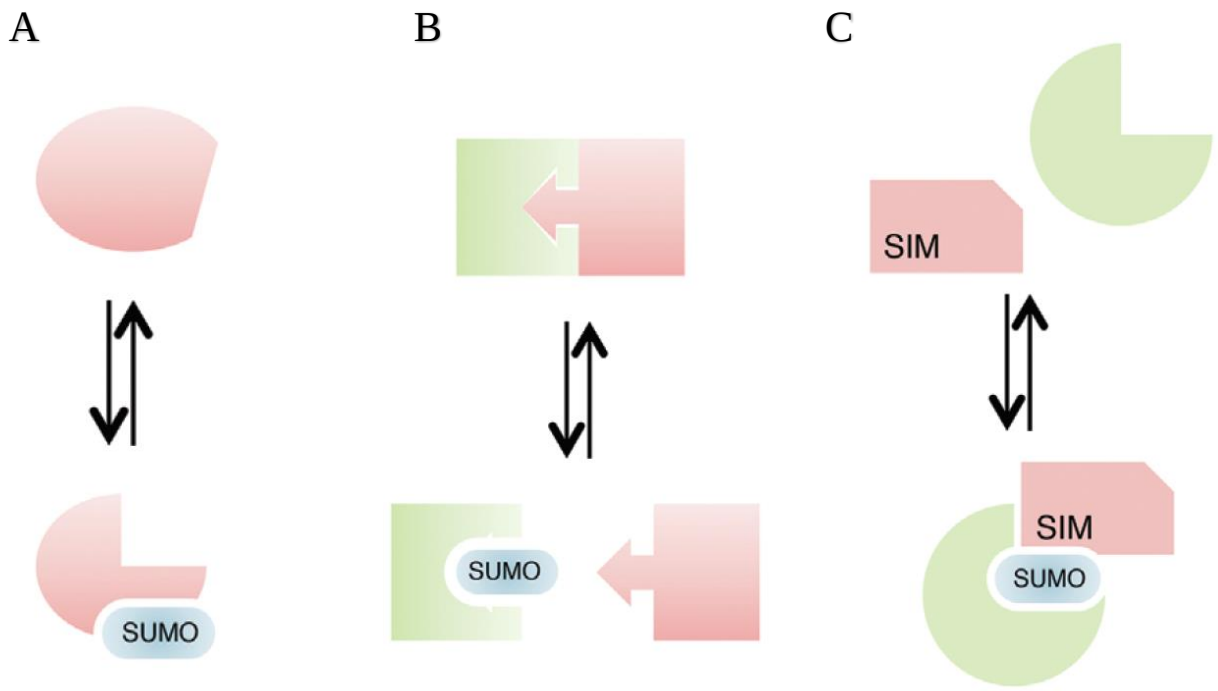

Figure 1.5: Schematic representation of how SUMO alters the binding site on the target surface. (A) SUMO conjugation induces conformational changes on the substrate. **(B)** Binding of SUMO prevents the interaction with a partner protein. **(C)** SUMO promotes protein-protein interaction through SUMO interacting motifs (SIM). Figure adapted from [13].

Sumoylation of thymine-DNA glycosylase (TDG) is thus far the best example to explain how SUMO modulates the target conformation (Figure 1.5A). During DNA repair, TDG binds to the DNA to execute base excision repair by removing the mutant base pair. After excision, TDG is modified by SUMO1 causing a conformational change. This change in structure reduces the affinity between TDG and DNA, thereby removing TDG from DNA [36]. This allows the binding of other proteins involved in DNA repair to then proceed with repairing the DNA lesion.

In addition to protein conformational change, SUMO can mask binding sites that are needed for other proteins to interact (Figure 1.5B). For example, transcription factor Sp1 is sumoylated by SUMO2/3 which prevents Sp1 interaction with the acetyl transferase p300 during ocular lens differentiation [37]. In addition, sumoylation of the ubiquitin-conjugating enzyme E2-25k inhibits the interaction with ubiquitin E1 enzyme, causing a decrease in ubiquitin conjugation [38]. In contrast, SUMO also provides binding sites/surface for other proteins to bind. Sumoylated proteins non-covalently interact with other proteins via SUMO interacting motif (SIM) (Figure 1.5C) [13, 20]. The SIM is made up of hydrophobic residues (V/I/L)-x-(V/I/L)-(V/I/L), where x is any amino acid [13]. Sumoylated proteins and proteins containing SIMs can bind to form protein complexes. For example, PML nuclear bodies (PML NBs) are made by forming complexes with sumoylated and SIM containing proteins such as PML, p53, sp100, CBP, and DAXX [39]. In fact, STUbLs bind to the poly-sumoylated protein using their SIMs, then mediate poly-ubiquitination for subsequent degradation (Figure 1.5C and Figure 1.3B) [27]. Along with PML (mentioned above), other proteins such as IκBα (Inhibitor the NF-κB transcription factor) and hypoxia-inducible factor 1α are also found to be degraded through similar methods [40, 41].

1.2.6 The SUMO Enigma

An interesting observation regarding sumoylation is that, although a small fraction of targets is sumoylated at a given time, this is enough for a maximum effect. This phenomenon has been referred to as 'SUMO enigma' [17]. The underlying mechanism for the SUMO enigma has not been determined, but there are some possible explanations regarding this effect. As sumoylation is a dynamic event, if proteases act constitutively and rapidly to de-sumoylate target proteins soon after their sumoylation, this might partly explain the low steady-state level of sumoylated proteins at a given time.

It is also possible that the effect of sumoylation persists even after de-sumoylation of the target proteins [17, 20, 42]. For example, sumoylation of some transcription factors was shown to be involved in transcriptional repression by recruiting proteins that repress transcription (detailed explanation below). There are two mechanisms proposed that explains how low levels of sumoylated transcription factors could repress transcription even after de-sumoylation (Figure 1.6) [20]. Sumoylated transcription factors might recruit chromatin remodelling proteins that have repressive activity. This will ensure that the chromatin stays in a repressive state even after SUMO is removed from the transcription factor (Figure 1.6A). Alternatively, sumoylation of a transcription factor initiates the formation of a repressive complex on chromatin and this complex will remain stable even after SUMO is removed from the transcription factor (Figure 1.6B) [20, 42]. In both cases, the transcription factor or the regulator need only to be sumoylated for a short period of time during which gene silencing is initiated. Regardless of the small fraction of sumoylated proteins found in cells, the functional outcomes of sumoylation in regulating target proteins are diverse.

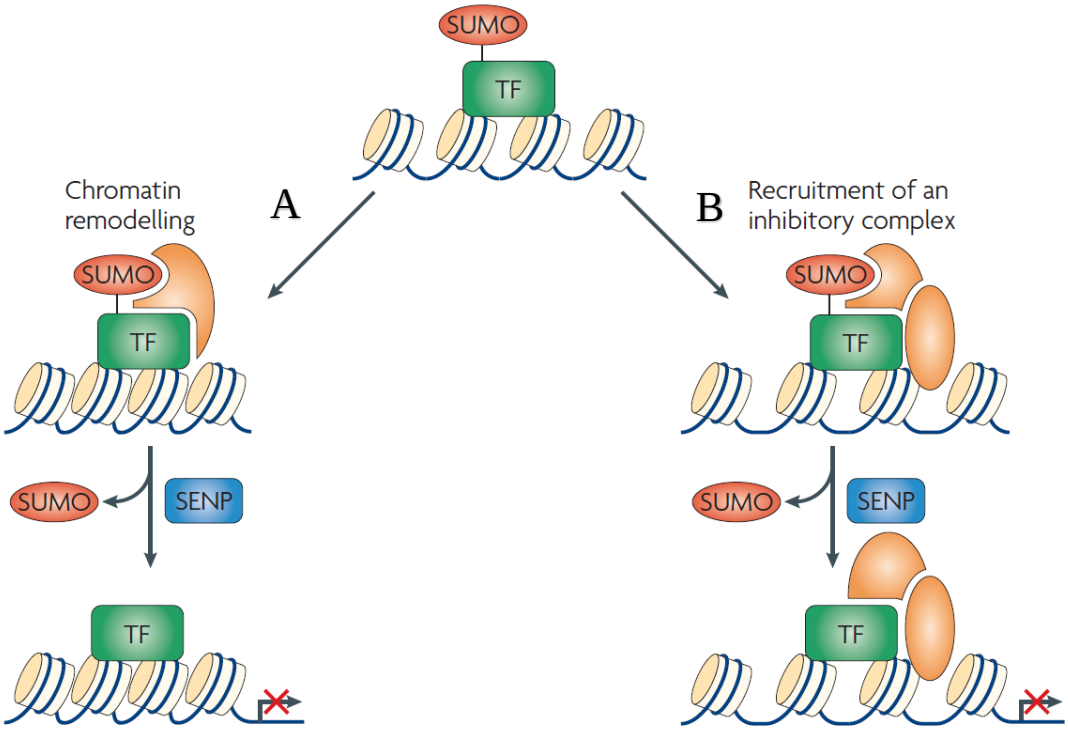

Figure 1.6: Methods explaining how sumoylation can have a prolonged effect on transcriptional repression even after de-sumoylation of the transcription factor. (A) Sumoylated transcription factors might recruit chromatin remodelling proteins that have repressive activity, such as deacetylation. This will ensure that the chromatin stays in a repressive state even after SUMO is removed from the transcription factor. **(B)** Sumoylation of a transcription factor initiates the formation of a repressive complex on chromatin and this complex will remain stable even after SUMO is removed from the transcription factor. TF: transcription factor. Figure adapted from [20].

1.2.7 SUMO and stress response

Although only a small fraction of targeted proteins are sumoylated at a given time, overall sumoylation levels are altered during stress conditions. Steady-state sumoylation levels are heavily elevated during stress conditions, and this scenario is called the SUMO stress response (SSR) [43]. SSR is common in mammalian, yeast and plant cells and is observed during several endogenous or environmental stress conditions such as DNA damage, osmotic stress, oxidative stress, hypoxia, and heat shock [12, 43-50]. In yeasts, the kinetics of SSR depends on the concentration and the type of the stressors [44]. For example, during severe hyperosmotic stress (1 M NaCl, 1 M KCl, or 1 M sorbitol) and oxidative stress (100 mM H_2O_2), the increase in sumoylation was noted within 2 min of introducing the stressors. In the presence of 10% ethanol, SSR was noted within 10-15 min of introducing the ethanol stress. SSR during severe osmotic only lasts about 10-45 min, but it lasts about 3 hours during severe oxidative and ethanol stress [44]. Similarly, in mammalian cells, elevated sumoylation was observed within 5 min of heat shock and lasted about 2 hours [45].

Many proteins are not detectably sumoylated during normal growth but display elevated sumoylation levels after exposure to stress. For example, in a yeast mass spectrometry study, H2B (a histone component), Bir (involved in kinetochore function), Sod1 and Tsa1 (antioxidant enzymes), proteins that are usually not modified by SUMO during normal growth, were found to be sumoylated during oxidative stress [46]. Similarly, in Hcr1 (related to translation), Adh1 (function in metabolism) and Bob3 (chromatin related), sumoylation was only observed during ethanol stress [46]. Moreover, an interesting observation in mammals was that during normal growth conditions, SUMO substrates were mostly conjugated by SUMO1, leaving a heavy abundance of free SUMO2/3. However, when cells were exposed to stress conditions, more

SUMO conjugations were observed with SUMO2/3, depleting the levels of free SUMO2/3, thus signifying its functional importance in SSR [47]. For example, during heat shock, 766 proteins were identified to be sumoylated by SUMO2 and in oxygen/glucose-deprivation-induced cells, 188 SUMO-3 substrates were identified [43, 45, 48].

There have been a few studies that examined mechanisms responsible for the SSR in cells. Among proteins that are sensitive to heat shock, it was observed that the activity of all SENPs (except SENP6), mammalian SUMO proteases, are heat-sensitive and inactive during heat shock. Therefore, the inactivation of SENP causes a decrease in de-sumoylation which explains the increased sumoylation observed during heat shock [49]. Similarly, severe oxidative stress inactivates SENP1 by forming a disulfide bond between two SENP1 molecules thereby interfering with the de-sumoylation pathway [43]. Related yeast studies suggest that the global increase in transcription during stress conditions is what causes the increase in sumoylated proteins during the SSR [44]. Since transcription related proteins are known to be heavily modified by SUMO (explained below), this study suggests that ongoing transcription and SSR are related. In fact, this study also demonstrated that SSR is not regulated by translation or proteasome-dependent degradation [44]. Moreover, SUMO conjugation during SSR is dependent on the Siz1 SUMO E3 ligase and the timely reversal of SSR is dependent on the SUMO-specific protease Ulp2 in yeast [44].

1.3 Biological functions of SUMO

Sumoylation is important for many cellular processes throughout the cell. Although early studies regarding SUMO functions focused more on nuclear processes, there are also many non-nuclear proteins that are modified by SUMO. The emerging evidence suggests that defects related to sumoylation have been observed in many disorders including cancer, Huntington's and Alzheimer's diseases [12].

1.3.1 Non-nuclear sumoylation

Sumoylated proteins are found on the plasma membrane, endoplasmic reticulum and mitochondria where they serve multiple functions in protein signaling, cytoskeletal organization, channel activity, receptor function, exocytosis, autophagy, mitochondrial dynamics and others [20, 51]. In fact, the first SUMO target discovered, Ran1GAP, is sumoylated in cytoplasmic filaments of the nuclear pore complex [11].

At the plasma membrane, SUMO targets receptors and plasma channels to exert its function. GluR6, a subunit of the kainate receptor, is sumoylated when induced by kainite to facilitate kainite-induced endocytosis [52]. In contrast, sumoylation of TRPM4, a cation channel, impairs its endocytosis function [53]. Sumoylation of RIM1α functions in synaptic vesicle exocytosis, causing clustering of calcium channels and enhancing the Ca2+ influx necessary for exocytosis [54]. In addition, sumoylation regulates the function of cytoskeletal proteins. For example, SUMO ligase PIAS3, sumoylates Rac1 a protein that regulates the actin cytoskeleton [55]. Rac1 sumoylation has been reported to function in Rac1 activation, cell migration, and lamellipodia formation in response to hepatocyte growth factor.

The first protein found to be sumoylated at the endoplasmic reticular membrane was protein-tyrosine phosphatase-1B (PTP1B). PTP1B negatively regulates growth-factor signaling by binding to receptor tyrosine kinases. SUMO E3 ligase-mediated sumoylation of PTP1B impairs its activity by reducing its protein stability [56]. SUMO has also been discovered to exert its functions in the mitochondria. Mitochondrial fission and fusion are balanced by the Drp1 protein and it is subjected to sumoylation by SUMO1, which causes mitochondrial fission [57]. Overexpression of SENP5 SUMO protease induces de-sumoylation of Drp1, which causes mitochondrial fusion [58]. However, the exact mechanism of how SUMO regulates this function is yet to be studied.

1.3.2 Nuclear sumoylation

The majority of SUMO related studies have focused on nuclear targets, and the sumoylated proteins identified in subnuclear structures such as polycomb group (PcG) bodies, PML nuclear bodies, and nucleoli, were found to play multiple roles in the DNA damage response (DDR), chromatin organization, and transcription [31]. DNA is constantly challenged by potential damage and the DDR is a critical mechanism in maintaining genomic integrity. Multiple proteins are sumoylated in the DDR pathway. For example, sumoylation of tumor suppressor PTEN is important for its nuclear retention and helps to function in homologous recombination [59]. Sumoylation of Rad52 enhances its stability by protecting it from ubiquitin mediated degradation [60]. Rad52 binds to the single stranded DNA and processes the ends of the DNA molecules, which can then be bound by Rad51. In addition to increasing its stability, sumoylation promotes Rad52 disassociation from DNA, thereby giving space for Rad51 to function [61]. A similar function was observed for Rad52 sumoylation in yeast [62]. Sumoylation of MDC1 is responsible for recruitment and complex formation of other proteins involved in the DDR, such as RNF4, RNF8-

UBC13, ABRA1 and BRCA1 [63]. In fact, BRCA1 is also subjected to sumoylation and this modification enhances its ubiquitin ligase activity 10-20 fold [64].

Along with DDR, telomere length must be maintained for proper genomic integrity. Yeast strains defective in SUMO, Ubc9, or SUMO E3 ligases demonstrated abnormal elongation of their telomeres [65, 66]. Supporting this, lengthened telomeres ware also observed in strains expressing non-sumoylatable Cdc3, an inhibitor of telomerase. However, in the presence of a Cdc3-SUMO fusion, telomere size was shortened, suggesting an inhibitory role for sumoylation in regulating telomere size [66]. Sumoylation also functions in maintaining heterochromatin structure. For example, during interphase, sumoylation of the heterochromatin factor HP1α is important for its localization into pericentric domains, and for the interactions with major α-satellite RNA transcripts, to promote heterochromatin formation in the centromere [67, 68].

1.4 Sumoylation and transcription

Large numbers of sumoylated proteins identified using proteomics and mass spectrometry are involved in transcription [1-3, 11-15]. This includes RNA polymerase subunits, transcription factors, coactivators, corepressors, histones, chromatin remodeling complexes, and components of PML NBs. Among these proteins, transcription factors represent the largest classes of SUMO substrates [69]. Furthermore, SUMO signals were observed on promoters of transcriptionally active genes such as constitutive and inducible genes, emphasizing the functional importance of SUMO in active transcription [70].

1.4.1 Transcriptional repression by SUMO

Sumoylation of proteins involved in transcription is most often associated with transcriptional repression. Indeed, transcription of inducible genes such as *STL1* (osmostress-induced gene) and *ARG1* (amino acid starvation-induced gene) was elevated when the function of Ubc9 was impaired [70]. Different mechanisms are used by SUMO to repress transcription. Among these mechanisms, in some cases, SUMO targets proteins involved in transcriptional activation for degradation. RNA polymerase (RNAP) III is involved in the expression of tRNAs, and one of its subunits, Rpc53, is targeted for sumoylation by the Siz1 E3 SUMO ligase. Sumoylated Rpc53 then triggers ubiquitination of Rpc160, the catalytic subunit of RNAPIII, followed by its degradation [71]. Similarly, transcription factors PEA3, Sp1 and c-Myc are degraded by the SUMO-ubiquitin-proteasome degradation pathway [72-74]. In another mechanism, SUMO enhances the activity of corepressors or their recruitment to chromatin. For example, in yeast, sumoylation of corepressor Tup1, part of Tup1-Cyc8 corepressor complex, was shown to be involved in transcriptional deactivation by promoting the removal of sumoylated transcription factor Gcn4 from DNA [75]. In mammals, sumoylation of the transcription factor PPARα creates a docking site for the nuclear receptor co-repressor NCoR, which represses transcription of PPARα-target genes [76].

Moreover, sumoylation of some transcription factors enhances their interaction with chromatin remodelling complexes. For example, sumoylation of transcription factors NFAT, Elk-1 and MafG enhanced their interaction with histone deacetylases (HDACs) [77-79]. This interaction induces deacetylation of histones followed by DNA condensation, and as a consequence, expression of target genes is suppressed. In fact, sumoylation of histone H4 also recruits HDACs and heterochromatin protein 1, in mammals [80]. Similarly, in yeast, all four core histones were found

to be sumoylated to repress transcription [81]. Interestingly, HDACs themselves have been shown to be sumoylated. HDAC1 is sumoylated by SUMO1 and this modification was shown to enhance its repressor activity [82]. Sumoylation is also known to be involved in the promoter clearance of some transcription factors such as yeast Gcn4 and mammalian c-Fos [83, 84]. Sumoylation of Gcn4 aids in its removal from the promoters of target genes through Srb10 phosphorylation-mediated degradation of sumoylated Gcn4 [83]. In contrast, the mechanism for c-Fos promoter clearance by its sumoylation remains to be studied [84].

1.4.2 Transcriptional activation by SUMO

Interestingly, emerging recent evidences suggests positive roles for sumoylation in regulating transcription. A genome-wide study suggested that SUMO signals were heavily enriched on tRNA genes and ribosomal protein genes [85]. This study demonstrated that SUMO at these genes is involved in transcriptional activation. Specifically, sumoylation of Rap1 transcription factor at these genes was found to promote the recruitment of general transcription factor TFIID to induce transcription of ribosomal protein genes [85]. In addition, in some cases, SUMO modification was shown to interfere with the corepressor function, or to block the interaction between transcription factors and co-repressors. For example, during hyperosmotic stress, the Cyc8 corepressor is sumoylated to form Tup1-Cyc8 inclusions, which allows the expression of genes that are important for hyperosmotic stress related adaptation [86]. In mammals, ErbB2 induces sumoylation of transcription factor MFZ1. Sumoylated MFZ1 is then activated by subsequent phosphorylation which assists in the dissociation of MFZI with its corepressors such as CTCF [87].

Furthermore, SUMO can enhance the interaction between transcription factors and DNA. For example, sumoylation of transcription factors CREB1 and Pax6 enhances their association with

DNA to induce transcription [88, 89]. Sumoylation also enhances the stability of proteins by preventing their ubiquitination-mediated degradation. For example, sumoylation competes with ubiquitin for the Lys residues on transcription factors such as delta-lactoferrin and ZHX1, to prevent ubiquitination [90, 91]. Moreover, some HDACs are sumoylated under certain conditions, which results in a loss of deacetylation functions and an increases in target gene expression. For example, HDAC2 sumoylation by SUMO1 is induced in the presence of cigarette smoke extract, which inhibits the HDAC2 activity and enhances the expression of the IL-8 gene [92].

1.5 Transcription

1.5.1 DNA cis-regulatory elements

The expression of protein coding genes requires the synthesis of mRNA in a process called transcription. Transcription involves the binding of transcription related proteins to DNA cis-regulatory sequences in a region usually upstream of the transcription start site (TSS) [93-99]. In yeast, the cis-regulatory sequence includes the core promoter, the upstream activator sequence (UAS), and potential silencer sequences [93] (Figure 1.7A). The yeast core promoter is located 20-30 base pairs upstream of TSS and may contain the TATA box element, which mostly differs from the consensus, TATAAA, by up to 2 bases [95].

Higher eukaryotic cis-regulatory sequences are more complex compared to the simple yeast system. Mammalian cis-regulatory sequences can include, in addition to the core promoter, multiple enhancer regions, silencers and insulators (Figure 1.7B). Enhancers can be situated upstream or downstream of the core promoter of the genes they regulate, and can even be found within introns [99]. The mammalian core promoter itself display significant complexity, with multiple sub-regulatory regions: (i) the TATA box that is located upstream of the TSS; (ii) the

initiator (INR) element that is located at, or immediately adjacent to, the TSS; (iii) the TFIIB recognition element (BRE) that is located 35 bp upstream of TSS; and (iv) the downstream promoter element (DPE) that is located 30 bp downstream of the TSS [96, 97].

Yeast UAS and the mammalian enhancers contain multiple gene-specific transcription factor recognition sites or binding sites through which various combinations of transcription factors are recruited to activate transcription [98, 99]. In contrast, silencers function to repress transcription, while insulators interfere with the function of both enhancers and silencers [98]. Insulators are further categorized as enhancer blockers, when the insulator blocks the enhancer function, and barriers, when the silencer function is blocked [98].

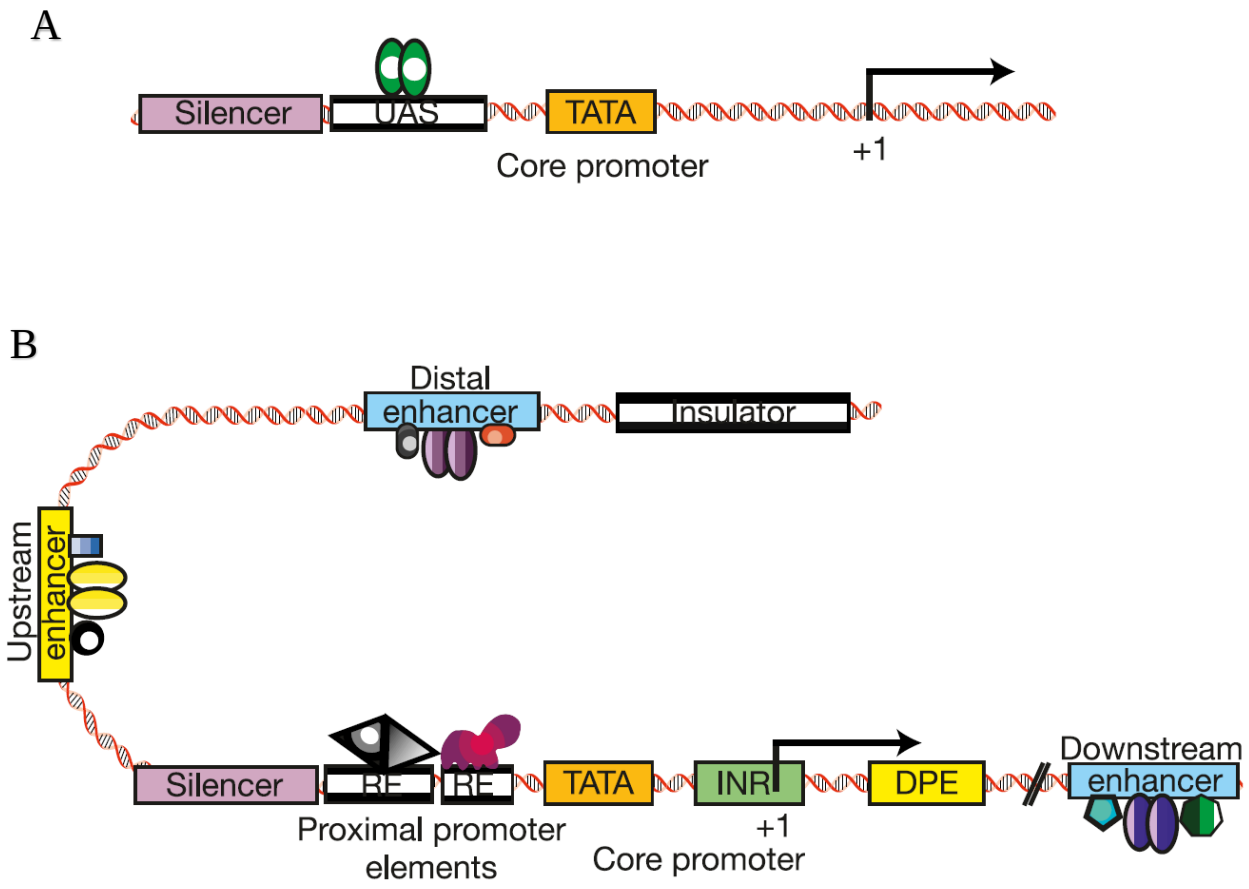

Figure 1.7: Schematic representation of the cis-regulatory sequences involved in transcription. (A) Yeast cis-regulatory sequences include a simple core promoter (e.g. TATA box), upstream activator sequence (UAS) and silencer element. **(B)** Complex eukaryotic promoter elements can include the TATA box (TATA), Initiator sequences (INR), and downstream promoter elements (DPE). In addition, multiple enhancer modules are found interspersed with silencer and insulator elements. Figure adapted from [99].

1.5.2 Transcription overview

Regulatory signals from gene-specific transcription factors converge at the core promoter to activate transcription [93] (Figure 1.8). Prior to initiation of transcription, RNA polymerase (RNAP) II and general transcription factors (GTFs: TFIID, TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH) assemble on promoter regions to form a pre-initiation complex (PIC) [93, 97-101] (Figure 1.8A). Formation of the PIC requires the concerted action of GTFs beginning with the binding of TFIID to the promoter. TFIID contains TATA-binding protein (TBP) and TBP-associated factors (TAFs), and the binding of TBP to the TATA element on the promoter induces a 90 degree bend in the DNA [97-100]. Not all core human and yeast promoter regions contain a TATA box. In fact only about 10% of the yeast promoters contain TATA [93]. However, TBP can associate with variants of TATA-like promoter regions [93, 100]. In mammals, TAF1 and TAF2 recognize the INR region and TAF6 and TAF9 recognize DPE region within the core promoter [97].

TFIIA and TFIIB are then recruited to stabilize the TBP-DNA complex. In mammals, TFIIB binds to the BRE region in the core promoter [97]. Next, an RNAPII-TFIIF complex binds to form the core PIC. TFIIF plays a role in TSS selection and prevents non-specific interaction of Pol II with DNA [100]. Finally, the binding of TFIIE and TFIIH completes formation of the PIC. TFIIE functions as a bridge between RNAPII and TFIIH [101]. In the presence of adenosine triphosphate (ATP), TFIIH uses its DNA-dependent ATPase activity to melt the DNA to make a ‘transcription bubble’ and eventually form the open promoter complex (Figure 1.8B) [100]. Next, nucleoside triphosphates (NTPs) are utilized to form the nascent RNA. Finally, GTFs (referred to as initiation factors in Figure 1.8B) dissociate from the promoter as RNAP II clears the promoter and associates with elongation factors during the elongation phase of transcription [100-102].

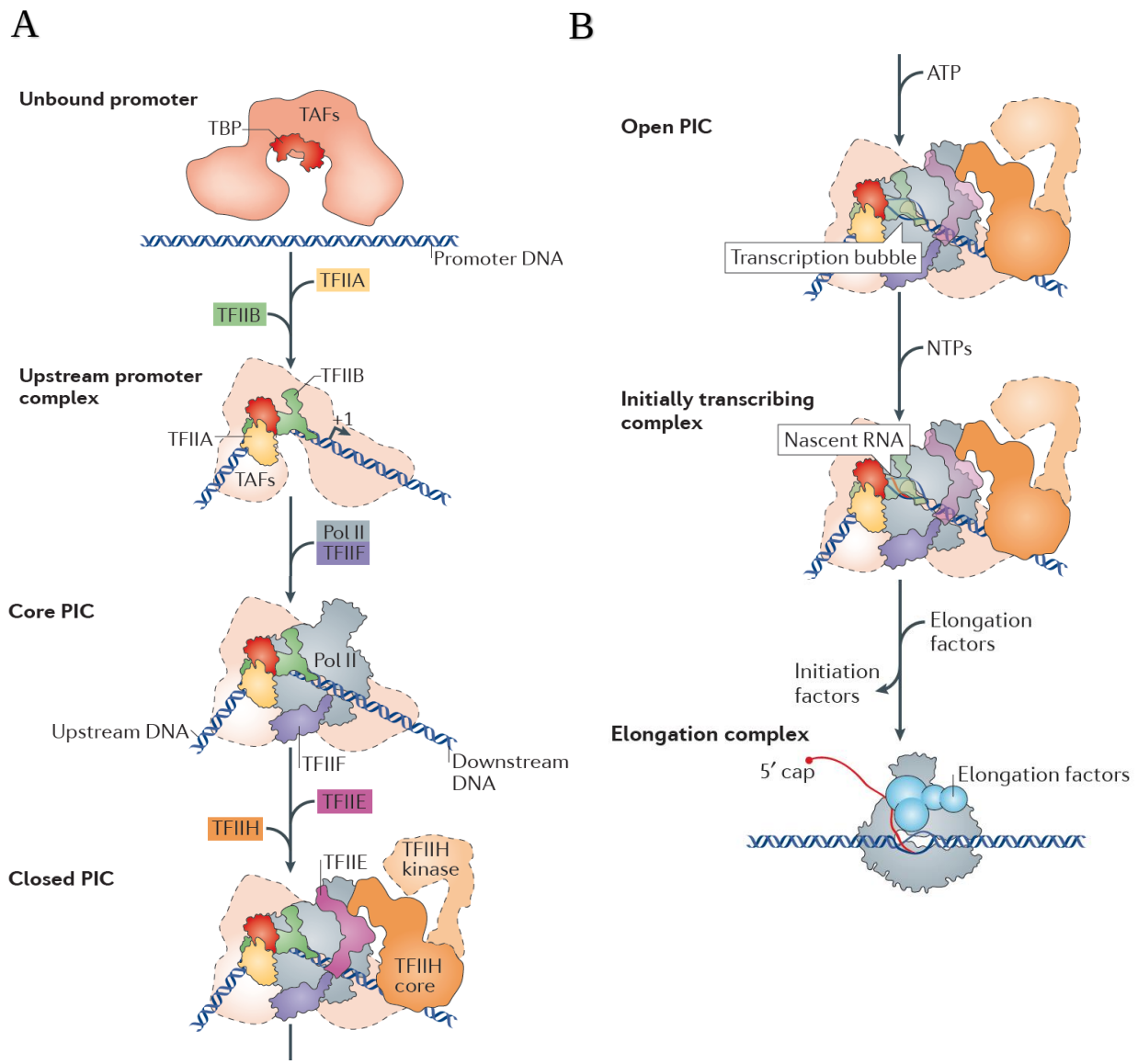

Figure 1.8: Schematic representation of the formation of transcription machinery. (A) The step-wise process of pre-initiation complex (PIC) formation is initiated by the recruitment of TFIID subunits, TATA box-binding protein (TBP) and TBP-associated factors (TAFs), to the unbound promoter. Then, TFIIB and TFIIA are recruited to stabilize TBP-DNA complex. Then, the core PIC is formed after the association of Pol II-TFIIF complex. Subsequent binding of TFIIE and TFIIH completes the formation of PIC. **(B)** In the presence of ATP, DNA is opened by TFIIH to form a 'transcription bubble' to initiate transcription. After the initiation of nascent RNA synthesis, initiation factors dissociate to enable the formation of an elongation complex. NTP, nucleoside triphosphate. Figure adapted from [100].

1.6 Transcription factors

Apart from the general transcription factors (mentioned above), there are multiple sequence-specific transcription factors (TFs) that bind to cis-regulatory sequences in DNA to control the expression of target genes [93, 103, 104]. By regulating gene expression they play an important function in cellular processes, including cell proliferation and differentiation, stress response and homeostasis. Generally, TFs contain a DNA-binding domain (DBD), effector domain (such as an activation or a repression domain), a nuclear localization sequence (NLS), and a regulatory domain [93].

TFs are referred to as transcriptional activators or transcriptional repressors depending on the presence of an activation domain or a repression domain, respectively [93]. In addition to having these effector domains, transcription factors interact with different cofactors (coactivators and corepressors) to decide the transcriptional outcome [93]. Some TFs including yeast TFs Ash1 and Sko1, are able to both activate and repress transcription, depending on the promoter that is targeted, cofactor association, and growth conditions [93, 103, 104]. TFs can be classified based on their binding sequence motifs and characterization of their DNA-binding domains (DBDs) [93, 103].

1.6.1 Transcription Factor binding motifs and DNA binding specificity

A single TF can recognize multiple DNA binding site sequences with varying binding affinity, and these sequences are collectively referred to as binding motifs [93]. Multiple methods are used to find DNA binding motifs for TFs, including genome-wide studies such as chromatin immunoprecipitation followed by next-generation sequencing (ChIP-Seq) [93, 103-105]. Interestingly, a single type of binding motif sequence can be found hundreds of times in a genome, but not all the sites will be occupied by the cognate TFs at a given time [105]. Some TFs, such as

Forkhead and C2H2-zf, can bind multiple binding motifs [104]. Therefore, apart from the binding motifs themselves, there are other factors that determine the DNA binding specificity of TFs. For example, an interaction between two TFs can be necessary to facilitate binding to a specific sequence in what is referred to as cooperative binding. As mentioned below, some families of TFs bind to DNA as partners, either as homodimers or heterodimers, and these cooperative bindings confer DNA binding specificity [103, 104, 106]. TF binding specificity can be determined by non-DNA binding cofactors and their recruitment motifs [104, 107]. Although non-DNA binding cofactors cannot directly contact DNA, in some cases, such recruitment motifs are required for their recruitment to target genes. For example, RYAAT, the recruitment motif for cofactors Met28 and Met4, which is situated near TF Cbf1 binding sites, is required for the association of the Met4–Met28–Cbf1 regulatory complex to target genes [107].

Moreover, DNA shape or DNA flexibility can guide TF-DNA interactions [103, 105]. Using propeller twist, a technique used to explain DNA flexibility based on it GC content, researchers found that TFs belonging to the homeodomain POU and Forkhead families prefer sequences with an enhanced negative propeller twist (more GC content), while TFs that belong to the C2H2 and ETS families prefer binding to sequences with a less negative propeller twist (more AT content) [105]. In addition, binding of one TF can influence the shape of the DNA in a manner that promotes the binding of a second TF. For example, binding of IFNβ enhanceosome facilitates the binding of eight other transcription factors and three non-binding coactivators [103].

1.6.2 Characterization of transcription factors based on DBD

Whereas TFs bind to their DNA binding motifs through DBDs, some, such as Swi6 and Met4, which do not contain a DBD, associate with DNA through a DNA binding partner [93]. For TFs

that do bind to the DNA directly, there are different types of DBDs. In fact, TFs can be grouped into different classes based on the type of DBD that they contain. Zinc finger (ZF), Homeodomain, basic helix-loop-helix (bHLH), basic leucine zipper (bZIP), and nuclear hormone receptor (NHR) are some examples [93, 103, 104].

1.7 bZIP transcription factors

Among the different types of TFs discovered, the basic leucine-zipper (bZIP) proteins form a large multifunctional family, which is conserved in all eukaryotes. There are about 53 bZIP proteins identified in humans and 14 bZIP proteins in yeast [108, 109, 112]. bZIP TFs contain a highly conserved 60-80 amino acid bZIP domain, consisting of a basic region, responsible for DNA binding, followed by a leucine zipper coiled-coil motif [108, 110]. bZIP dimers form a chopsticks-like structure by dimerization through their leucine-zipper segments (Figure 1.9A). bZIP proteins can either form homodimers or heterodimers. As dimers, they regulate transcription by binding short DNA target sites, often in the form of 8-base pair palindromes [110]. There are different groups of families within bZIP proteins whose members have similarity in their protein sequences and DNA binding preferences. This includes activator protein-1 (AP-1), activating Transcription Factor (ATF), cAMP Response Element-Binding Protein (CREB) and CCAAT-enhancer binding protein (CEBP). Members within these groups can bind together to form heterodimers [108-110].

Among these different groups of families, ATF/CREB members have been extensively studied and are used as models to understand transcription factor-DNA interaction. Although ATF and CREB proteins were discovered individually, these two families bind to identical sequences in DNA, and are usually referred to as the ATF/CREB family. They bind to the cAMP responsive

element (CRE), TGACGTCA, in DNA [11]. There are multiple subfamilies in the ATF/CREB family that function in a variety of cellular process during non-stress and stress conditions.

In yeast, transcription factors belonging to ATF/CREB family are Aca1, Cst6 (also known as Aca2), Sko1 (also known as Acr1), and Hac1 [112]. These transcription factors share some similarities and differences in their structures as illustrated in Figure 1.9B. All four proteins are highly similar in their basic domain, and they all contain a Lys residue within the basic region, at a position that is conserved with a Lys in ATF/CREB, but not in AP-1 [112]. Among these proteins, Aca1 and Cst6 share a stretch of an 80 amino acid region (known as Aca-specific region), which is not present in the other two proteins. Although these proteins belongs to the same group, they have distinct biological functions and targets genes as described below for Sko1 and Cst6.

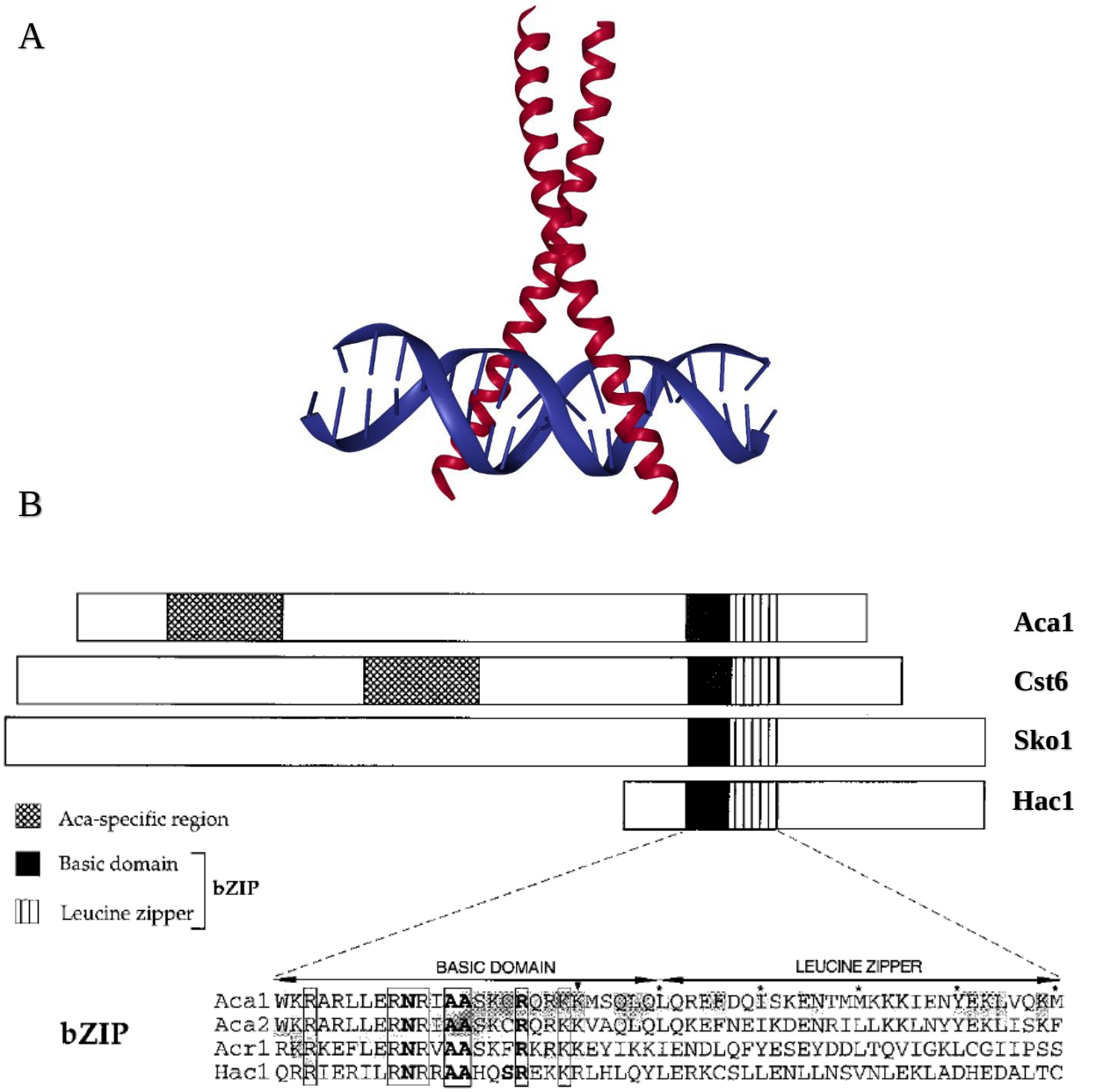

Figure 1.9: bZIP transcription factor structures and comparisons. (A) Structure of bZIP transcription factor. Yeast Gcn4 bZIP domain structure is shown, as obtained from the protein data bank (PDB ID: 2DGC). **(B)** Schematic structural comparison between yeast ATF/CREB bZIP transcription factors. bZIP domains (striped and shaded boxes) and the Aca-specific region (hatched boxes) are indicated. Conserved residues in the basic region are bolded and boxed. Figures adapted from [93] and [112], respectively.

1.7.1 Suppressor of Kinase Overexpression 1 (Sko1)

Sko1, initially identified as ATF/CREB repressor 1 (Acr1), is constitutively expressed and composed of 647 amino acids [113, 114]. It belongs to the ATF/CREB family due to the fact that its bZIP domain is highly similar to the bZIP domains of mammalian TFs CREB and ATF2, although the Sko1 bZIP domain is less similar to the bZIP domains of yeast AP-1 TFs Gcn4 and Yap1 [113]. Using this domain, Sko1 binds as a homodimer to the CRE region in its target genes [114]. It is not required for mitotic growth, mating, sporulation, spore germination, or filamentous growth [113]. However, Sko1 is associated with promoters of genes involved in glycolysis, transmembrane transporters, transcription regulators and stress response [113-121]. In particular, Sko1 is the main TF that regulates the expression of osmotic and oxidative stress response genes, including the expression of transcription factors and phosphatases that mediate the general stress response and the expression of genes that encode proteins that directly relieve osmotic and oxidative stress [114, 117].

Sko1 plays a unique bi-functional role in transcription: during normal growth, it represses the transcription of osmotic and oxidative stress-inducible genes and upon stress, it activates the transcription of these same defense genes (Figure 1.10) [114-116]. Its transcriptional repressor function is dependent on the recruitment of Tup1-Cyc8 corepressor complexes to its target promoters (Figure 1.10A) [114-116]. Tup1 binds directly to the N-terminus of Sko1 to induce the repressor function [114, 118]. The switch from being a repressor to an activator during osmotic stress relies specifically on the phosphorylation of the Hog1 MAP kinases and the Hog1-dependent recruitment of the coactivator SAGA, SWI/SNF nucleosome remodeling complexes and RNAPII to induce the transcription of target genes (Figure 1.10B) [116-120]. Studies have shown that the

interaction between Sko1 and Tup1-Cyc8 corepressor complexes is unaffected in the presence of Hog1 during osmotic stress [115, 116]. In fact, ChIP experiments on Sko1 target genes, such as *GRE2* and *AHP1*, have shown that the recruitment of Ada2, a subunit of the SAGA complex, and Swi2, subunit of SWI/SNF complex, are reduced in the absence of Tup1 indicating the importance of Tup1 function during osmotic stress [116]. In addition to the phosphorylation by Hog1, Sko1 is phosphorylated by the PKA kinase near its bZIP domain [115, 118]. PKA phosphorylation is important for the nuclear localization of Sko1, for enhancing the repression activity of Sko1 during normal growth, and for slightly enhancing the affinity between Sko1 and the CRE element [118].

Previous studies have shown that the amount of Sko1 occupied on the promoter region varies depending on the target promoter and growth conditions [119, 121]. To date, there are four different Sko1 DNA binding patterns that have been reported during osmotic stress: (i) constant binding at all time points during osmotic stress and during normal growth conditions; (ii) rapid binding during osmotic stress, when Sko1 binding reached its maximum after 15 min; (iii) slow and gradual binding of Sko1, observed after 5 min of osmotic stress, when the maximum occupancy level was reached after 30 min; and (iv) transient binding with only a minor increase in Sko1 occupancy after osmotic stress compared to normal growth. Along with the osmotic stress condition-dependent Sko1 occupancy, the binding of other proteins, such as other TFs, on Sko1 target promoters also determines the binding pattern of Sko1 [119, 121]. For example, TFs Yap4 and Yap6 are bound on promoters where Sko1 is constantly bound. In addition, some of the promoters where Sko1 occupancy reaches maximum after 30 min of osmotic stress were also bound by the TF Smp1. Importantly, Sko1 is at the top of the transcription network and it associates with many other targets including Msn2, Msn4, Yap4, and Yap6 during normal growth and

osmotic stress conditions to regulate gene expression, which emphasizes its importance in yeast cells [121].

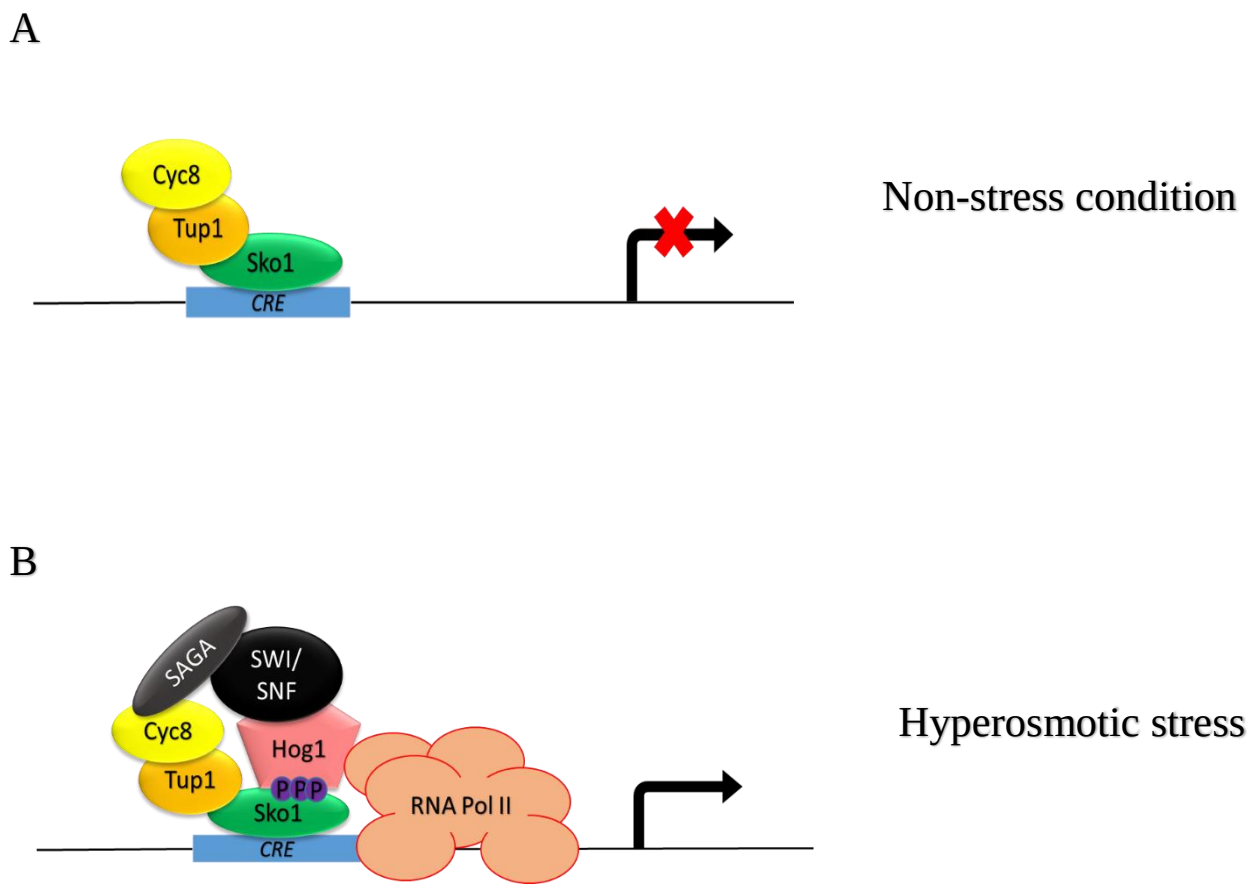

Figure 1.10: Sko1 is a bifunctional transcription factor. (A) During non-stress conditions, it functions as a transcriptional repressor by recruiting Tup1 and Cyc8 co-repressor complexes to the target promoters. **(B)** However, during hyperosmotic stress, Sko1 functions as a transcriptional activator by interacting with the Hog1-MAP kinase. Hog1 phosphorylates Sko1 and recruits co-activator SAGA, the SWI/SNF nucleosome remodeling complexes and RNAPII to induce transcription of target genes. CRE: cAMP response element, P: Phosphate group.

1.7.2 Chromosome stability 6 (Cst6)

Cst6, also known as ATF/CREB activator 2 (Aca2), functions as a transcriptional activator and is composed of 587 amino acids. Similar to Sko1, it recognises and binds to the CRE-like promoter sequence [112]. Cst6 binds to DNA as a homodimer or a heterodimer with the structurally similar bZIP transcription factor Aca1 [112]. Interestingly, a recent study showed that the Cst6 binding site has an additional conserved guanine nucleotide (5′-GTGACGT-3′) at the 5′ end of the consensus CRE motif, indicating possible different preferences of DNA binding between Cst6 and other bZIP transcription factors [122].

Genome-wide and phenotypic studies have revealed multiple functions for Cst6 including transcription, cellular respiration, gluconeogenesis, stress response, and pseudohyphal growth [122]. Cst6 is essential for survival during ethanol stress and has roles in the utilization of carbon sources such as glycerol, ethanol, and raffinose [112, 122]. *CST6* deleted yeast cells show poor growth or no growth when faced with ethanol stress or when ethanol was used a sole carbon source. This is because, in the presence of ethanol, yeast fermentation is affected and the production of CO_2 is slow. During this growth condition, Cst6 activates *NCE103*, a gene that encodes a carbonic anhydrase, to convert CO_2 to HCO_3^-, a reaction necessary for yeast cell survival. Under elevated CO_2 levels, Cst6 is phosphorylated by Sch9 kinase at serine 266 to repress *NCE103* activation [123].

In addition, Cst6 regulates the expression of oxidative and heat shock stress induced genes [112, 124, 125]. In fact, a previous study has suggested that the derepression of *GRE2*, an osmotic and oxidative stress-induced gene, in the absence of Sko1 depends on the function of Cst6 and Aca1 [115]. Phosphorylation of Cst6 at Ser-399 and Thr-401 is induced by oleate and mutation at these

sites increased the expression of β-oxidation genes [126]. In addition, a *CST6* deletion mutant showed poor ability to maintain 2 μm plasmids, while its overexpression resulted in chromosome instability [127]. Although Cst6 occupancy was identified on promoters of genes with multiple functional outcomes, Cst6 related studies in budding yeast are very limited.

1.8 Rationale and objectives of this study

Sumoylation is a key regulatory protein modification and deregulation of sumoylation is implicated in various pathological disorders such as, cancer, neurodegenerative diseases and heart diseases [12]. There are many sumoylated targets identified to date that suggest that sumoylation plays a vital role in many cellular processes. Specifically, proteins involved in transcription are one of the largest classes of SUMO substrates [1-3, 11-15]. In fact, proteomic studies have identified multiple sumoylated proteins from the transcription machinery including transcription factors, RNAPII, transcriptional co-regulators and a variety of chromatin regulatory factors [1-3, 11-15]. Among these, sequence specific DNA-binding TFs represent the largest group of SUMO conjugates [69]. Previous work showed that SUMO accumulates on transcriptionally active genes, including constitutive genes and activated inducible genes [70]. In addition, the recruitment of Ubc9, the SUMO E2 conjugating enzyme, was observed on promoters of inducible genes. However, the promoters of repressed or un-induced genes did not show an accumulation of either SUMO or Ubc9 [70]. These findings indicates that sumoylation functions in regulating active transcription: to either repress or to activate the target gene expression.

Numerous studies have been carried out to determine the role sumoylation plays in regulating different TFs. The majority of previous studies suggested that transcription factor sumoylation was involved in transcriptional repression or deactivation [13, 69]. This is mostly achieved by

recruiting various repressor complexes to chromatin, by interfering with protein modifications that promote transcription, or by facilitating promoter clearance of transcription factors. However, some recent discoveries also suggest that transcription factor sumoylation is involved in transcriptional activation by enhancing transcription factor binding to target promoters [13, 69, 85].

Based on the previous findings, we know that the effects of sumoylation vary with different target TFs. Regardless of these findings, it is still not clear whether sumoylation has a general role in regulating TFs. In fact, there have been more TFs that were identified to be potential SUMO substrates, which still need to be investigated in order to shed more light on sumoylation functions. In addition, majority of earlier studies were conducted in normal growth conditions. However, a drastic accumulation of sumoylated proteins were observed during stress conditions, as explained by the SUMO stress response [46]. Therefore, it is important to study whether the function of sumoylated TFs changes from a normal growth condition to a stress condition.

A prior work in our lab showed that the bZIP TF Gcn4 is sumoylated during amino acid starvation, and this modification aids in its removal from the promoters of target genes through Srb10 phosphorylation-mediated degradation of sumoylated Gcn4 [83]. To extend the investigation on whether sumoylation play a general role in regulating TF association with chromatin, my PhD dissertation focused on determining roles for sumoylation in regulating the function of two other yeast (*S. cerevisiae*) gene-specific bZIP TFs, Sko1 and Cst6. The experiments were conducted during normal growth conditions and stress conditions, to elucidate more functions for SUMO. Large scale proteomic studies discovered Sko1 and Cst6 as potential SUMO targets and the first step of these projects was to validate sumoylation of both these proteins [128-130]. Once

sumoylation was confirmed, changes in sumoylation under different stress conditions were tested to determine which stress condition to use for additional studies. Then, we identified the SUMO-site(s) on these two TFs to generate mutants to block sumoylation. Then, the normal and non-sumoylateable forms of these two TFs were used in a variety of experiments, such as growth analysis/cell fitness, protein stability, expression of target genes, and ChIP, to explicitly determine whether sumoylation affects the association of Sko1 and Cst6 with chromatin.

CHAPTER 2

SUMOYLATION OF DNA-BOUND TRANSCRIPTION FACTOR SKO1 PREVENTS ITS ASSOCIATION WITH NONTARGET PROMOTERS

Veroni S. Sri Theivakadadcham[1], Benjamin G. Bergey[1], Emanuel Rosonina[1]

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[1]Department of Biology, York University, Toronto, Ontario, Canada

Author Contributions

Project planning and experimental design: V.S.S and E.R.

Experiments and data acquisition: V.S.S. performed all experiments shown; B.G.B. assisted with the ChIP-seq experiments.

Data analysis: Data was analyzed by V.S.S. with assistance from E.R. Bioinformatics analyses were performed by E.R. and B.G.B.

Writing – original draft: V.S.S. and E.R.

Writing – review and editing: V.S.S., B.G.B., E.R.

2.1 ABSTRACT

Sequence-specific transcription factors (TFs) represent one of the largest groups of proteins that is targeted for SUMO post-translational modification, in both yeast and humans. SUMO modification can have diverse effects, but recent studies showed that sumoylation reduces the interaction of multiple TFs with DNA in living cells. Whether this relates to a general role for sumoylation in TF binding site selection, however, has not been fully explored because few genome-wide studies aimed at studying such a role have been reported. To address this, we used genome-wide analysis to examine how sumoylation regulates Sko1, a yeast bZIP TF with hundreds of known binding sites. We find that Sko1 is sumoylated at Lys 567 and, although many of its targets are osmoresponse genes, the level of Sko1 sumoylation is not stress-regulated and the modification does not depend or impinge on its phosphorylation by the osmostress kinase Hog1. We show that Sko1 mutants that cannot bind DNA are not sumoylated, but attaching a heterologous DNA binding domain restores the modification, implicating DNA binding as a major determinant for Sko1 sumoylation. Genome-wide chromatin immunoprecipitation (ChIP-seq) analysis shows that a sumoylation-deficient Sko1 mutant displays increased occupancy levels at its numerous binding sites, which inhibits the recruitment of the Hog1 kinase to some induced osmostress genes. This strongly supports a general role for sumoylation in reducing the association of TFs with chromatin. Extending this result, remarkably, sumoylation-deficient Sko1 binds numerous additional promoters that are not normally regulated by Sko1 but contain sequences that resemble the Sko1 binding motif. Our study points to an important role for sumoylation in modulating the interaction of a DNA-bound TF with chromatin to increase the specificity of TF-DNA interactions.

2.2 INTRODUCTION

Sumoylation is an essential eukaryotic post-translational modification that functions in many, predominantly nuclear, cellular processes, such as DNA repair and transcription, by regulating target protein localization, stability, or interactions with other proteins or with chromatin [1-5]. The modification involves the covalent attachment of a ~12-kDa SUMO (*S*mall *U*biquitin-like *Mo*difier) peptide to specific lysine residues on substrate proteins through a three-enzyme cascade that is analogous to ubiquitination [1]. In contrast to ubiquitination, however, the sole SUMO E2 conjugating enzyme Ubc9 can recognize its substrates via the consensus sequence ΨKxD/E, where Ψ is a hydrophobic residue, K is the modified lysine and x is any amino acid [6]. Although thousands of proteins have been identified as potential SUMO targets, modification levels of individual proteins are typically low and can be controlled by the desumoylating action of SUMO-specific isopeptidases (SUMO proteases), including the SENP family in mammals, and Ulp1 and Ulp2 in budding yeast [3,7,8]. On a global level, cellular sumoylation can be coordinately regulated, which is exemplified by the SUMO stress response, a rapid increase in overall SUMO conjugation that is observed in both yeast and mammalian cells in response to various stress conditions, including temperature, oxidative, and osmotic stress [4, 9-12].

Chromatin immunoprecipitation (ChIP) analyses, both genome-wide and on individual genes, have demonstrated that sumoylated proteins are detected specifically at promoter regions of constitutively active and induced genes, suggesting that the modification is important for regulating early steps of transcription [13-17]. Supporting this, proteomics studies have identified subunits of the general transcription factors (GTFs), RNA Polymerase II (RNAP II), and Mediator as SUMO conjugates in yeast, Drosophila, and human cells [18]. Moreover, one of the largest groups of SUMO substrates, with over 300 substrates identified in human SUMOylome analyses,

is sequence/gene-specific transcription factors (TFs) [3, 19]. Numerous studies have characterized the role of sumoylation on individual TFs, and in most cases the modification is associated with a repressive effect on transcription of target genes [5, 19-21]. For example, upon sumoylation, NFAT, Elk-1 and MafG interact with histone deacetylases (HDACs) to condense DNA and restrict access of the transcription machinery, whereas sumoylation of the Forkhead Box TF FoxM1b promotes its cytoplasmic retention and ubiquitin-mediated degradation, thereby limiting its access to target genes [22-25]. Why a transcriptionally repressive mark like SUMO is enriched at promoter regions of transcriptionally active genes remains unknown.

Providing a possible explanation, studies on two basic leucine zipper (bZIP) motif-containing TFs, yeast Gcn4 and human FOS (c-Fos), showed that promoter-associated sumoylation is important for regulating their occupancy levels [26-28]. DNA-bound Gcn4 and FOS are sumoylated during active transcription, and the modification promotes their clearance from DNA. This limits the association of these TFs with their target genes once they are activated, which likely serves to prevent excessive gene expression. Consistent with this, ChIP studies have shown that sumoylation modulates the chromatin occupancy of multiple other TFs, including human TFs FOXA1, MITF, c-Maf, and the androgen and glucocorticoid nuclear receptors [29-33]. How sumoylation affects TF occupancy is not always known, but in some cases SUMO modifications directly affect DNA binding. For example, modification of SP1, specifically by the SUMO2 isoform, reduces its DNA binding activity in an in vitro assay [34]. These studies have led to speculation that sumoylation has a general role in controlling the interaction of TFs with their target sites on chromatin [19]. This can serve to regulate the expression levels of target genes, but another possible function for SUMO-mediated modulation of TF-chromatin interactions is in binding site selection. Multiple factors, in addition to DNA sequence, play a role in determining what genomic sites are recognized

and bound by TFs, including genomic context, DNA modifications, the nature of flanking DNA sequences, and interaction with cofactors [35, 36]. However, it is not known whether sumoylation plays a role in regulating binding site selection for TFs across eukaryotes.

To address this, we examined how sumoylation regulates the bZIP TF Sko1 (*S*uppressor of *K*inase *O*verexpression-1) from the budding yeast, *Saccharomyces cerevisiae*. Sko1 was identified as a putative SUMO target in multiple proteomics screens, suggesting that its activity or association with chromatin is regulated by sumoylation [37-39]. It was first characterized as a repressor of cAMP response element (CRE)-containing genes, such as *HIS3* and *ENA1*, that are induced after exposure to osmotic stress [40-43]. Repression by Sko1 involves recruitment of the Tup1/Cyc8 corepressor complex, and relief of repression occurs during osmotic stress as a result of phosphorylation by the activated MAP kinase Hog1 [40, 41, 44-47]. In addition to functioning as a repressor, Sko1 was subsequently shown to be involved in the induction of some stress response genes, through the Hog1-dependent recruitment of the SAGA and SWI/SNF nucleosome remodelers to promoter regions bound by Sko1/Tup1/Cyc8 complexes [45, 47, 48]. Genome-wide analyses showed that Sko1 is constitutively associated with >250 gene promoters and that osmotic stress results in a general redistribution of Sko1, with some genomic sites showing stress-dependent gain or loss of Sko1, while occupancy at other sites remains unchanged [49-51]. Nonetheless, many Sko1 binding sites are not associated with genes specifically involved in the osmotic stress response, and *sko1Δ* cells show no growth defect in high osmolarity medium, suggesting that the TF plays a wider role in gene regulation [45, 49].

Here, we demonstrate that Sko1 is indeed a SUMO target and that the modification functions primarily in preventing Sko1 from associating with nonspecific sites on the genome. We identify

Lys 567 as the major SUMO modification site on the TF and show that Sko1 sumoylation is a constitutive modification whose levels do not significantly change under different growth conditions, including osmotic stress. DNA binding activity is necessary for Sko1 sumoylation in vivo, implying that the modification takes place after Sko1 associates with chromatin. Significantly, our genome-wide analysis shows that a sumoylation-deficient form of Sko1 shows increased occupancy levels at its binding sites and is found at numerous additional promoter regions across the genome, when compared with sumoylatable Sko1. Intriguingly, although the consensus Sko1 binding motif is largely absent from these additional sites, most contain sequences that resemble the motif. Taken together, our results imply that a key function for sumoylation is in controlling the association of a DNA-bound TF with chromatin to increase its binding site specificity.

2.3 MATERIALS AND METHODS

Yeast strains and plasmid

Yeast strains used in this study are listed in S1 Table. Sko1- and Hog1-tagged strains were epitope tagged using homologous recombination as previously described [69]. Strains with a *SKO1* deletion were generated using a KanMX deletion cassette by homologous recombination. Plasmid Gal4DB-K450E was generated by PCR-based sub-cloning of the *sko1-K450E* coding sequence into vector pGBT9 at the SmaI restriction site. It was then transformed into the HF7c yeast strain, which contains two genomic reporter genes with Gal4 binding sites.

Yeast growth assay ("spot assay")

Yeast cultures were grown overnight in appropriate liquid medium and diluted to an optical density (OD at 595 nm) of 0.2. Then, cells of each strain were spotted side-by-side in five-fold serial dilutions on solid-media plates with or without indicated osmotic stress conditions. Plates were incubated at 30˚C and images were taken after the indicated durations.

Preparation of yeast lysates and immunoprecipitation (IP)

Yeast cultures (40–50 mL) were grown in appropriate liquid medium to mid-log phase (OD595 nm of 0.5 to 0.7). Osmotic stress was induced by adding NaCl to a final concentration of 0.4M for the indicated time points. Cells were then harvested by centrifugation at 3000 g for 5 min, followed by a wash with IP buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Nonidet P-40 (NP40), 1X yeast protease inhibitor cocktail (BioShop), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2.5 mg/mL N-ethylmaleimide (NEM)). Cells were lysed with glass beads in IP buffer containing 0.1 mM dithiothreitol for 30 min at 4˚C. The lysed material was isolated from the beads and

centrifuged twice to remove insoluble materials. Samples were either analyzed by immunoblot or used for IP. For IP experiments, an aliquot of yeast lysate was retained as input sample, and the remainder was incubated overnight with anti-HA agarose beads or Protein G agarose with HA antibody at 4˚C. IPs were washed three times with ice-cold IP buffer plus 0.1% NP40, and twice with IP buffer alone. Beads were then resuspended and boiled with SDS loading buffer for 3 min, prior to analysis by the indicated immunoblots.

Denatured immunoprecipitation (IP)

Yeast cultures (50 mL) were grown in YPD medium to an OD595 nm of ~0.65. Cells were harvested by centrifugation at 3000 g for 5 min and washed with 20% trichloroacetic acid (TCA). Washed cells were then lysed with glass beads in 20% TCA. Lysates were precipitated and washed with 5% TCA and resuspended with modified SDS buffer (60 mM Tris pH 6.7, 5% 2-mercaptoethanol, 1% SDS, few drops of a bromophenol blue solution) prior to boiling for 5 min. Boiled samples were centrifuged at room temperature for 10 min. An aliquot (40 μL) of the supernatant was retained as input sample and the remainder was diluted with denaturing IP buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP40) containing 0.5 mg/mL of bovine serum albumin. Diluted samples were then incubated overnight with anti-HA agarose beads at 4˚C. IPs were washed three times with ice-cold denaturing IP buffer. Beads were then resuspended and boiled with SDS loading buffer for 3 min prior to analysis by the indicated immunoblots.

Chromatin immunoprecipitation (ChIP)

Yeast cultures (50 mL) were grown in YPD medium and induced by osmotic stress as indicated for IP procedure. Cells were then cross-linked with 1% formaldehyde for 20 min, followed by 5 min of quenching with 282 mM of glycine. Cells were pelleted by centrifugation and washed twice with ice-cold TBS (20 mM Tris-HCl, pH 7.5 and 150 mM NaCl), then in ChIP buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate and 0.1% SDS). Washed samples were resuspended in ChIP buffer and lysed with glass beads using a mini bead beater. The lysed materials were isolated from glass beads and sonicated to yield an average DNA fragment size of 300 to 500 bp in length. Samples were then centrifuged at 14,000 g for 5 min. To the isolated supernatants, additional NaCl was added to a final concentration of 225 mM. For IP, salt-adjusted supernatants were incubated with 15 µL magnetic Protein G beads (Dynabeads, Thermo Fisher Scientific) prebound with 2 µg rabbit anti-HA (Novus) or mouse anti-MYC (NEB) antibodies. IPs were washed with four different buffers for 4 min in the following order: (1) ChIP buffer with 275 mM NaCl; (2) ChIP buffer with 400 mM NaCl; (3) A buffer containing 10 mM Tris-HCl, pH 8, 0.25MLiCl, 1mM EDTA, 0.5% NP-40 and 0.5% sodium deoxycholate; (4) Tris-EDTA buffer (10 mM Tris-HCl, pH 8 and 1 mM EDTA). Washed beads were then incubated in ChIP elution buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA and 1% SDS) for 10 min at 65˚C. Bound material was isolated on a magnet, treated with RNase for 30 min at 37˚C, then with proteinase K at 42˚C for 1 h. To reverse crosslinking, samples were incubated overnight at 65˚C. DNA was purified and recovered using GeneJet Gel Extraction Kit (Thermo Fisher) followed by a quantitative PCR using primers listed in S2 Table. Results from qPCR were normalized to an untranscribed region of Chromosome V for Figure 2.2C, then an internal control gene, *PMA1*, for other ChIP analyses. ChIP experiments were repeated at least three times and the

averages were plotted with the standard deviations shown as error bars. Pair-wise statistical analyses were performed by Student's *t*-test (Figures 2.2C, 2.6A, 2.6D, 2.6E, 2.6F and 2S6), whereas statistical comparison of sets of data in 2S4B and 2S5 Figures were performed by ANOVA. Asterisks indicate a significant difference with *P*-values less than 0.05 for ChIP and RT qPCR analyses, whereas pairs and sets with *P*-values greater than 0.05 are unmarked or marked as "n/s."

Isolation of RNA and reverse transcription (RT)

RNA was isolated as previously reported from 10 mL yeast culture and subjected to DNase treatment [70]. Reverse transcription was performed on 1 μg of DNA-free-RNA using iScript cDNA synthesis (Bio-Rad) followed by qPCR. mRNA levels were normalized to an internal loading control, 25S rRNA. Primer sequences used for these experiments are listed in S2 Table. Error bar represents the standard deviation of three replicates.

ChIP-seq and analysis

For ChIP-seq analysis, ChIP was performed as described above, but scaled up as appropriate. Namely, culture volumes were 200 mL in rich (YPD) medium, and 6 μg of HA antibody was pre-bound to 40 μL of Dynabeads. Two replicates of the ChIP-seq experiment described above were performed, with sequencing libraries generated for both IP and input samples for Replicate 1, but only IP samples were sequenced for Replicate 2. Libraries were prepared using the NEBNext Ultra II DNA library prep kit (New England Biolabs), and paired-end sequencing (two times 126 bases) was performed using an Illumina HiSeq 2500 instrument at The Centre for Applied Genomics (TCAG) at the Hospital for Sick Children (Toronto). Raw and processed sequencing data files

have been uploaded to the NCBI Gene Expression Omnibus (GEO) with accession number GSE118655.

For differential binding analysis, which was performed at TCAG, sequencing reads were aligned, and peaks assigned as follows. Sequencing adaptors were trimmed using Trim Galore! (version 0.0.4) running Cutadapt (version 1.10) with the following parameters: quality score cut-off of 25, six nucleotides were removed from 50 ends, Illumina universal adapter sequences were removed, stringency setting of 5, sequences shorter than 40 nt after trimming were discarded, and only pairs of reads were retained. Trimmed forward and reverse reads were then aligned to the sacCer3 reference genome using Bowtie2 (version 2.3.2) [71]. Peak calling was performed using the MACS2 peak assignment tool (version 2.1.1) [72] in paired-end mode, with a genome size of 1.2e7, and using corresponding input samples derived from Replicate 1 as controls for peak calling for both Replicates 1 and 2. The default statistical significance cutoff was applied: 0.05 for the *q*-value, which is the false-discovery rate (FDR)-adjusted *p*-value, calculated using the Benjamini-Hochberg correction. Fold enrichment (FE) is calculated as the fold enrichment for each peak summit against a random Poisson distribution with local lambda. A set of consensus peaks was assembled using DiffBind (version 2.2.12), such that each peak was identified in at least two independent samples from both replicates, or as otherwise noted. Binding affinities were determined and compared using DiffBind based on the number of ChIP read counts (log2 of normalized ChIP read counts with input read counts subtracted) (S5 Table). Because input samples were derived only for Replicate 1, and Replicate 2 had consistently lower read counts, the replicate number was used as a blocking factor for batch correction in the statistical model used by DiffBind when calculating the affinity scores. Peaks were annotated with all genomic features within 5 kb of consensus peaks using ChIPpeakAnno (version 3.12.7; Bioconductor Package) [73], with the

reference genome annotation package TxDb.Scerevisiae.UCSC.sacCer3.sgdGene. Differential binding data provided by TACG was then used to generate heatmaps to display pair-wise fold changes in binding occupancy levels (Figure 2.6B) using the Heatmapper web tool [74].

For peak number analysis (Figures 2.3, 2.4, 2.5 and 2S2), sequence read alignments and peak calling were performed as described above, with the following notes and exceptions. Peak assignment normalization for Replicate 2 was performed using either the input controls from Replicate 1, or using local genomic bias from the ChIP samples, themselves (i.e. without a control) [72]. Both methods produced similar results, with the latter used to produce the data shown in Figures 2.4, 2.5 and 2S2. Some computations were performed using the Niagara supercomputer at the Sci- Net HPC Consortium. Lists of identified peaks, excluding peaks found with the mitochondrial genome, are presented in 2S3 Table. Peak analysis, including identifying overlapping peaks among data sets (findOverlapsOfPeaks function; 2S4 Table) and the distribution of peaks across gene features (assignChromosomeRegion function) were performed using ChIPpeakAnno. Frequency of motif occurrences within peak sets (Figures 2.4D and 2.5C) was determined using the summarizePatternInPeaks function within the ChIPpeakAnno toolset. De novo motif discovery was performed using the MEME analysis tool for indicated peak sets using 41 nt surrounding peak summits and restricted to motifs of 6 to 12 nt in length [75]. An analysis with 81 nt surrounding peak summits was also performed, which resulted in similar results. Visualization of peak alignments (Figure 2.6C) was performed using the Integrative Genomics Viewer (Broad Institute) [76].

2.4 RESULTS

Sko1 is sumoylated at Lys 567 in yeast grown in normal conditions

Previous studies demonstrated that for two different bZIP TFs, Gcn4 in budding yeast and human FOS, sumoylation acts to restrict their association with DNA thereby preventing excessive expression of target genes [26-28]. We examined published lists of sumoylated proteins that were generated through proteomics analyses and identified 28 additional bZIP motif-containing TFs that are probable SUMO targets, four in *S. cerevisiae* and 24 human proteins (Figure 2.1A) [4,8]. This represents almost half of known bZIP TFs in these species and suggests that sumoylation is a common mechanism of regulating their functions. To explore this, we selected for further study the yeast bZIP TF Sko1 which was identified as a SUMO target in large-scale studies, but the effects of its sumoylation have not yet been reported [37-39].

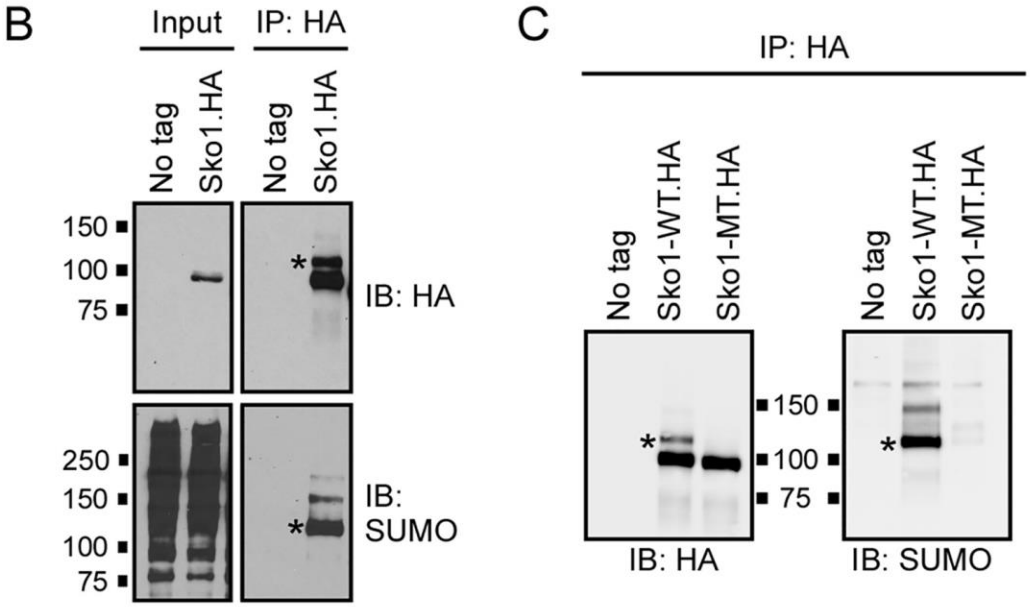
A yeast strain was generated that expresses a C-terminal 6xHA-tagged form of Sko1 (Sko1.HA) from its natural locus and we confirmed that the presence of the tag does not affect cell growth under normal and osmotic stress conditions (2S1A Figure). Cell lysates were analyzed using the same procedures that were previously used for examining sumoylated proteins in yeast, i.e. HA immunoprecipitation (IP) followed by HA and SUMO immunoblotting (see *Materials and Methods*) [27, 28, 52]. Protein bands of the molecular weights expected for sumoylated Sko1 were detected in the SUMO immunoblot, including a “ladder” of bands that is typical for proteins that are multi- or poly-sumoylated, confirming that Sko1 is sumoylated (Figure 2.1B). Supporting that the sumoylated species detected in the immunoblots correspond to modified Sko1 specifically, we repeated the analysis using protein samples that were prepared under denaturing conditions (i.e. precipitation with trichloroacetic acid, TCA), which again showed bands of the expected size in

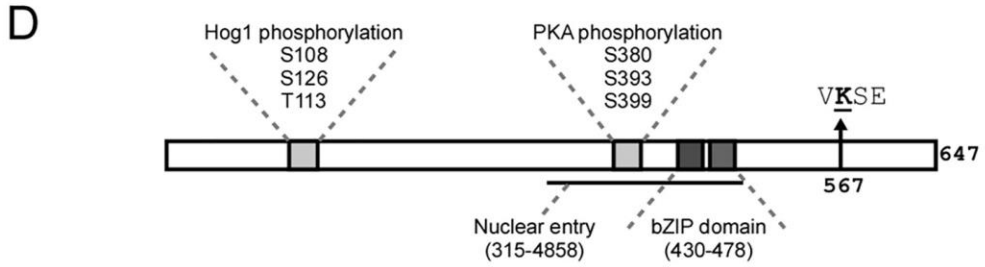
the SUMO immunoblot (2S1B Figure). These results confirm the observation made in multiple proteomics studies that Sko1 is sumoylated in yeast grown in normal conditions [37-39].

We analyzed the polypeptide sequence of Sko1 using a SUMO-site prediction tool, GPS-SUMO [53], and identified Lys 567, which is within a SUMO consensus motif, as the most likely site of SUMO modification. To test this, we generated a yeast strain expressing only a K567R-mutant form of Sko1.HA from the *SKO1* locus (hereafter referred to as Sko1-MT), and examined its sumoylation by IP-immunoblot. As shown in Figure 2.1C, Sko1-MT is expressed at normal levels, but the mutation virtually abolishes its sumoylation, indicating that Lys 567 is the major site of sumoylation on the protein. This residue lies in the C-terminal part of the protein, significantly downstream of the bZIP DNA binding motif, the Hog1 and PKA phosphorylation sites, and the region of the protein required for nuclear entry, and therefore defines a novel regulatory region on Sko1 (Figure 2.1D) [54]. A higher molecular weight form of Sko1 that is detectable in HA immunoblots of Sko1-WT (asterisks in Figures 2.1B, 2.1C, 2.1E and 2S1B), but is not present in the Sko1-MT IP lane in Figure 2.1C, corresponds in size to monosumoylated Sko1, implying that both unmodified Sko1 and its major sumoylated form can be detected on the same HA immunoblot. Taking advantage of this, we used densitometry to measure the intensity of bands corresponding to unmodified and monosumoylated Sko1 and determined that at least 13% of Sko1 is sumoylated in normally growing yeast. Nonetheless, sumoylation of Sko1 is not required for cell viability as the *sko1-MT* strain grew as well as strains expressing Sko1-WT on rich (YPD) or synthetic complete (SC) media (2S1C Figure). This result is not surprising since *SKO1* itself is not required for normal yeast growth (e.g. *sko1Δ* in 2S1C Figure and [44]), but our data indicates that, at any given time, a significant fraction of Sko1 polypeptides are modified by sumoylation.

A

S. cerevisiae	Human				
Cin5	ATF3	CEBPA	FOS	JUNB	NFE2
Cst6	ATF4	CEBPB	FOSL2	MAF	NFE2L2
Gcn4	ATF6	CEBPD	CREB3L2	MAFA	NFIL3
Met4	ATF7	CEBPE	JUN	MAFG	NRL
Sko1	BACH2	CREB1	JDP2	MAFK	XBP1





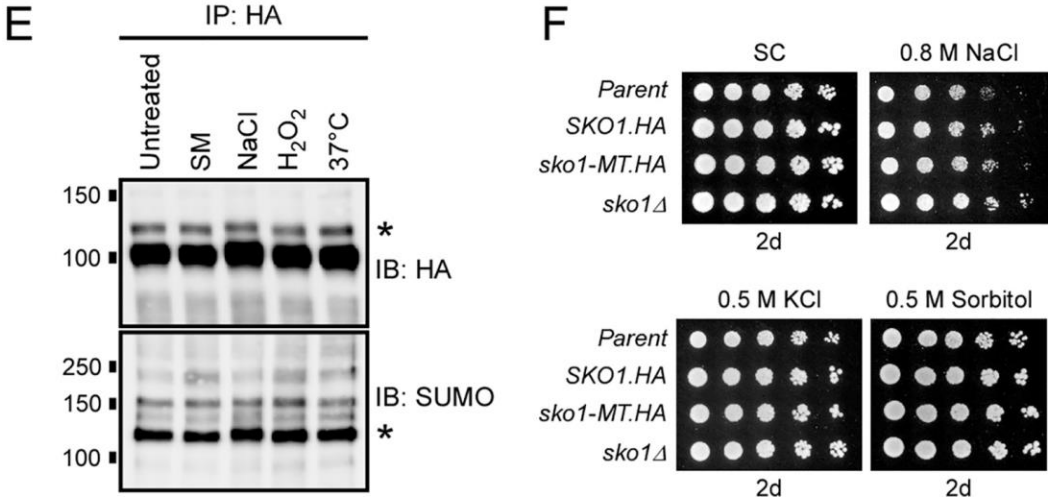


Figure 2.1: Sko1 is constitutively sumoylated at Lys 567. (A) List of yeast and human bZIP motif-containing proteins that have been identified as putative SUMO targets in published proteomics studies. **(B)** Lysates from yeast strains expressing 6xHA-tagged Sko1 from its chromosomal locus, or a strain with no HA-tagged proteins (*No tag*), were examined by HA IP and immunoblot analysis with HA and SUMO antibodies. Inputs represent approximately 5–10% of the IPed material. **(C)** A yeast strain was generated with a chromosomal *sko1* K567R mutation (Sko1-MT.HA) and used for IP-immunoblot analysis, as in *(B)*, alongside No tag and Sko1-WT.HA controls. **(D)** Schematic of the Sko1 protein indicating bZIP DNA binding domain, regions targeted by Hog1 and PKA kinases, region required for nuclear entry [54], and the primary site of sumoylation, Lys 567, which is found within a SUMO consensus motif, VKSE. **(E)** IP-immunoblot analysis of lysates from the *SKO1-WT.HA* strain grown in SC medium ("Untreated"), or SC medium treated for 20 min with 0.5 μg/mL sulfometuron methyl (SM, amino acid starvation), 0.4M NaCl (osmotic stress), 1 mM H2O2 (oxidative stress), or incubated at 37˚C (temperature stress), as in *(B)*. **(F)** Spot assay comparing growth of indicated strains in various types of osmotic stress. Approximately 10,000 cells were spotted on the left-most position and serial five-fold dilutions were spotted on adjacent spots to the right in each panel. Media plates were imaged after two days. Asterisks (*) indicate position of the major (mono-) sumoylated form of Sko1 in each immunoblot.

Sko1 sumoylation is not affected by osmotic stress

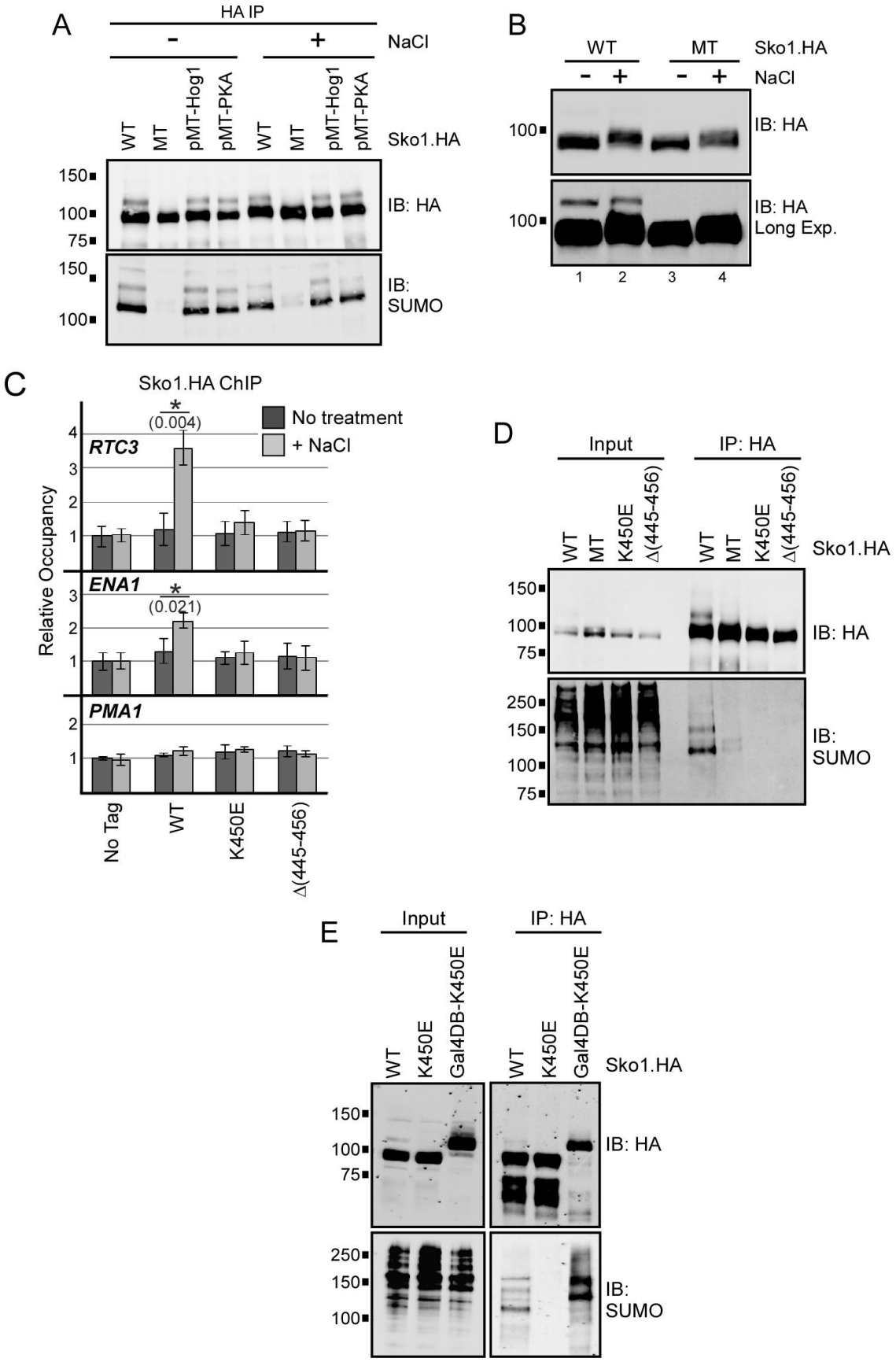
To examine whether sumoylation might regulate Sko1 in response to high osmolarity or other types of stress, the *SKO1-WT* strain was grown in different conditions, and the level of Sko1 sumoylation was determined in each condition by IP-immunoblot analysis. As shown in Figures 2.1E and 2S1D, compared to growth in normal medium ("untreated"), Sko1 sumoylation levels were essentially unchanged after exposure to osmotic, oxidative, or temperature stress, or during amino acid starvation (through addition of sulfometuron methyl, SM, to growth medium) [55]. This indicates that sumoylation of Sko1 does not occur as part of a stress response but that the modification regulates Sko1 constitutively. Cellular levels of Sko1 in *SKO1-WT* and *sko1-MT* strains were approximately the same under normal growth conditions, and remained constant during osmotic stress, implying that sumoylation does not act to regulate Sko1 stability or abundance (2S1E Figure). Moreover, the *sko1-MT* strain grew as well as the strain expressing Sko1-WT on a variety of osmotic stress media, indicating that Sko1 sumoylation is not required for cell survival during osmotic stress (Figure 2.1F). However, Sko1 itself is not required for survival during osmotic stress (*sko1Δ* in Figure 2.1F and [45]), reflecting that its role in regulating the transcription of target stress response genes has more subtle consequences, and that the effects of sumoylation on Sko1 function are likely intricate (see below) [48].

Sko1 sumoylation does not depend on or influence its phosphorylation

Many SUMO modifications show codependence or interference with other types of posttranslational modifications, including phosphorylation, on the same protein [19, 56]. To determine whether prior phosphorylation by Hog1 or PKA is required for Sko1 sumoylation, we generated yeast strains expressing mutant forms of Sko1.HA, including Sko1 (pMT.Hog1), which

contains Ala substitutions at the three Hog1 target residues (Ser 108, Ser 126, and Thr 113), and Sko1 (pMT.PKA), which has Ala substitutions at the three PKA target Ser residues (380, 393, and 399) [44]. The strains were then grown untreated or treated with NaCl for 10 min prior to the preparation of lysates and subsequent examination by IP-immunoblot. As shown in Figure 2.2A, normal levels and patterns of Sko1 sumoylation were observed in the SUMO immunoblot analysis of both the Sko1 (pMT.Hog1) and Sko1 (pMT.PKA) forms of Sko1.HA. This indicates that sumoylation of Sko1 occurs independently of its prior phosphorylation by Hog1 or PKA.

Next we examined whether sumoylation can influence subsequent phosphorylation of Sko1 by Hog1, which occurs in response to osmotic stress [44]. HA immunoblot analysis of Sko1-WT from cells grown in osmotic stress showed a mobility shift that is consistent with its phosphorylation and similar to a mobility shift that was previously reported (Figure 2.2B) [44]. The NaCl-dependent shift occurred for both the prominent band in the immunoblot, which corresponds to unsumoylated Sko1, as well as for the monosumoylated form of Sko1-WT (compare lanes 1 and 2 in upper and lower panels of Figure 2.2B). Sko1-MT also showed a NaCl-dependent shift in migration during osmotic stress (compare lanes 3 and 4). To examine this further, we repeated the HA immunoblot analysis using a phosphate-binding compound present in the acrylamide mix that enhances detection of phosphorylated protein isoforms during SDS-PAGE ("Phos-tag") [57]. As shown in 2S1F Figure, prominent higher-molecular weight bands appeared after treatment of either *SKO1-WT* or *sko1-MT* strains with NaCl ("Sko1-P"), which we attribute to Hog1-mediated phosphorylation since this shift was not observed in a *hog1Δ* strain. Together, these analyses indicate that sumoylation is not a pre-requisite for Sko1 phosphorylation during osmotic stress but suggests that both unsumoylated and sumoylated Sko1 can be phosphorylated by Hog1.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SUMO
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RTC3
ENA1
*
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PMA1
Relative Occupancy
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Δ(445-456)
D
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MT
K450E
Δ(445-456)
WT
MT
K450E
Δ(445-456)
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150
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IB: HA
250
150
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IB:
SUMO
E
Input
IP: HA
WT
K450E
Gal4DB-K450E
WT
K450E
Gal4DB-K450E
Sko1.HA
150
100
75
IB: HA
250
150
100
IB:
SUMO

Figure 2.2: Sko1 sumoylation shows no interdependence with phosphorylation but requires DNA binding. (A) Yeast strains expressing WT, MT, Hog1 kinase site-mutant ("Sko1(pMT.Hog1)"; S108,126A, T113A), or PKA kinase site-mutant ("Sko1(pMT.PKA)"; S380,393,399A) forms of Sko1.HA were grown in untreated medium or with 0.4MNaCl for 10 min. Lysates were then prepared and subjected to IP-immunoblot analysis as in Figure 2.1B. **(B)** HA immunoblot analysis of lysates from *SKO1-WT.HA* and *sko1-MT.HA* strains that were grown in untreated medium, or with 0.4M NaCl for 10 min. A longer exposure is shown to highlight the differential migration of the sumoylated (higher molecular weight) form of Sko1.HA in the first two lanes. **(C)** Yeast strains expressing no HA-tagged proteins or WT, K450E or Δ(445–456) forms of Sko1.HA, were grown in normal conditions or treated with 0.4M NaCl for 5 min, then cross-linked for HA ChIP analysis. qPCR was performed on promoter regions of two Sko1 target genes, *RTC3* and *ENA1*, and a non-Sko1 target gene, PMA1. Error bars represent standard deviation of three independent experiments. Asterisks (*) indicate that the compared data pairs are statistically different ($P < 0.05$; see *Materials and Methods*). **(D)** IP-immunoblot analysis of lysates from yeast strains expressing indicated forms of Sko1.HA, performed as in Figure 2.1B. **(E)** IP-immunoblot analysis of lysates from strains expressing WT, K450E, or Gal4-DNA binding domain-fused Sko1-K450E (GalDB-K450E) forms of Sko1.HA.

DNA binding is necessary and sufficient for Sko1 sumoylation

To investigate whether Sko1 becomes sumoylated prior to, or after binding target DNA sequences, we tested if its DNA binding activity is necessary for the modification to take place. Lys 450 of Sko1 is at a conserved position in the bZIP motif, and a Lys-to-Glu mutation of the corresponding Lys in the human bZIP TF CREB1 abolished its ability to bind DNA [43, 58]. We constructed yeast strains that expresses an analogous K450E mutant form of Sko1.HA, or Sko1.HA with a 12-amino acid residue deletion surrounding Lys 450, Δ(445–456). To confirm that these mutant forms of Sko1 are indeed defective in DNA binding, we performed ChIP analysis at the promoter regions of the *RTC3* and *ENA1* genes, both of which are bound by Sko1 during osmotic stress [49, 50], and *PMA1*, which is not targeted by Sko1 [48]. As expected, treatment of the *SKO1-WT* strain with NaCl led to rapid recruitment of Sko1 to the RTC3 and ENA1 promoters, but not to the promoter of *PMA1* (Figure 2.2C). For both Sko1-K450E and Sko1 Δ(445–456), however, no recruitment was detected, indicating that both forms of Sko1 are indeed defective in DNA binding. WT, MT, and the DNA-binding-deficient forms of Sko1.HA were then examined by IP-immunoblot, and, as shown in Figure 2.2D, even though the K450E and Δ(445–456) forms were expressed and IPed at levels comparable to Sko1-WT, neither showed a signal on the SUMO immunoblot. This indicates that Sko1 that is not able to bind DNA is not sumoylated and suggests that the modification takes place only after the TF binds to its target DNA sites.

To determine whether DNA binding itself can trigger Sko1 sumoylation, we constructed an expression plasmid that generates the DNA binding-deficient form of Sko1.HA (K450E) fused to the DNA binding domain of the transcription activator Gal4 (construct Gal4DB-K450E). Strikingly, when introduced into a yeast strain that contains multiple Gal4 binding sites, the fusion

protein showed high levels of sumoylation (Figure 2.2E). This indicates that DNA binding itself can restore SUMO modification to the Sko1-K450E mutant, and strongly implies that DNA binding is a major determinant for Sko1 sumoylation.

Genome-wide identification of Sko1-WT and Sko1-MT binding sites

To explore whether sumoylation regulates the association of Sko1 with chromatin at its binding sites genome-wide, we performed HA ChIP followed by next-generation sequencing (ChIP-seq) for both *SKO1-WT* and *sko1-MT* strains, under normal growth and after exposure to osmotic stress (0.4M NaCl for 5 min). Sko1 binding sites (peaks) were identified, using the MACS2 software tool, through detection of genomic regions with statistically significant Sko1 occupancy in IPed samples relative to background levels from input samples (statistical significance cut-off of $q < 0.05$). Numerous peaks were identified for both Sko1-WT and Sko1-MT in both untreated and osmotic stress-treated (+NaCl) samples (Figure 2.3A and 2.3B and 2S3 Table), including peaks near many known Sko1-regulated genes (Figure 2.3C and see 2S5 Table). NaCl treated samples had somewhat fewer identified peaks than untreated samples for both Sko1-WT and Sko1-MT, which likely reflects the osmotic stress-associated binding dynamics of Sko1 in which its levels are reduced on some targets [49-51]. Consistent with previous genome-scale binding studies, occupancy levels of Sko1-WT range widely among its binding sites, with about half showing relatively high occupancy levels (at least a two-fold enrichment in read density compared to input, FE > 2; Figure 2.3A and 2.3C), including binding sites for some known Sko1 target genes, as indicated in Figure 2.3C [48-51]. Supporting the effectiveness of this ChIP-seq experiment, we compared it with a previous Sko1 ChIP-seq study using the ChIPPeakAnno analysis toolkit and found that 80% of the peaks in our Sko1-WT set were also identified in that study [50].

Furthermore, a search for recurring sequences in the Sko1-WT peak set from our analysis produced the previously reported Sko1 binding motif, ATGACGT, with very high confidence (Figure 2.3D) [50,59].

Sko1-MT binds more promoter regions than Sko1-WT

Intriguingly, the ChIP-seq analysis showed that Sko1-MT binds dramatically more sites than Sko1-WT, in both untreated and +NaCl samples (66% and 47% more peaks, respectively; Figure 2.3A and 2.3B, and 2S3 Table). To confirm this observation, we performed an independent ChIPseq replicate experiment (Replicate 2), which again showed more binding sites for Sko1-MT than Sko1-WT in untreated and +NaCl conditions (2S2A and 2S2B Figures). Overall, Replicate 2 showed consistently lower normalized read counts (i.e. occupancy levels) than Replicate 1 and it consisted of many peaks of low fold enrichment that were largely absent from Replicate 1 (2S2A and 2S2C Figures). As we did not produce an input control set for Replicate 2, this might reflect that peak assignment normalization was performed by other methods for this replicate (see *Materials and Methods*). Nonetheless, a large number of overlapping peaks were identified in each analysis between the two replicates (2S2C Figure, left and S3 Table), and, when only peaks from Replicate 2 that have high fold enrichment (FE > 2) were considered, 75% to 91% were found to overlap with peaks from Replicate 1 (2S2C Figure, right). Importantly, each replicate identified more peaks bound by Sko1-MT compared with Sko1-WT, regardless of whether yeast were grown in normal or osmotic conditions (compare Figure 2.3A with 2S2A Figure). To increase confidence in our results, the peak analyses described below were performed using only binding sites that were identified in both replicates for each sample ("overlapping peak sets"). Notably, the overlapping peak sets also showed dramatically higher numbers of peaks for Sko1-MT than for

Sko1-WT in untreated and +NaCl conditions (2S2D and 2S2E Figures). Lists of peaks identified in each replicate and in the overlapping peak sets are presented in 2S3 and 2S4 Tables, respectively.

We next performed a detailed analysis of the Sko1-WT and Sko1-MT peaks derived from normally growing yeast (untreated) from the overlapping peak sets. Nearly all the 207 Sko1-WT binding sites were also bound by Sko1-MT, but Sko1-MT was found at an additional 277 sites (Figure 2.4A). This is not the result of random binding of Sko1-MT at positions across the genome because ~90% of the peaks that are unique to Sko1-MT ("MT only") are found near promoter regions, which is only slightly higher than the ratio of promoter-associated peaks in the Sko1-WT set (Figure 2.4B). Promoter regions include 2000 nt upstream, and 200 nt downstream, of transcriptional start sites (TSSs), which encompasses the upstream activation sequences (UASs) to which TFs typically bind [60]. Peaks that are unique to Sko1-WT ("WT only") showed a somewhat different distribution, with about one third appearing in regions immediately downstream of gene ends, but this might be skewed by the small number of peaks (20), and its significance is not known (Figure 2.4B). For a more detailed analysis of the position of peaks, we plotted their distribution around TSSs (Figures 2.4C and 2S3A). MT-only peaks show a slightly more focused distribution than peaks that are common to both the Sko1-WT and MT sets ("WT & MT"), but they are predominantly situated around 400 to 500 bp upstream of TSSs, which is similar to the distribution of WT & MT peaks. These results indicate that Sko1 that cannot be sumoylated binds to numerous additional promoter regions in normally growing yeast, thereby implicating sumoylation in binding site specificity.

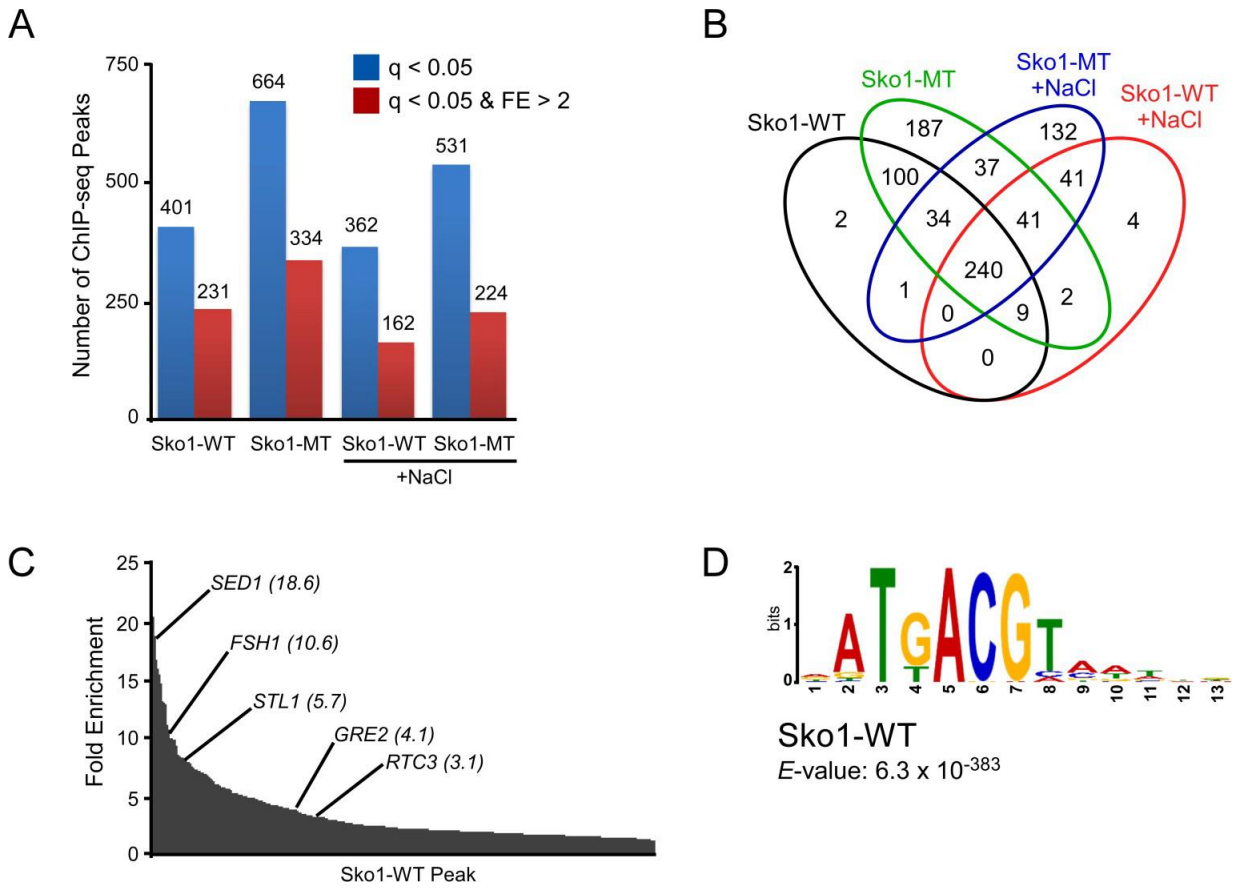


Figure 2.3: Binding site analysis from Sko1-WT and Sko1-MT ChIP-seq experiment. (A) Number of binding sites (peaks) identified from Replicate 1 of ChIP-seq analysis of *SKO1-WT.HA* and *sko1-MT.HA* strains, either untreated or treated with 0.4 M NaCl for 5 min, with a *q*-value less than 0.05 (blue bars). Subset of identified peaks having a *q*-value less than 0.05 and a fold enrichment (FE) value greater than 2 are also indicated, in red. **(B)** Venn diagram showing number of peaks ($q < 0.05$) shared among the four samples tested. When generating Venn diagrams throughout this study, if two peaks in one sample both overlap with the same peak in another sample they are counted as a single overlapping peak. This results in slightly fewer total peaks for each sample in the Venn diagram compared with the number of identified peaks listed in 2S3 and 2S4 Tables and in panel *(A)*. **(C)** Distribution of fold enrichment values for the peaks identified in the untreated Sko1-WT sample, with peaks of select known Sko1 target genes indicated with FE values shown in brackets. **(D)** Motif discovery analysis, using MEME tool, of the sequences surrounding the summit of the peaks from the untreated Sko1-WT sample, identified the indicated consensus sequence.

We then examined the nature of the Sko1-WT and Sko1-MT peaks to determine whether binding sites unique to Sko1-MT have common or distinguishing features. Genes situated nearest the Sko1-WT peaks are involved in diverse pathways, but Gene Ontology (GO) term analysis indicates that glucose, hexose, and ethanol metabolic processes are significantly enriched among these ($P < 1.0 \times 10^{-4}$), which matches the results of a previous examination of the Sko1 regulon [49]. GO term analysis for peaks unique to Sko1-MT, however, showed no GO term enrichment, indicating that there is no apparent bias in the distribution of MT-only binding sites with respect to target gene function.

To explore whether the SUMO-site mutation might alter the Sko1 binding sequence specificity, we determined the frequency at which the consensus Sko1 binding motif appears in Sko1-WT and Sko1-MT peak sets. About 60% of Sko1-WT peaks contain the sequence ATGACGT, whereas it is found in only ~12% of MT-only peaks (Figure 2.4D), strongly suggesting that blocking sumoylation alters Sko1 binding specificity. When we repeated this analysis with a less-specific version of the Sko1 binding site, TKACG (where K is G or T; based on the logo in Figure 2.3D), we found that this sequence is present in >80% of both Sko1-WT and MT-only peaks (Figure 2.4D). This implies that Sko1-MT recognizes Sko1 binding motif-like sequences, but with less stringency than Sko1-WT. In support of this, in a de novo motif discovery analysis, the only significant recurring motif identified in the MT-only peak set is a slightly weaker match to the consensus Sko1 motif, and it is found in a smaller fraction of peaks, when compared to the most recurring motif in the WT & MT set (2S3B Figure). Taken together, our analysis suggests that sumoylation functions in preventing the association of Sko1 with non-specific binding sites that show some sequence similarity to its consensus binding motif.

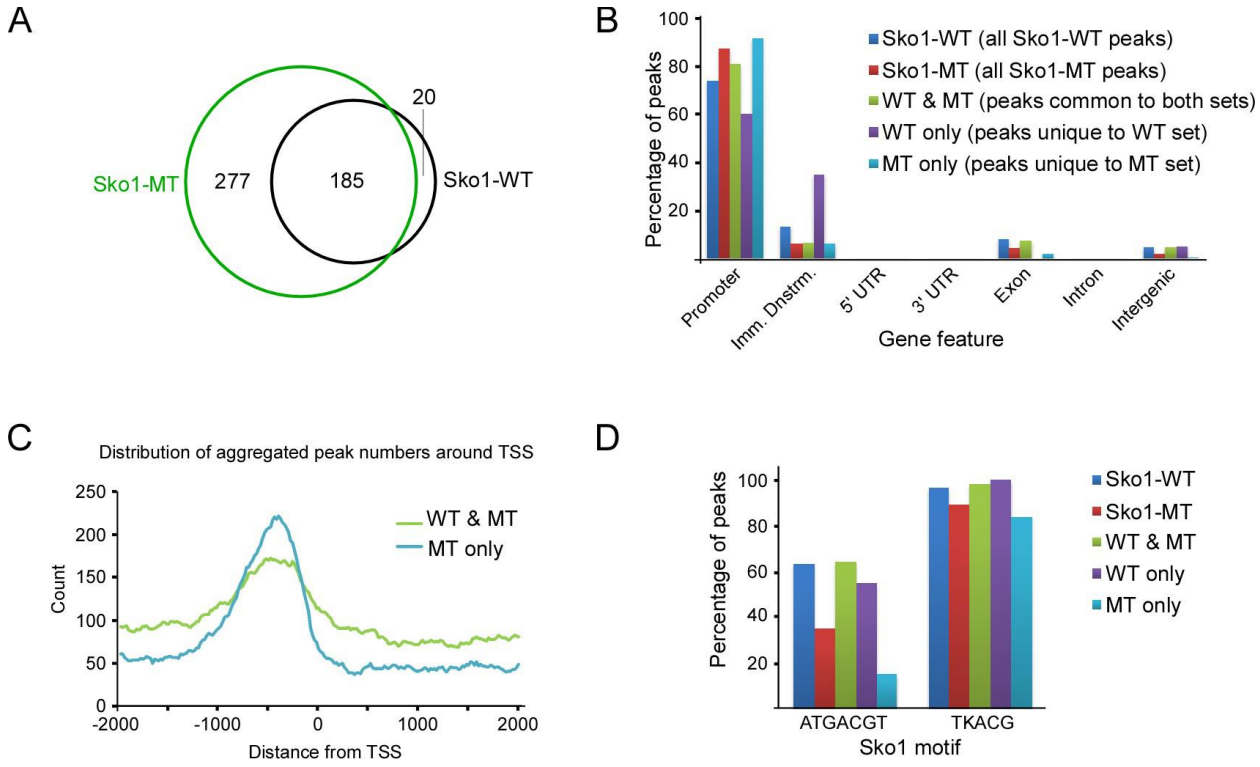


Figure 2.4: Sko1-MT binds dramatically more promoter regions than Sko1-WT. (A) Venn diagram showing number of common and unique peaks from the untreated Sko1-WT and Sko1-MT ChIP-seq analyses. Peaks identified in both ChIP-seq replicates (i.e. overlapping peak sets) were examined. **(B)** Distribution of ChIP-seq peaks from untreated Sko1-WT and Sko1-MT samples across indicated gene features. "Imm. Dnstrm." refers to the 1000 bases immediately downstream of the 3´ UTR-encoding sequence. "WT & MT" refers to peaks shared between the Sko1-WT and Sko1-MT sets, whereas "WT only" and "MT only" refer to peaks that are unique to the respective peak sets. **(C)** Distribution of aggregate peak numbers around the nearest transcriptional start site (TSS) for "WT & MT" and "MT only" peak sets. **(D)** Percentage of peaks, within indicated peak sets, that contain the major Sko1 consensus motif, ATGACGT, or a less-specific motif, TKACG, where K is G or T.

Sko1-MT binds more promoter regions than Sko1-WT during osmotic stress

In both *SKO1-WT* and *sko1-MT* strains, osmotic stress resulted in a partial redistribution of Sko1 with many binding sites gained and several lost, which demonstrates that high osmolarity influences Sko1 binding site selection (Figures 2.3B, 2S2B and 2S2E). We analyzed the overlapping peak sets obtained by ChIP-seq after treatment with NaCl, which again indicated that Sko1-MT binds significantly more sites than Sko1-WT (240 peaks for MT versus 122 for WT; Figure 2.5A). Approximately 80% of all +NaCl Sko1-bound sites, including the 129 bound only by Sko1-MT and the 111 bound by both WT and MT, are near promoters (Figure 2.5B). De novo motif discovery indicates that the consensus Sko1 binding site is the most significantly recurring motif for both the WT & MT and MT-only +NaCl peak sets (2S3B Fig), with the consensus motif appearing in ~45% of MT-only peaks and in ~60% of WT & MT peaks (Figure 2.5C). This is a greater fraction of MT-only peaks that contain the Sko1 binding site than in the untreated set (~45% vs ~12%; compare Figures 2.4D with 2.5C), which suggests that the effect of osmotic stress on Sko1 binding site selection outweighs the tendency for Sko1-MT to interact with degenerate binding sites. Consistent with this, high osmolarity likely influences Sko1 redistribution through phosphorylation of Sko1 by Hog1 [50], which we have shown occurs independently of its sumoylation (see above). Our results indicate that Sko1-MT binds many more sites than Sko1-WT in both untreated and +NaCl conditions, but binding appears to be more stringent for Sko1-MT under osmotic conditions, as more of the additional sites match the Sko1 consensus motif than under normal conditions.

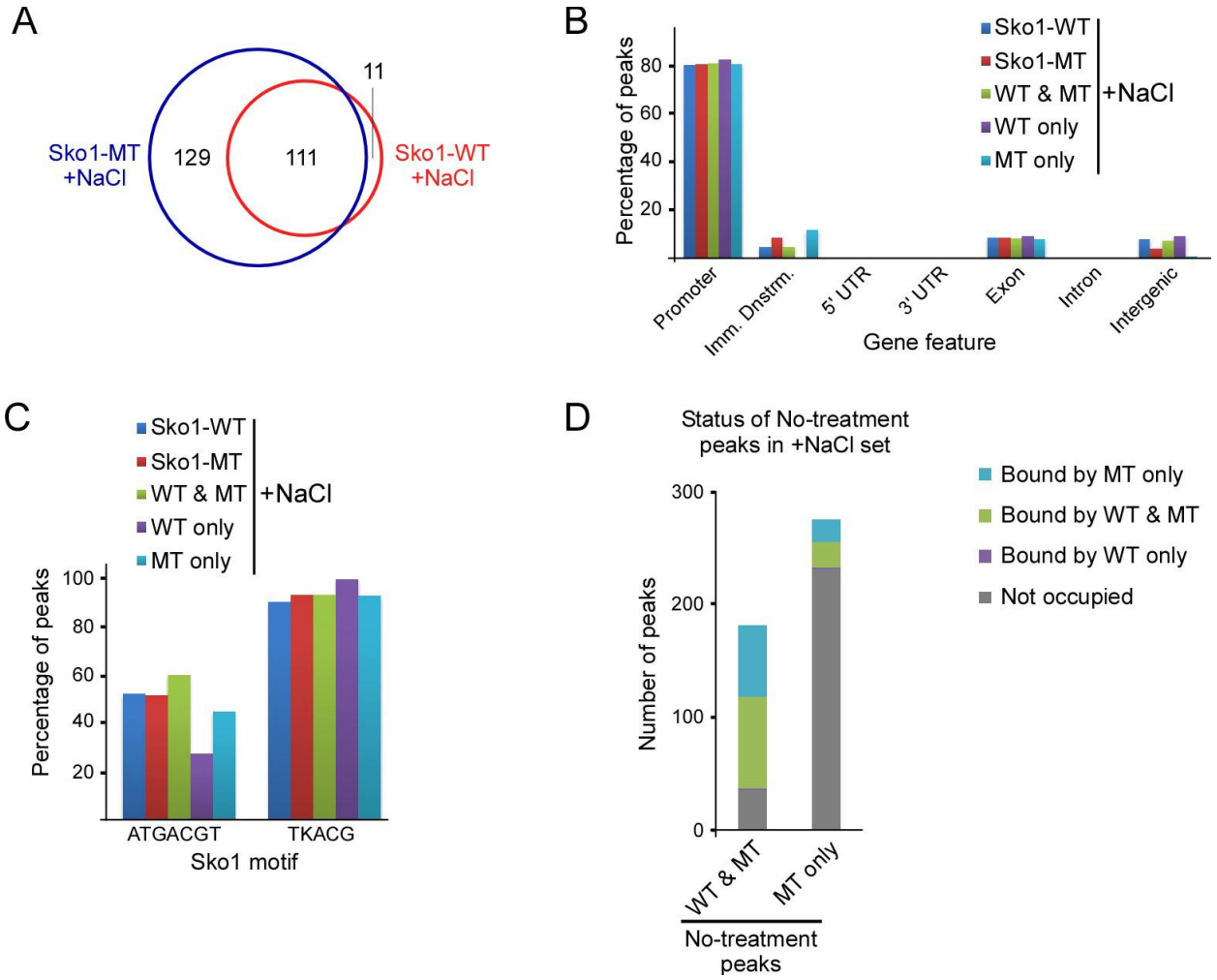


Figure 2.5: Sko1-MT binds more promoter regions than Sko1-WT after treatment with NaCl. (A) Venn diagram showing number of common and unique peaks from the NaCl-treated Sko1-WT and Sko1-MT ChIP-seq analysis. Peaks identified in both ChIP-seq replicates (overlapping peak sets) were examined. **(B)** Distribution of ChIP-seq peaks from NaCl-treated Sko1-WT and Sko1-MT samples across indicated gene features, as in Figure 2.4B. **(C)** Percentage of peaks, within indicated peak sets, that contain the major Sko1 consensus motif, ATGACGT, or a less-specific motif, TKACG, where K is G or T. **(D)** Status of "WT & MT" and "MT only" Sko1 binding sites from ChIP-seq analysis of untreated samples in the NaCl-treated set.

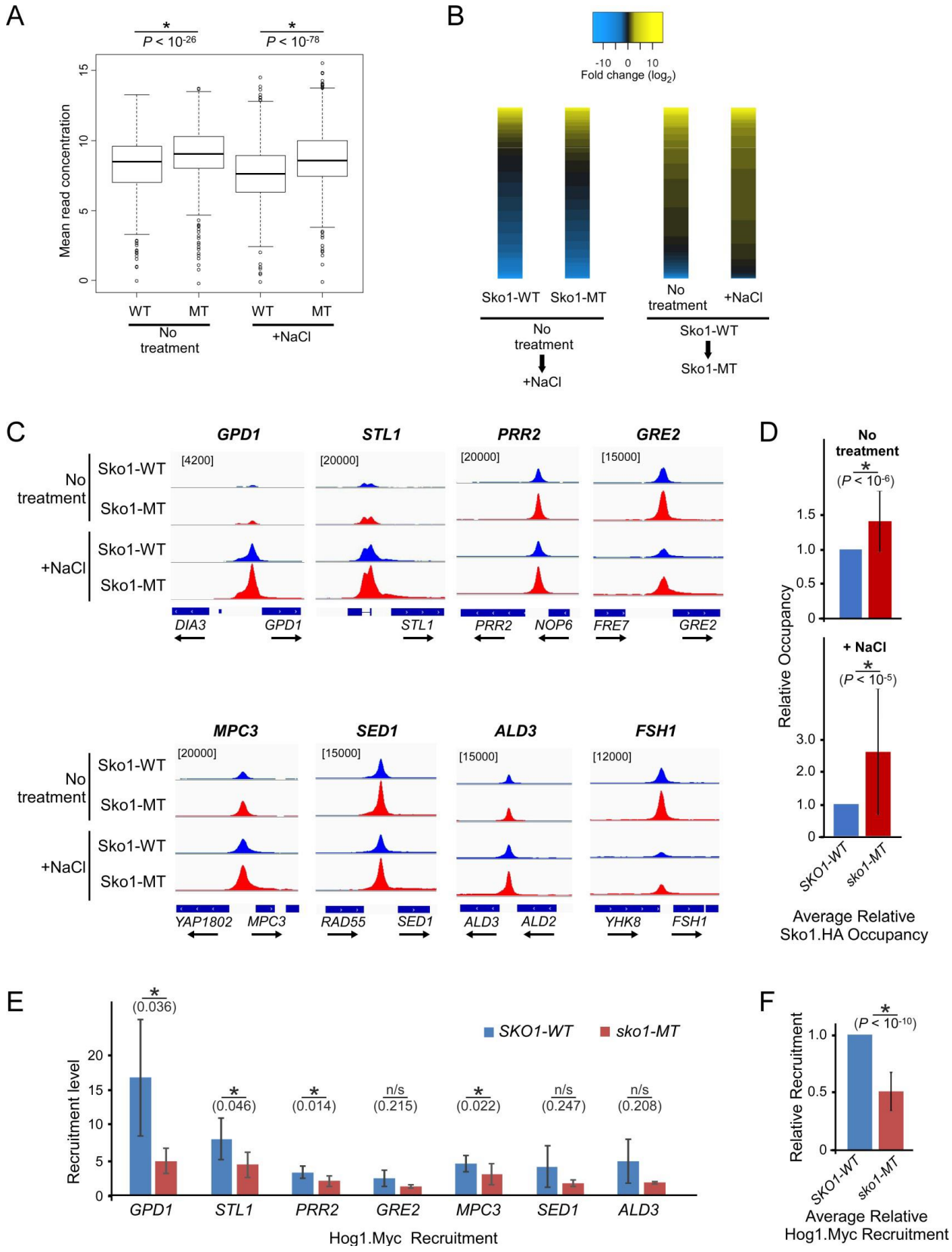
To explore the effects of osmotic stress on Sko1 binding site redistribution, we examined the status of peaks from the untreated WT & MT and MT-only samples in the +NaCl peak sets (Figure 2.5D). About 75% of sites that were common to both Sko1-WT and Sko1-MT sets during normal growth remained bound during osmotic stress (Figure 2.5D). Strikingly, however, 83% of sites bound only by Sko1-MT during normal growth were unbound after treatment with NaCl. This indicates that Sko1-MT is dissociated from most of its nonspecific binding sites after exposure to NaCl, which suggests that binding to these sites can be less stable than to actual Sko1 binding sites. Altogether, our analysis supports the notion that sumoylation acts to restrict Sko1 to appropriate stable binding sites, even during osmotic stress.

Sumoylation-deficient Sko1 shows higher occupancy levels at its binding sites

To investigate whether sumoylation can influence the occupancy level of Sko1, we next compared Sko1-WT and Sko1-MT occupancy levels at common binding sites across the genome. For this analysis, the DiffBind analysis tool was applied, which used a normalized number of sequence reads as a measure of occupancy across a consensus set of 630 peaks. The consensus peak set consists of peaks that were found in at least two of the four independent ChIP-seq analyses from both replicates, as listed in 2S5 Table. Significantly, as shown in the boxplots in Figure 2.6A and in 2S5 Table, Sko1-MT had overall higher occupancy levels than Sko1-WT in both untreated and +NaCl sets. To be sure that the effect is not only a result of the high number of MT-only peaks in the consensus peak set, two additional peak sets were also examined: (i) the set of 52 peaks that are present in both replicates of all four ChIP-seq analyses and, (ii) the 212 peaks that are present in both replicates of the Sko1-WT analyses in either the untreated or +NaCl sets, which can be

considered normal Sko1 binding sites. In all cases, Sko1-MT showed significantly higher occupancy levels than Sko1-WT (2S4A Figure).

To visualize the differences in occupancy levels at each of the 630 consensus peaks, heatmaps were generated (Figure 2.6B). For both Sko1-WT and Sko1-MT, osmotic stress resulted in an overall similar redistribution, with some sites showing increased binding (shown in yellow), but slightly more sites showing reduced binding (blue; Figure 2.6B; left pair of heatmaps). When comparing occupancy levels of Sko1 in *SKO1-WT* versus *sko1-MT* strains, however, most sites showed higher levels of Sko1-MT, and the effect was similar but more pronounced after treatment with NaCl (yellow; Figure 2.6B; right pair of heatmaps). Increased occupancy levels can also be seen in the unnormalized peak alignments shown in Figure 2.6C near eight selected representative Sko1 target genes, including those that show Sko1 recruitment (*GPD1*, *STL1*, *MPC3*, and *ALD3*), release of Sko1 (*GRE2*, *FSH1*), or no observable change in Sko1 occupancy in response to NaCl treatment (*PRR2*, *SED1*). To validate these results, we performed independent ChIP experiments followed by qPCR analysis of the promoter regions of the eight representative Sko1 target genes. Indeed, most genes showed statistically significantly higher levels of Sko1-MT compared with Sko1-WT in untreated and NaCl-treated samples (2S4B Fig). When averaged across all eight genes, Sko1-MT occupancy was ~1.4 times greater than Sko1-WT for untreated samples, and ~2.6 times greater in the +NaCl samples (Figure 2.6D). Taken together, both our ChIP-seq and ChIP-qPCR experiments strongly indicate that sumoylation-deficient Sko1 shows increased occupancy at the majority of its binding sites and suggest that sumoylation decreases the binding affinity of Sko1.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P < 10-78
Mean read concentration
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WT
MT
No treatment
+NaCl
B
-10 0 10
Fold change (log2)
Sko1-WT
Sko1-MT
No treatment
+NaCl
No treatment
Sko1-WT
Sko1-MT
C
GPD1
STL1
PRR2
GRE2
MPC3
SED1
ALD3
FSH1
No treatment
+NaCl
Sko1-WT
Sko1-MT
DIA3
NOP6
FRE7
YAP1802
RAD55
ALD2
YHK8
D
No treatment
+ NaCl
Relative Occupancy
SKO1-WT
sko1-MT
Average Relative Sko1.HA Occupancy
E
SKO1-WT
sko1-MT
Recruitment level
Hog1.Myc Recruitment
F
Relative Recruitment
Average Relative Hog1.Myc Recruitment

Figure 2.6: Differential binding analysis indicates that Sko1-MT has higher occupancy levels than Sko1-WT. (A) Boxplots comparing mean read concentrations (log2 normalized ChIP read counts) for the 630 consensus peaks in each of the four indicated ChIP-seq analyses. **(B)** At left, heatmap of differential binding analysis showing changes in occupancy levels (fold change; log2) in Sko1-WT or Sko1-MT after treatment with NaCl, for the 630 consensus peaks. Yellow indicates increased binding after NaCl, whereas blue indicates reduced binding. At right, heatmap of differential binding analysis comparing occupancy levels in Sko1-WT with Sko1-MT in untreated or NaCl-treated samples, at the 630 consensus peaks. Yellow indicates higher occupancy levels for Sko1-MT, whereas blue indicates higher occupancy for Sko1-WT. In all cases, black indicates no significant difference in occupancy level. Each heatmap is independently sorted by magnitude of occupancy level difference. **(C)** Sko1 occupancy peaks were aligned to the yeast genome and eight representative peak alignments are shown for the four samples analyzed from Replicate 1, as plotted using the Integrative Genomics Viewer (IGV). Numbers in square brackets refer to maximum data range (of sequence reads) for the tracks in each column. (D) Validation of ChIP-seq was performed at eight genes by qPCR analysis of five independent ChIP experiments (as summarized in 2S4B Fig). The average occupancy of Sko1-MT, relative to Sko1-WT occupancy, across all tested genes and replicates, is shown, in untreated samples (top), and in samples treated with 0.4M NaCl for 5 min (bottom). Error bars represent standard deviation. **(E)** Recruitment level of Hog1.Myc, as determined by ChIP analysis (see 2S6 Figure), at indicated promoter regions in *SKO1-WT* and *sko1-MT* strains. Recruitment level was calculated as the ratio of occupancy at 5 min to occupancy at 0 min after treatment with NaCl. Error bars represent standard deviation of three independent replicates. **(F)** Average Hog1.Myc recruitment level in *sko1-MT* relative to its level in *SKO1-WT*. The level of Hog1.Myc recruitment in *sko1-MT*, calculated relative to recruitment in *SKO1-WT*, was averaged across all seven Hog1 target genes tested by ChIP-qPCR (panel *(E)* and 2S6 Fig). Error bars represent standard deviation for three replicates across all seven genes. P-values from Student's *t*-test analysis are indicated. Asterisks (*) indicate that the compared data pairs are statistically different ($P < 0.05$; see Materials and Methods).

Blocking Sko1 sumoylation reduces Hog1 recruitment to target promoters

To explore the consequences of increased occupancy of Sko1-MT compared to Sko1-WT, we examined expression levels of some Sko1 target genes in *SKO1-WT* and *sko1-MT* cells, in both normal growth conditions and at various time points after treatment with NaCl. Blocking Sko1 sumoylation (*sko1-MT*) resulted in reduced RNA levels of one gene, *FSH1*, both prior to and after NaCl treatment, but had no significant effect on expression of the inducible genes *STL1*, *PRR2*, or *MPC3* (2S5A Figure), suggesting that the consequences of Sko1 sumoylation on target gene expression are gene-specific. We also determined the mRNA levels of a selection of genes that were not bound by Sko1-WT but were bound by Sko1-MT in our ChIP-seq analyses. For all eight of these genes, there was no significant difference in expression level in the *SKO1-WT* and *sko1-MT* strains, both under normal conditions and 10 min after treatment with NaCl (2S5B Figure). This indicates that binding of Sko1-MT alone is not sufficient to significantly alter the expression of genes that it does not normally regulate.

We then examined whether Sko1 sumoylation can influence the recruitment of Hog1, which associates with many Sko1 target genes during osmotic stress [47, 50]. Strains expressing a 3xMyc-tagged version of Hog1 were generated in the *SKO1-WT* or *sko1-MT* backgrounds, and we performed Myc ChIP analysis over a NaCl-treatment time-course. The analysis showed robust but transient recruitment of Hog1 to seven of its known target genes in *SKO1-WT* cells after addition of NaCl, but not to *FSH1*, which is not regulated by Hog1 (2S6 Figure). Two genes, *STL1* and *PRR2*, showed significantly reduced levels of Hog1 occupancy in the *sko1-MT* strain at 5 min after addition of NaCl. More significantly, however, in the *sko1-MT* strain, recruitment of Hog1, which we calculated as the ratio of occupancy at 5 min to 0 min post NaCl treatment, showed a

statistically significant reduction at four of the seven genes (Figures 2.6E and 2S6). Indeed, when the average recruitment level was calculated across all seven genes, *sko1-MT* cells showed only 50% as much Hog1 recruitment as in *SKO1-WT* cells (Figure 2.6F). These observations suggest that sumoylation prevents excessive binding of Sko1 at target sites, and that elevated Sko1 binding affects expression of some genes and can significantly impair the recruitment of Hog1.

2.5 DISCUSSION

SUMO is a predominantly nuclear modifier that targets a large number of chromatin-associated proteins, including chromatin remodelers, general and sequence-specific transcription factors, and proteins involved in mRNA transport and processing [3, 4, 19, 61]. Potentially then, the expression of numerous genes can be controlled by the cumulative effects of sumoylation of multiple substrates. Unlike ubiquitination, sumoylation in yeast and mammals involves a single E2 conjugating enzyme and a small number of E3 ligases and proteases [1]. This suggests that regulating the activity of only one enzyme in the SUMO pathway, such as Ubc9, can have widespread consequences for the cell since so many substrates may be coordinately affected. This scale of vast protein regulation, particularly on chromatin, might be necessary for achieving a specific goal, such as the dramatic refocusing of the transcription machinery to specific genes after exposure to heat stress [62]. However, unstressed cells show substantial levels of sumoylation and SUMO modification of numerous chromatin-associated proteins is constitutive, implying that this modification functions primarily in the mechanisms of gene expression under normal growth conditions [3, 8]. Our analysis of Sko1 sumoylation supports this. Although osmotic stress resulted in the redistribution of Sko1 across the genome, its sumoylation level did not change, and blocking its sumoylation increased the number of its binding sites both in the presence or absence of NaCl stress.

Altogether, our data point to a role for SUMO modification in preventing Sko1 from binding to nontarget sites, genome-wide, which supports a novel function for the modification. Intriguingly, nonspecific sites bound by sumoylation-deficient Sko1 are not randomly distributed across the genome but are mostly situated near gene promoters that contain Sko1 binding site-like sequences. This might reflect the intrinsic accessibility of chromatin around promoters and the frequent occurrence of such sequences in many promoter regions [63]. Indeed, binding motifs for Sko1 and the related TFs Aca1 and Cst6 are highly similar, and it has been proposed that these TFs compete with each other for binding to promoter elements [40, 45, 64]. Based on the results of our study, we propose a model in which unbound Sko1 has high affinity for sequences that generally resemble the Sko1 motif. This might be necessary to ensure that all functional Sko1 target sites become occupied by the TF, even though nonspecific sites are initially bound, as well. Our model then posits that subsequent sumoylation of Sko1 relaxes its interaction with bound DNA, resulting in release from inoptimally bound nonspecific sites but allows sustained association with actual Sko1-regulated genes. Key to this model is that sumoylation takes place only after Sko1 has bound DNA, which is supported by our finding that DNA binding mutations eliminate Sko1 sumoylation. Similarly, DNA binding mutations have been shown to prevent sumoylation of other TFs such as Gcn4 and Ikaros, as well as non-TF DNA-binding proteins, including yeast Yku70, human TDG and viral ULFF, which are involved in DNA damage repair [28, 65, 66]. This suggests that the sumoylation apparatus can distinguish between DNA-bound and unbound forms of many chromatin-associated proteins, possibly as a result of conformational changes that may occur after binding DNA, or due to the proximity of the sumoylation machinery with chromatin. Supporting this, ChIP analyses have shown that Ubc9 is associated with chromatin, including promoter regions

of transcriptionally activated genes [14, 15]. Further studies are necessary to test our model and explore the consequences of Sko1 sumoylation on its structure and DNA binding affinity.

Consistent with the idea that sumoylation reduces Sko1 DNA binding affinity, blocking Sko1 sumoylation also resulted in increased occupancy levels at most of its target genes. However, not all target genes showed altered expression in the *sko1-MT* strain. The increase in Sko1 levels at these sites might not have been sufficiently dramatic to noticeably alter their gene expression levels, but this supports the previous finding that Sko1 occupancy levels on target genes generally do not correlate with their expression levels [49]. Instead, its context dependent roles involve regulating the recruitment or release of repressors, such as Tup1/Ssn6, chromatin remodelers SAGA and SWI/SNF, and the kinase Hog1 [44, 47, 50]. Hog1 is activated in response to osmotic stress which triggers its nuclear localization and association with promoters of osmo-regulated genes where it phosphorylates a number of transcription-related substrates, including Sko1 and RNAP II, resulting in efficient induction of the genes [67, 68]. Whereas Sko1 itself is required for the recruitment of Hog1 to at least some target genes during osmotic stress [47, 50], our results suggest that increased binding of Sko1 in *sko1-MT* cells generally hinders the recruitment of Hog1 to its targets. Increased Sko1 binding in these cells might inhibit promoter-associated rearrangements that are necessary for induction of osmoregulated genes [47], or Sko1 sumoylation itself might stimulate the recruitment of Hog1 through unknown mechanisms. In any case, our results point to a role for Sko1 sumoylation in controlling its association with chromatin not only to ensure binding site specificity, but also to prevent excessive binding at its authentic target sites.

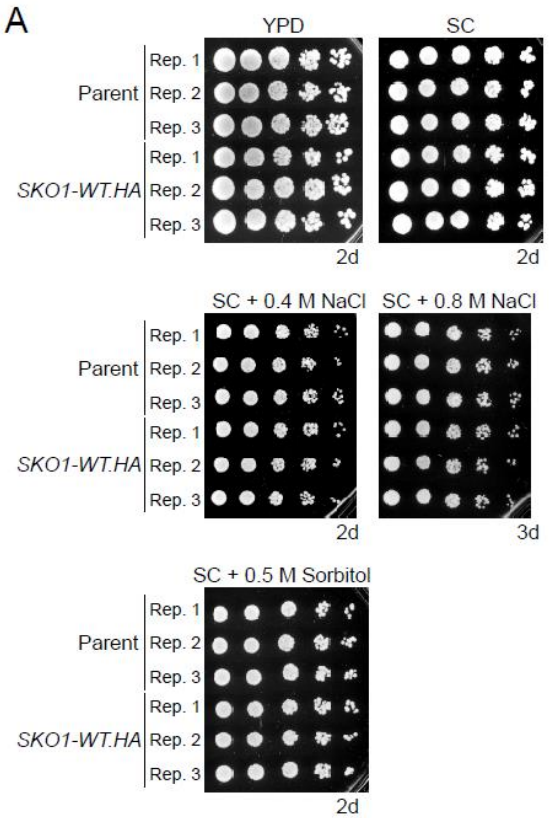
Few studies to date have examined the role of sumoylation on the genome-wide occupancy TFs, and, when considered with our results, the conclusions of these studies strongly point to a

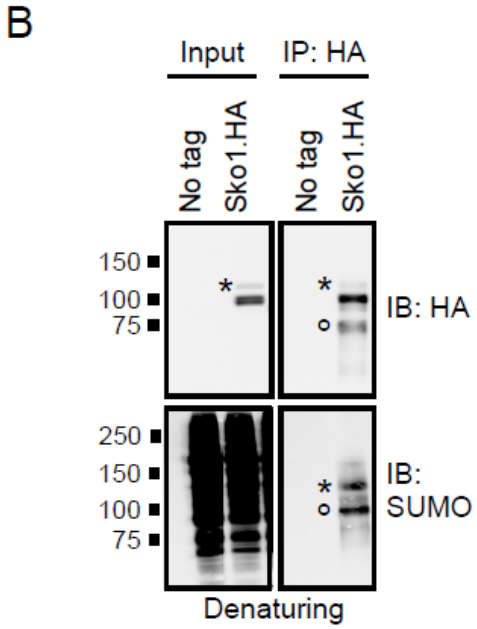
conserved role for sumoylation in regulating TF binding specificity. In the first such study, it was reported that a germline mutation in the human TF MITF that is associated with increased coincidence of melanoma and renal cancer also significantly reduces sumoylation of the protein [30]. ChIP-seq analysis was performed in human melanoma cell lines expressing either WT MITF or the sumoylation-deficient form, MITF-E318K. As in our analysis with Sko1, dramatically more sites across the genome were bound by the sumoylation-deficient form of MITF, and occupancy levels were higher compared with WT MITF at sites that were bound by both the mutant and WT proteins. Recent ChIP-seq studies of the glucocorticoid and androgen nuclear receptors again showed that SUMO-site mutation led to altered genome occupancy patterns for both TFs, with the mutant proteins binding to significantly more sites than the wild-type counterparts [32,33]. The parallel observations of these analyses of different TFs in evolutionarily distant organisms strongly suggests a common function for sumoylation across eukaryotes. Considering the vast number of eukaryotic TFs that have been reported as SUMO targets and the close association of the modification with chromatin, we anticipate that future additional genome-wide studies will reveal that, indeed, a major role for sumoylation is in regulating the association of DNA-bound TFs with chromatin in order to restrict their activity to appropriate target genes.

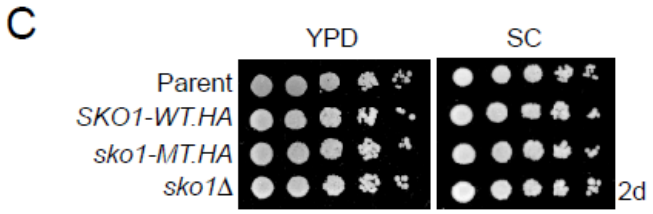
2.6 ACKNOWLEDGMENTS

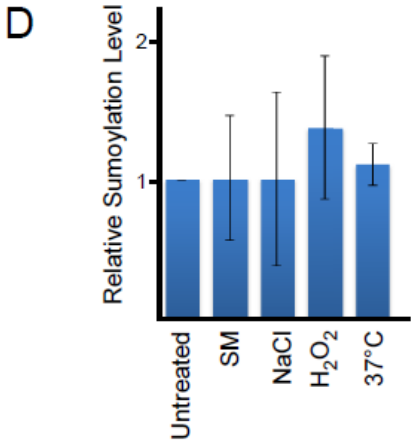
We are grateful to Jennifer Haynes and J. Bryan McNeil for their insightful comments and critical review of the manuscript and to Jeffrey Fillingham for technical advice.

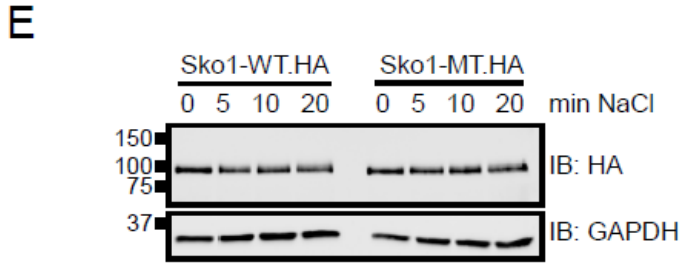
2.7 SUPPLEMENTARY DATA











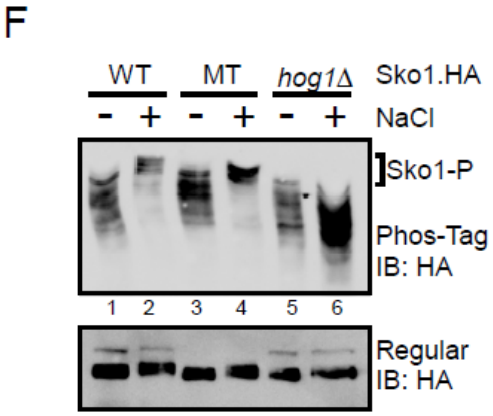


Figure 2 S 1: (A) C-terminal 6xHA tag on Sko1 does not affect cell growth in normal or osmotic conditions. Spot assays (as in Figure 2.1F) in which indicated yeast strains were grown in triplicate on rich (YPD) or synthetic complete (SC) medium, or supplemented with indicated levels of NaCl or sorbitol. Plates were photographed after two or three days, as indicated. **(B) Detection of Sko1 sumoylation in lysates prepared under denaturing conditions.** IP-immunoblot analysis was performed with protein samples prepared under denaturing conditions (TCA precipitation). Asterisks (*) indicate position of the major (mono-) sumoylated form of Sko1 in each immunoblot. Open circles (◦) indicate position of putative Sko1 degradation products, detectable in both HA and SUMO immunoblots. **(C) A yeast strain expressing sumoylation deficient Sko1 shows no growth defect.** Spot assays in which growth of indicated yeast strains were compared on rich (YPD) or synthetic complete (SC) medium, as in Figure 2.1F. Growth was for two days (2d). **(D) Sko1 sumoylation levels are unaffected by stress.** Relative Sko1 sumoylation levels were quantified after IP-immunoblot analyses as in Figure 2.1E by dividing Sko1 SUMO signals by the Sko1-HA signals in the respective blots. Data is presented relative to the untreated sample, with error bars indicating standard deviation of three experiments. By Student's *t*-test, there is no significant statistical difference among the samples. **(E) Blocking Sko1 sumoylation does not affect its abundance.** HA and GAPDH immunoblot analysis of lysates from *SKO1-WT.HA* or *sko1-MT.HA* strains grown in SC medium treated with 0.4M NaCl for indicated times. Sumoylated forms of Sko1.WT cannot be seen in this short exposure. **(F) Blocking Sko1 sumoylation does not prevent its Hog1-mediated phosphorylation.** HA immunoblot analysis, as in Figure 2.2B, using Phos-Tag acrylamide to enhance detection of phosphorylated forms of Sko1.HA, indicated as "Sko1-P." A strain lacking *HOG1* and expressing Sko1.HA was included as a control. Analysis using standard SDS-PAGE analysis is shown at bottom.

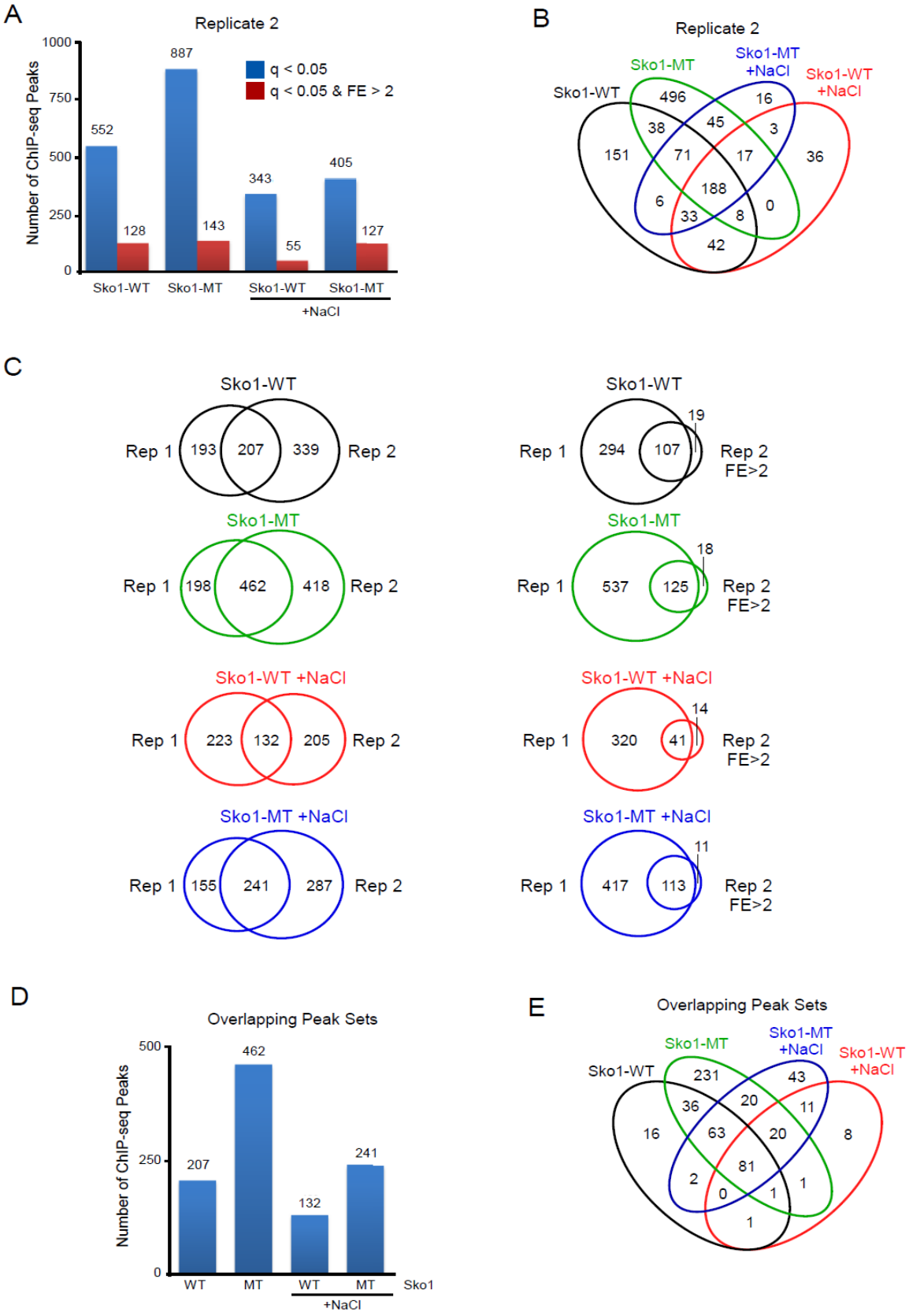
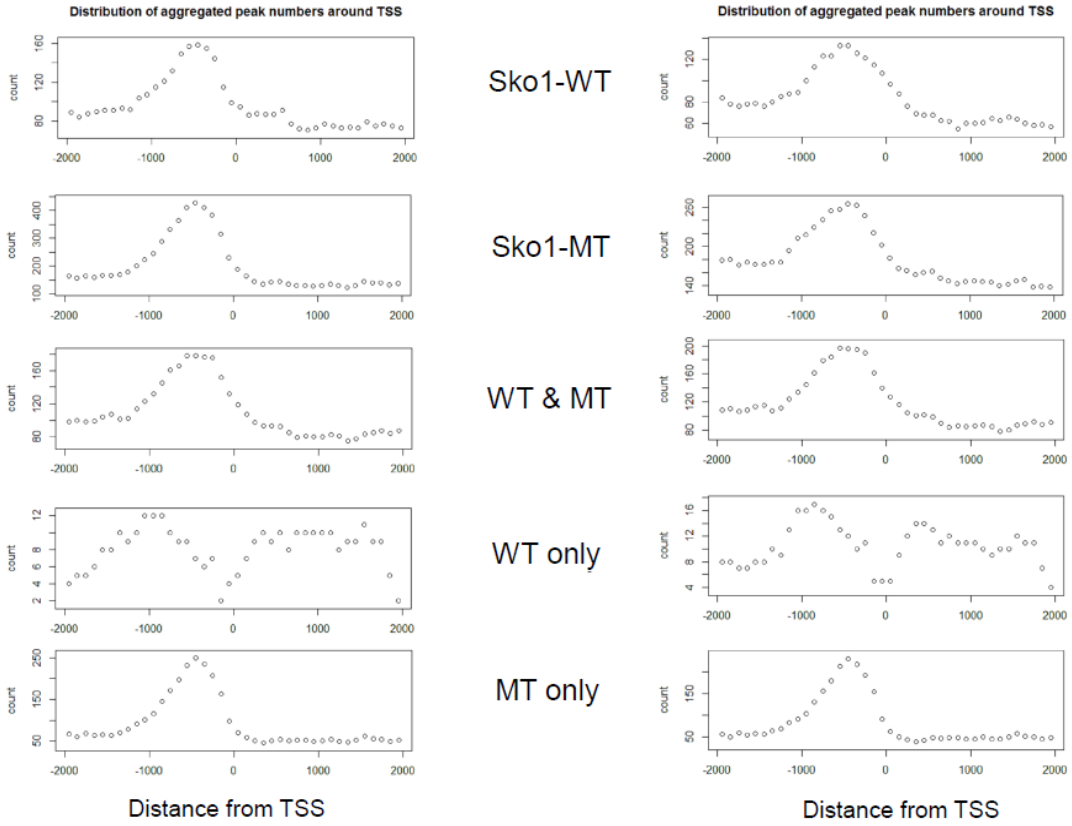

A
Replicate 2
Number of ChIP-seq Peaks
1000
750
500
250
0
q < 0.05
q < 0.05 & FE > 2
552
128
887
143
343
55
405
127
Sko1-WT
Sko1-MT
Sko1-WT
Sko1-MT
+NaCl
B
Replicate 2
Sko1-WT
Sko1-MT
Sko1-MT +NaCl
Sko1-WT +NaCl
496
16
38
45
3
151
71
17
36
188
6
33
8
0
42
C
Sko1-WT
Rep 1
193
207
339
Rep 2
Sko1-WT
Rep 1
294
107
19
Rep 2 FE>2
Sko1-MT
Rep 1
198
462
418
Rep 2
Sko1-MT
Rep 1
537
125
18
Rep 2 FE>2
Sko1-WT +NaCl
Rep 1
223
132
205
Rep 2
Sko1-WT +NaCl
Rep 1
320
41
14
Rep 2 FE>2
Sko1-MT +NaCl
Rep 1
155
241
287
Rep 2
Sko1-MT +NaCl
Rep 1
417
113
11
Rep 2 FE>2
D
Overlapping Peak Sets
Number of ChIP-seq Peaks
500
250
0
207
462
132
241
WT
MT
WT
MT
Sko1
+NaCl
E
Overlapping Peak Sets
Sko1-WT
Sko1-MT
Sko1-MT +NaCl
Sko1-WT +NaCl
231
43
36
20
11
16
63
20
8
81
2
0
1
1
1

Figure 2 S 2: Binding site analysis of Sko1-WT and Sko1-MT ChIP-seq experiment for Replicate 2 and for peaks overlapping in both replicates. (A) Number of binding sites (peaks) identified from Replicate 2 ChIP-seq analysis of *SKO1-WT.HA* and *sko1-MT.HA* strains, either untreated or treated with 0.4M NaCl for 5 min, with a q-value less than 0.05 (blue bars). Subset of peaks having a *q*-value less than 0.05 and a fold enrichment (FE) value greater than 2 are also indicated (red bars). **(B)** Venn diagram, as in Figure 2.3B, showing number of peaks ($q < 0.05$) shared among the four samples in Replicate 2. **(C)** Venn diagrams indicating numbers of peaks identified in both Replicate 1 and 2, for each of the four samples. Peaks found in both replicates (i.e. intersects) for each sample constitute the "Overlapping Peak Sets." At right, similar analysis comparing peaks from Replicate 1 and the subset of peaks from Replicate 2 that have an FE greater than 2. All analyzed peaks have *q*-values less than 0.05. **(D)** Number of binding sites for each of four samples in the overlapping peak sets, which includes only peaks identified in both replicates (as indicated in *(C)* left). **(E)** Venn diagram showing number of common and unique peaks in the overlapping peak sets from the four samples.

A

No Treatment

+NaCl



B

No Treatment

WT & MT

E value = 1.9e-207
Sites: 172 (of 185)

MT only

E value = 6.0e-062
Sites: 150 (of 277)

+NaCl

WT & MT

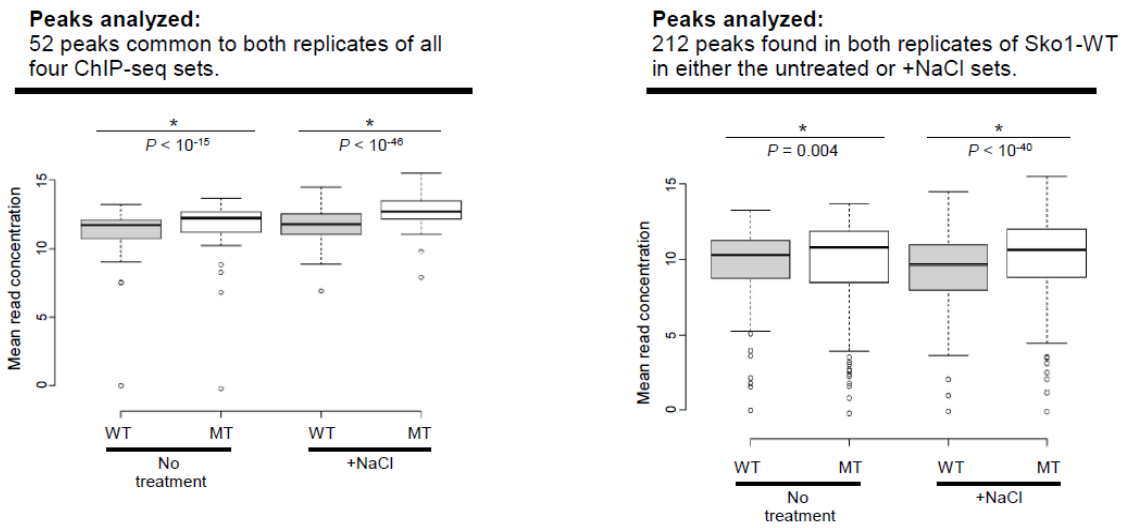
E value = 4.0e-087
Sites: 82 (of 111)

MT only

E value = 4.0e-079
Sites: 83 (of 129)

Figure 2 S 3: Distribution and motif analysis of ChIP-seq peak sets. (A) Distribution of aggregate ChIP-seq peak numbers (for overlapping peak sets) around the nearest transcriptional start site (TSS) for indicated peak sets in untreated and NaCl-treated samples. **(B)** De novo motif discovery was performed, using the MEME analysis tool, for "WT & MT" and "MT only" peak sets, in untreated and NaCl-treated samples. Only one significant motif was identified for each of the untreated and +NaCl MT-only peak sets (with an *E*-value less than 1e-005), and the most significant motifs for the WT & MT peak sets are shown (additional motifs discovered for the WT & MT set have *E*-values greater than 1e-32 and are present in fewer than 40 peaks). Number of peaks contributing to the motif ("Sites") is indicated in each case.

A


Peaks analyzed:
52 peaks common to both replicates of all four ChIP-seq sets.
*
P < 10-15
*
P < 10-46
Mean read concentration
0
5
10
15
WT
MT
WT
MT
No treatment
+NaCl
Peaks analyzed:
212 peaks found in both replicates of Sko1-WT in either the untreated or +NaCl sets.
*
P = 0.004
*
P < 10-40
Mean read concentration
0
5
10
15
WT
MT
WT
MT
No treatment
+NaCl

B

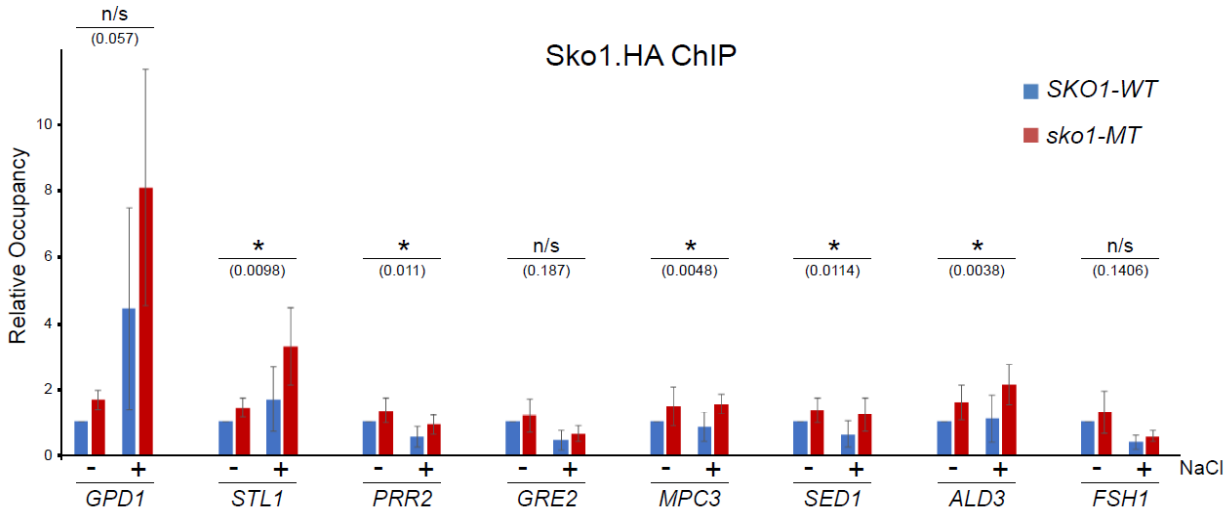

Sko1.HA ChIP
SKO1-WT
sko1-MT
Relative Occupancy
0
2
4
6
8
10
n/s
(0.057)
*
(0.0098)
*
(0.011)
n/s
(0.187)
*
(0.0048)
*
(0.0114)
*
(0.0038)
n/s
(0.1406)
- +
NaCl
GPD1
STL1
PRR2
GRE2
MPC3
SED1
ALD3
FSH1

Figure 2 S 4: (A) Differential binding analysis of different groups of peaks. Boxplots comparing mean read concentrations (log2 normalized ChIP read counts) in each of the four indicated ChIP-seq analyses for the 52 peaks that are found in both replicates of all four ChIP-seq sets (left) or the 212 peaks that are found in both replicates of the Sko1-WT sets in both untreated and +NaCl conditions (right). **(B) Validation of ChIP-seq analysis.** Five independent standard ChIP experiments were performed with *SKO1-WT* and *sko1-MT* strains. Sko1.HA occupancy levels at promoter regions of eight representative genes were determined by qPCR, at 0 or 5 min after the addition of 0.4MNaCl. For each gene, occupancy is shown relative to Sko1-WT in untreated samples. Error bars represent standard deviations. *P*-values from twofactor ANOVA analysis of WT vs MT sets for each gene are shown. Asterisks (*) indicate that the two data sets (WT and MT) are statistically different ($P < 0.05$; see *Materials and Methods*).

qRT-PCR

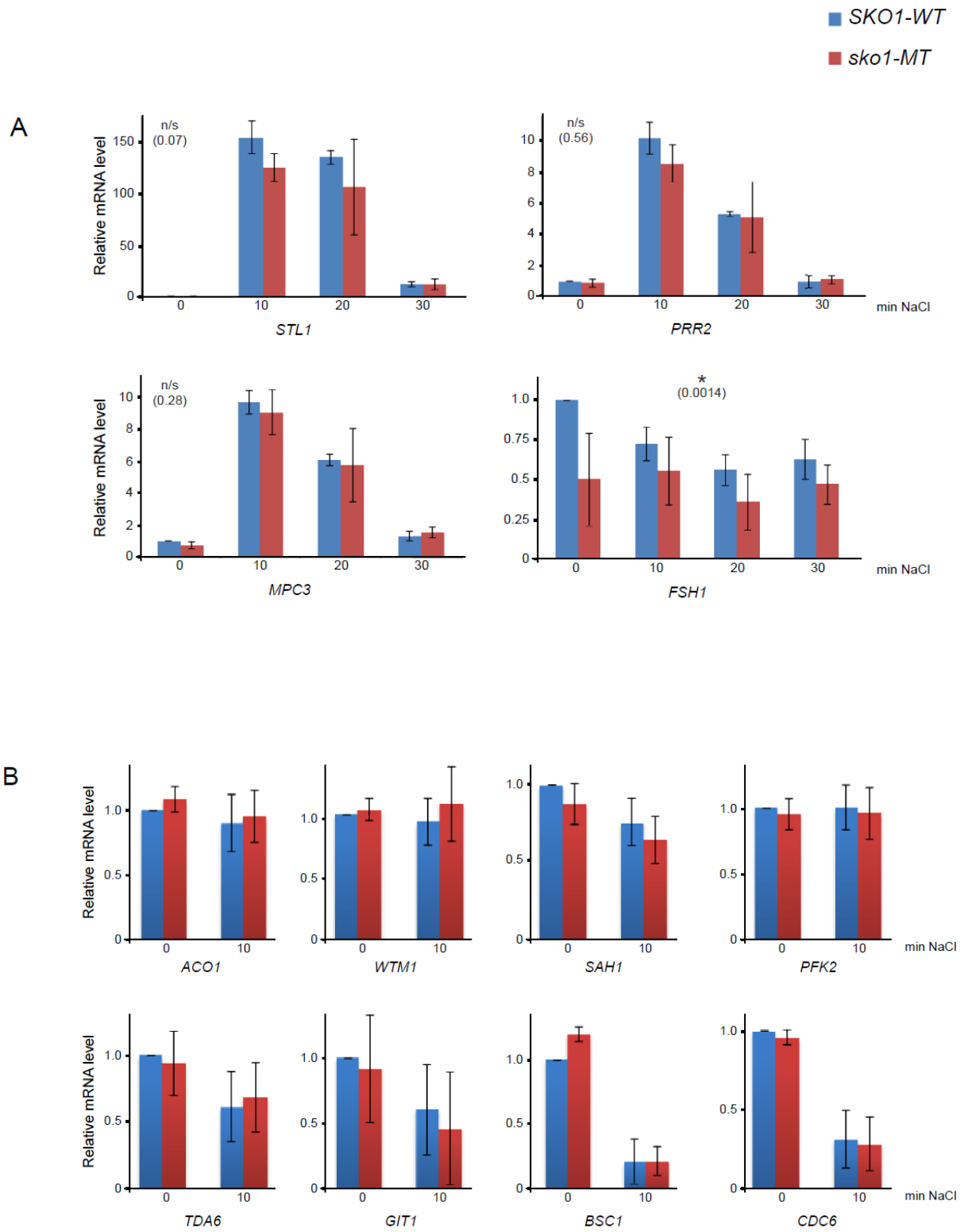

SKO1-WT
sko1-MT
A
Relative mRNA level
n/s
(0.07)
STL1
n/s
(0.56)
PRR2
n/s
(0.28)
MPC3
*
(0.0014)
FSH1
min NaCl
B
ACO1
WTM1
SAH1
PFK2
TDA6
GIT1
BSC1
CDC6

Figure 2 S 5: Effects of elevated Sko1 binding on steady-state expression levels of target genes in the sko1-MT strain. **(A)** Quantitative RT-PCR analysis of mRNA levels of indicated representative Sko1-target genes at 0, 10, 20 and 30 min after treatment of *SKO1-WT* or *sko1-MT* cultures with 0.4M NaCl. Error bars represent standard deviations of three independent replicates. *P*-values from two-factor ANOVA analysis of WT vs MT sets for each gene are shown. Asterisks (*) indicate that the two data sets (WT and MT) are statistically different ($P < 0.05$; see *Materials and Methods*). **(B)** Quantitative RT-PCR analysis of mRNA levels of a selection of genes that are bound by Sko1-MT, but not Sko1-WT, at 0 and 10 min after treatment of *SKO1-WT* or *sko1-MT* strains with 0.4MNaCl. Statistical analysis indicates no significant difference between WT and MT sets. Error bars represent standard deviations of four independent replicates.

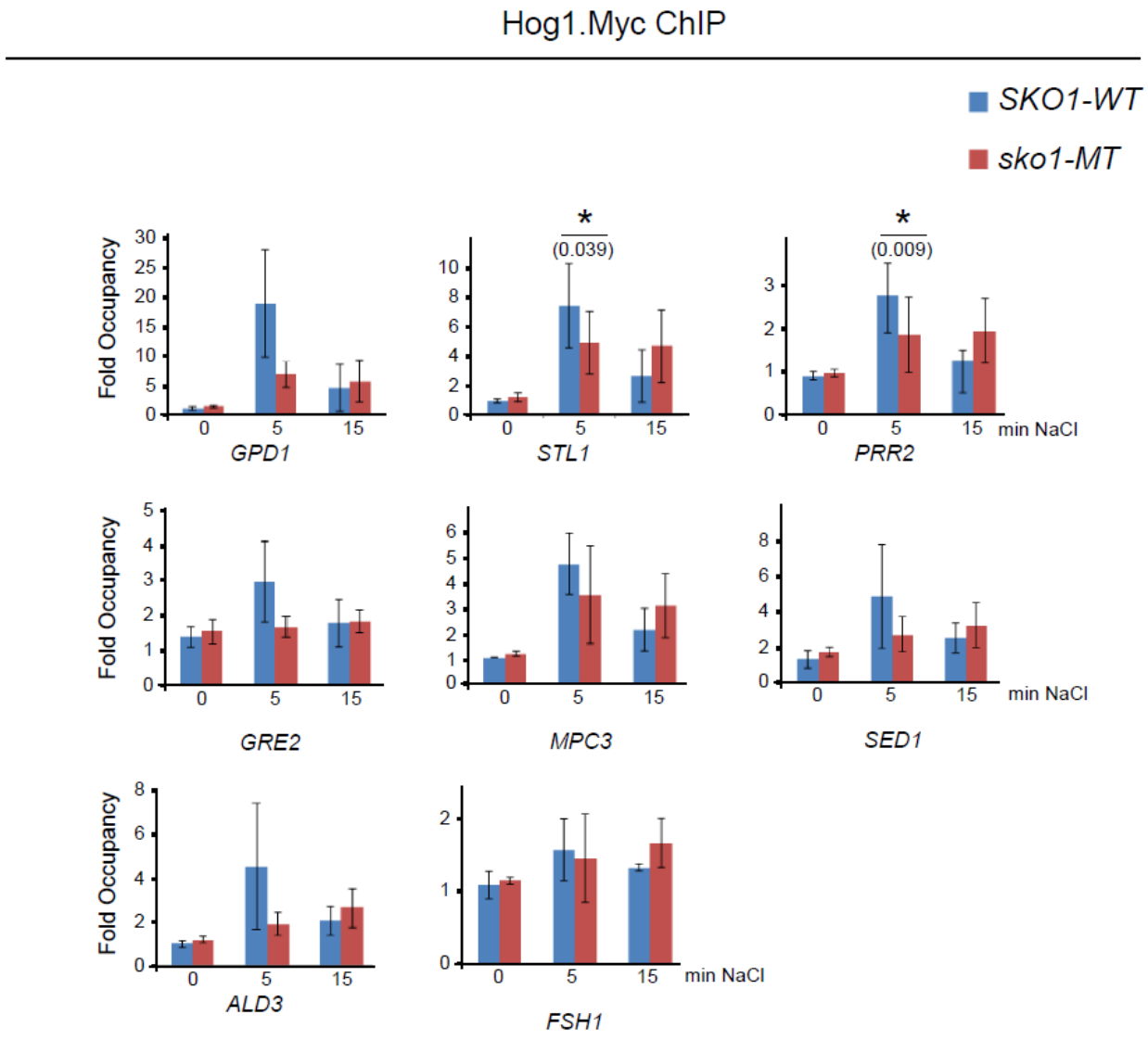


Figure 2 S 6: Effects of blocking Sko1 sumoylation on recruitment of Hog1 to target genes during osmotic stress. ChIP-qPCR analysis of Hog1.Myc occupancy at indicated genes in *SKO1-WT* and *sko1-MT* strains at 0, 5, or 15 min after treatment with NaCl. Data are represented as fold occupancy (relative to occupancy at the *PMA1* locus which is not targeted by Hog1 or Sko1). Error bars represent standard deviations of three independent replicates. Asterisks (*) indicate that the compared data pairs are statistically different ($P < 0.05$; see *Materials and Methods*). Statistical comparison of Hog1.Myc recruitment is shown in Figure 2.6E.

Table 2 S 1: Yeast strains used in this study

Strain	Parental	Genotype
Background Strain		
W303a		*MAT a ura3-52 trp1Δ2 leu2-3_112 his3-11 ade2-1 can1-100*
HF7c		*MAT a ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, LYS2::GAL1UAS-GAL1TATA-HIS3, URA3::(GAL 17mers) 3-Cyc1TATA-lacZ*
Derived Strains		
YVS003C	W303a	*SKO1-6HA::kl TRP1*
YVS007K	W303a	*sko1-K567R-6HA::KlTRP1*
YVS008A	W303a	*sko1Δ::kanMX*
YVS035B	W303a	*sko1-K450E-6HA:: KlTRP*
YVS036B	W303a	*sko1-Δ(445-456)-6HA:: KlTRP*
YVS052A	HF7c	*[pGAL4DBD-Sko1-K450E-6HA]*
YVS047B	W303a	*sko1-S108,126A,T113A-6HA:: KlTRP*
YVS051E	W303a	*sko1-S380,393,399A-6HA:: KlTRP*
YVS041A	YVS003C	*SKO1-6HA::KlTRP hog1 Δ:: KanMX*
YVS011A	YVS003C	*SKO1-6HA:KlTRP1 HOG1-3MYC::KanMX6*
YVS021A	YVS007K	*sko1-K567R-6HA::KlTRP1 TUP1-3MYC::KanMX6*

Table 2 S 2: Oligonucleotide primers used in this study.

Gene	Oligonucleotide sequence(s)
Primers for quantitative PCR analysis of ChIP samples	
RTC3 promoter	Forward: 5′- AAGATTTCCCGTTGCGCTAT -3′
	Reverse: 5′- GGAGAAGAGACACGGAGTAGGA -3′
ENA1 promoter	Forward: 5′- GGTCTTAAACATCGCCGTGC -3′
	Reverse: 5′- CCCTGCCCTAACAAACGTCA-3′
PMA1 promoter	Forward: 5′- CAATTATGACCGGTGACGAAAC -3′
	Reverse: 5′- AATCGAAACTAATGGAGGGGAG -3′
GPD1 promoter	Forward: 5′-CCCACCCACACCACCAATAC -3′
	Reverse: 5′-CCCATTCTGATACTTGTTGTGC -3′
STL1 promoter	Forward: 5′-TTGTCCCACTATTCCACCGC -3′
	Reverse: 5′-GGACAAAGTCGGACCCTTCA -3′
PRR2 promoter	Forward: 5′-ATCGGAGCTACTTTTCCGCA -3′
	Reverse: 5′-CCCCAATATGCTAACAGCCG -3′
GRE2 promoter	Forward: 5′-AACAATTGGCCCTCACCTCT -3′
	Reverse: 5′-ACTTCCGCGAGAAAATTCCGTA -3′
MPC3 promoter	Forward: 5′-CCCGCTTTTATTTCTCCCGC -3′
	Reverse: 5′-GAGCCTTTCGGTTTTGCGTG -3′
SED1 promoter	Forward: 5′-ACCACTGATTGCTCCACGTC -3′
	Reverse: 5′-AATGACCGTGTGTGCTCTGG -3′
ALD3 promoter	Forward: 5′-CTGCATATGACGTCTGTTCTTC -3′
	Reverse: 5′-AAATGCACTAAAGGGCGTGG -3′
FSH1 promoter	Forward: 5′-CGCCGTATGCATGGGATGAT -3′
	Reverse: 5′-TCTTTGGCCTATGCGTGTTG -3′
Primers for quantitative RT-PCR analysis	
STL1	Forward: 5′-TGAAACCGCCGGAAGAAGTT -3′
	Reverse: 5′-CCATGGTTGAGTGCCATCCT -3′
PRR2	Forward: 5′-CACCAGGAAAGCACAGTTGC -3′
	Reverse: 5′-TGATCCAGTTGAGACTGGCG -3′
MPC3	Forward: 5′-CCAACTTTGAAGTGGGGGCT -3′
	Reverse: 5′-TGATGACAAACGACCAACGC -3′
FSH1	Forward: 5′-GGTGCCGCATTGTCCTCTAT -3′
	Reverse: 5′-CCAGGATGTTCTGGGTCTGG -3′
ACO1	Forward: 5′-TGGTGTTGACACCTTCTCCG -3′
	Reverse: 5′-TACCACGACCAGTTGCTTCC -3′
WTM1	Forward: 5′-TGAAGCTGCCACCACTGATT -3′
	Reverse: 5′-GTCGACAACAGAATCACCGC -3′
SAH1	Forward: 5′-CGGTGATGTCGGTAAGGGTT -3′
	Reverse: 5′-TCGGTAACCAAGACACGAGC -3′

PFK2	Forward: 5′-CCGCTTCTTCAACCAGAGGT -3′
	Reverse: 5′-TGAGCACCAACAGCCAAAGA -3′
TDA6	Forward: 5′-CGGAAACGCATATCCCCAGT -3′
	Reverse: 5′-GCGCCTTAGAGTTGTAGCCT -3′
GIT1	Forward: 5′-AGCAACCGCTGTTAGAGGTG -3′
	Reverse: 5′-GGGTTGGAAACATTCGACGC -3′
BSC1	Forward: 5′-CTGACGGTTGCACAGTTTGG -3′
	Reverse: 5′-AATCGAAGGTGGTTGTCCCC -3′
CDC6	Forward: 5′-ATGGCCATCAAATTCGCAGC -3′
	Reverse: 5′-AGGTCACCCGTATTTCCAGC -3′
25S	Forward: 5′- TCTAGCATTCAAGGTCCCATTC -3′
	Reverse: 5′- CCCTTAGGACATCTGCGTTATC -3′

Table 2 S 3: List of peaks identified in ChIP-seq peak analysis for each of the four samples over two replicates (Replicates 1 and 2). (XLSX file)
https://doi.org/10.1371/journal.pgen.1007991.s009

Table 2 S 4: Annotated list of peaks that are present in both replicates for each sample/analysis ("Overlapping Peak Sets"). (XLSX file)
https://doi.org/10.1371/journal.pgen.1007991.s010

Table 2 S 5: Differential binding analysis (performed with DiffBind) at consensus peaks for pair-wise comparisons of ChIP-seq data sets. Data relates to Figure 2.6. (XLSX file)
https://doi.org/10.1371/journal.pgen.1007991.s011

Table 2 S 6: Numerical values for graphical data presented throughout, listed by figure number as separate sheets/tabs. (XLSX file)
https://doi.org/10.1371/journal.pgen.1007991.s012

CHAPTER 3

ETHANOL STRESS STIMULATES SUMOYLATION OF TRANSCRIPTION FACTOR CST6 WHICH RESTRICTS EXPRESSION OF ITS TARGET GENES

Veroni S. Sri Theivakadadcham[1], and Emanuel Rosonina[1]

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[1]Department of Biology, York University, Toronto, Ontario, Canada

Author Contributions

Project planning and experimental design: V.S.S and E.R.

Experiments and data acquisition: V.S.S. performed all experiments shown.

Data analysis: Data was analyzed by V.S.S. with assistance from E.R.

Writing – original draft: V.S.S. and E.R.

Writing – review and editing: V.S.S. and E.R.

3.1 ABSTRACT

Sumoylation is an essential post-translational modification that functions in multiple cellular processes, including transcriptional regulation. Indeed, transcription factors represent one of the largest groups of proteins that are modified by the SUMO peptide. Multiple roles have been identified for sumoylation of transcription factors, including regulation of their activity, interaction with chromatin, and binding site selection. Here, we examine how Cst6, a bZIP-containing sequence-specific transcription factor in *Saccharomyces cerevisiae*, is regulated by sumoylation. Cst6 is required for survival during ethanol stress and has roles in the utilization of carbon sources other than glucose. We find that Cst6 is sumoylated to appreciable levels in normally growing yeast at Lys residues 139, 461 and 547, and that its sumoylation level increases in ethanol and oxidative stress conditions, but decreases if ethanol is used as the sole carbon source. To understand the role of Cst6 sumoylation during ethanol stress, we generated a yeast strain that expresses a non-sumoylatable mutant form of Cst6. Cellular levels of the mutant protein are moderately reduced compared to the wild-type form, implying that sumoylation promotes Cst6 stability. Although the mutant can bind DNA, chromatin immunoprecipitation (ChIP) analysis shows that its occupancy level is significantly reduced on promoters of some ethanol stress-regulated genes, suggesting that Cst6 recruitment is attenuated or delayed if it can not be sumoylated. Furthermore, impaired Cst6 sumoylation in the mutant strain correlates with elevated expression of some target genes, either constitutively or during induction by ethanol stress. This is most striking for *RPS3*, which shows dramatically increased expression in the mutant strain. Together, our results suggest that sumoylation controls multiple properties of Cst6 to limit the expression of its target genes.

3.2 INTRODUCTION

Transcription factors (TFs) maintain cellular functions by integrating external signal information into gene expression programs that are transmitted through signalling pathways. Often, these signalling pathways orchestrate the function of TFs through post-translational modifications (PTMs) including acetylation, phosphorylation, and ubiquitination [1]. In recent years, regulation of TFs by sumoylation has gained increased attention. Sumoylation, a reversible post-translational modification that plays an essential role in many cellular processes, involves the covalent attachment of a ~12 kDa SUMO (*S*mall *U*biquitin-like *Mo*difier) peptide to specific lysine residues of substrate proteins [2, 3]. Similar to ubiquitination, sumoylation is a dynamic process that requires catalysis by three classes of enzymes: activation of SUMO by an E1 enzyme, conjugation to target proteins by an E2 enzyme (Ubc9), and facilitation of transfer by E3 ligases [4]. In contrast to ubiquitination, in which E3 ubiquitin ligases are the primary determinants of substrate specificity, Ubc9 can directly specify its targets via the SUMO site consensus sequence ΨKxD/E, where Ψ is a hydrophobic residue, K is the lysine to be modified, x is any amino acid, and D/E represents an acidic residue [5]. Sumoylation is a reversible modification, in which SUMO can be removed from substrate proteins by SUMO-specific isopeptidases (SUMO proteases), including a family of sentrin/SUMO-specific proteases (SENPs) in mammals and the Ubl- specific proteases (Ulp1 and Ulp2) in budding yeast [4, 6].

Proteomics studies, in which SUMO-conjugated proteins were isolated and identified through mass spectrometry, have determined that within the sumoylome of yeast and mammalian cells, proteins involved in transcription are among the largest classes of SUMO targets [7-10]. This includes TFs, RNA polymerase II, transcriptional co-regulators and a variety of chromatin regulatory factors. Among these, sequence specific DNA-binding TFs represent the largest group

of SUMO conjugates [11]. In most cases, sumoylation of these TFs has been found to associate with transcriptional repression or deactivation. This can be achieved by recruiting various repressor complexes to chromatin, as for TFs NFAT, Elk-1 and MafG [12-14], or by facilitating their clearance from promoters of induced genes, which is the case for TFs Gcn4 and c-Fos [15,16]. Genome-wide chromatin immunoprecipitation (ChIP) analyses showed that sumoylation-deficient forms of three human TFs, MITF, glucocorticoid receptor, and androgen receptor, occupied far more genomic sites than their wild-type counterparts [17-19]. Similarly, we recently demonstrated that the sumoylation of the budding yeast bZIP TF Sko1 is required to prevent it from binding to numerous non-target promoters [20]. Together, these observation have led to a model in which controlling binding-site specificity has been proposed as a conserved and general role for sumoylation in regulating TFs [21].

Whereas the studies mentioned above demonstrate that sumoylation can promote the dissociation of TFs from DNA at inappropriate genomic sites or during deactivation, for some TFs, sumoylation has been shown to facilitate their association with DNA. For example, sumoylation enhances the DNA-binding ability of Pax-6 and CREB1, thereby positively regulating transcription of their target genes [22, 23]. Furthermore, sumoylated proteins are detected at promoters of transcriptionally active genes, including constitutive and activated inducible genes, indicating that TF sumoylation can function during active transcription [24]. The individual effects of TF sumoylation, therefore, can vary with substrate and context, as sumoylation can function during the initial stages of transcription activation, during active transcription, during the deactivation process, or to promote transcriptional silencing.

To extend our understanding of TF sumoylation, we focused on the budding yeast bZIP TF, Cst6 (*C*hromosome *St*ability-*6*), which was identified as a potential SUMO target in large-scale proteomics studies [10, 25]. Cst6, also known as Aca2, is essential during ethanol stress and in the presence of non-optimal carbon sources [26, 27]. It contains a bZIP DNA binding domain, binds to DNA as a homodimer or a heterodimer with Aca1, another yeast bZIP TF, and it recognises cAMP response element (CRE)-like promoter sequences [27]. A recent study showed that the Cst6 binding site (5′-GTGACGT-3′) has an additional guanine nucleotide at the 5′ end of the consensus CRE motif (5′-TGACGT-3′), implying that it has a somewhat different binding site preference than other bZIP TFs [28]. Through genome-wide binding site analysis, the study also identified 59 protein-coding genes as putative targets for Cst6 regulation. Gene ontology (GO) analysis of these genes revealed that Cst6 controls different cellular processes including transcription, cellular respiration, gluconeogenesis, stress response, and pseudohyphal growth, signifying the importance of its function in budding yeast [28].

In the present study, we demonstrated that Cst6 is multi-sumoylated on three lysine residues (K139, K461, and K547), and that the level of its SUMO modification increases during ethanol and oxidative stress. Whereas the Cst6 sumoylation increases with increased duration and dose of ethanol stress, it is significantly reduced if ethanol is used as the sole carbon source in the medium. We further demonstrate that a mutant, non-sumoylatable form of Cst6 shows reduced or delayed association with its target promoters during ethanol stress. This might be partly explained by a moderate decrease in the abundance of Cst6 in the absence of its sumoylation. Finally, we find that the Cst6 mutant strain shows elevated expression of Cst6 target genes either constitutively, or during exposure to ethanol stress. Collectively, these results suggest that sumoylation enhances Cst6 binding to ethanol-induced target promoters to limit the transcription of its target genes.

3.3 MATERIALS AND METHODS

Yeast strains

All *Saccharomyces cerevisiae* strains used in this study are listed in Table S1. Cst6 was epitope-tagged with a 6xHA tag (along with a *K. lactis TRP1* marker gene) using homologous recombination, as previously described by Knop *et al.*, [29]. PCR-based point mutagenesis was used to generate strains expressing mutant forms of Cst6-HA. The Cst6-deleted strain was constructed using a *KanMX* deletion cassette, and all strains are derived from the W303a background strain.

Media and cultivation

Yeast cultures were grown overnight in either rich (1% yeast extract, 2% peptone and 2% glucose) or Synthetic Complete (SC; 0.17% YNB, and 0.5% ammonium sulfate) medium (containing either 2% glucose or 1% ethanol as carbon source, or as otherwise noted) at 30°C and diluted to an optical density (OD_{595nm}) of 0.2. Strains were then cultivated on a rotary shaker at 200 rpm until the OD reached 0.5-0.7 at which point, where appropriate, stressors were added directly to the culture as indicated in the figure captions or legends. Cells were then harvested by centrifugation at 3000 *g* for 5 min, followed by a wash with an experiment-specific buffer. For growth assays on agar plates ("spot assays"), approximately 10,000 cells of each strain were spotted side-by-side in the first position, and five-fold serial dilutions were spotted on adjacent positions, on plates with or without indicated stress conditions. Plates were incubated at 30°C and images were recorded daily for up to three days.

Preparation of yeast lysate and Immunoprecipitation (IP)

Yeast cultures (30-40 mL) were grown in appropriate liquid medium to an OD_{595nm} of 0.5-0.7. Cultures were then treated with stress conditions, if appropriate, and harvested by centrifugation, followed by a wash with IP buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 2.5 mg/mL *N*-ethylmaleimide, 0.1% Nonidet P-40 (NP40), 1X yeast protease inhibitor cocktail (BioShop), and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Cells were then broken up with glass beads in IP buffer containing 0.1 mM dithiothreitol for 30 min at 4°C with a 5 min ice incubation halfway through. The lysed materials were isolated from the beads and separated from cell debris by centrifugation three times at 14,000 *g* for 5 min. The soluble samples were either analyzed by immunoblot or used for IP. If proceeding with immunoblot experiments, the samples were diluted with an equal volume of 2X SDS-PAGE sample buffer (4% sodium dodecyl sulfate, SDS; 20% glycerol; Bromophenol Blue; 10% 2-Mercaptoethanol; and 140 mM Tris-HCl pH 8) and boiled for 4 min prior to analysis by the indicated immunoblots. For IP experiments, 50 µL yeast lysate was retained as input sample, and the remainder was incubated for 2 h with anti-HA agarose beads at 4˚C. IPs were then washed three times with ice-cold IP buffer containing 0.1% NP40, and twice with IP buffer alone. Beads were then resuspended with 2X SDS-PAGE sample buffer and boiled for 4 min prior to analysis by the indicated immunoblots. Quantification of Cst6 sumoylation levels were obtained using ImageJ program. First, on the SUMO immunoblot, intensity of signals below the non-specific band were quantified, then these were normalized to the intensity of corresponding HA IP signals. Finally the normalized values plotted relative to the control (first) sample. Statistically significant differences, calculated using Student's *t*-test ($P < 0.05$), are denoted with an asterisk on graphs.

Denatured immunoprecipitation (IP)

Denatured IP samples were prepared using a method previously described by Sri Theivakadadcham *et al.*,[20]. Briefly, 50 mL of yeast cultures were grown in rich medium, harvested by centrifugation, and washed with 20% Trichloroacetic acid (TCA). Lysed, precipitated and washed materials were resuspended with modified SDS buffer (60 mM Tris pH6.7, 5% 2-mercaptoethanol, 1% SDS, and few drops of bromophenol blue) prior to boiling for 5 min. These samples were then centrifuged to remove insoluble material. The supernatant was separated into a new tube and an aliquot (40 μL) of this supernatant was retained as input sample and the remainder was diluted with denaturing IP buffer (50 mM Tris pH 7.4, 150 mM NaCl, and 0.5% NP40) containing 0.5 mg/mL of bovine serum albumin. Diluted samples were then incubated overnight with anti-HA agarose beads at 4˚C. The next day, IP samples were washed with ice-cold denaturing IP buffer. Beads were then resuspended with 2X SDS-PAGE sample buffer and boiled for 3 min prior to analysis by the indicated immunoblots.

Chromatin immunoprecipitation (ChIP)

ChIP samples were prepared using a method previously described by Sri Theivakadadcham *et al.*, [20]. 50 mL yeast cultures were grown in rich medium and ethanol stress was induced by adding absolute ethanol to a final concentration of 5% for the indicated time points. Cells were then cross-linked with 1.1% of formaldehyde for 20 min, followed by 5 min of quenching with 282 mM of glycine and occasional mixing. Cells were harvested by centrifugation at 3000 *g* for 5 min and washed twice with ice-cold TBS (20 mM Tris-HCl, pH 7.5 and 150 mM NaCl). Washed samples were resuspended in cold ChIP buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1% sodium deoxycholate and 0.1% SDS) containing 1 mM PMSF

and 1X yeast protease inhibitor cocktail. Cells were disrupted and lysed with glass beads using a mini bead beater. The homogenized sample was isolated from glass beads and sonicated to obtain an average DNA fragment size of ~500 bp in length. Samples were then centrifuged for 5 min at 14, 000 *g*, and the supernatant was transferred to a new tube. Then, NaCl was added to the supernatant to a final concentration of 225 mM prior to overnight incubation with 15 µL of magnetic Protein G beads (Dynabeads, Thermo Fisher Scientific) pre-bound with 2 µg rabbit anti-HA (Novus). On the next day, IPs were washed with four different buffers for 4 min each in the following order: (1) ChIP buffer with 275 mM NaCl; (2) ChIP buffer with 500 mM NaCl; (3) 10 mM Tris-HCl, pH 8, 0.25 M LiCl, 1 mM EDTA, 0.5% NP-40 and 0.5% sodium deoxycholate; (4) Tris-EDTA buffer (10 mM Tris-HCl, pH 8 and 1 mM EDTA).Washed beads were then incubated in ChIP elution buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA and 1% SDS) for 20 min at 65°C. HA bound materials were isolated on a magnet stand and were then treated first with RNase for 30 min at 37°C and then again with proteinase K for 1 hour at 42°C. To reverse crosslinking, samples were incubated overnight at 65°C. Next day, DNA was purified and recovered using the GeneJet Gel Extraction Kit (Thermo Fisher). Purified DNA was used for quantitative PCR using primers listed in Table 3S2. Results from qPCR were normalized to an internal control gene, an untranscribed region of Chromosome V. Error bars represent the standard deviation of three replicates and the asterisks indicate a significant difference with *P*-values less than 0.05 obtained from a Student's *t*-test.

Isolation of RNA and reverse transcription (RT)

Yeast cultures (10 mL) were grown in rich medium and ethanol stress was induced by adding absolute ethanol to a final concentration of 5% for the indicated time points. RNA was isolated as previously reported [30]. Briefly, samples were harvested by centrifugation at 3000 *g* for 3 min and washed twice with chilled AE buffer (50 mM sodium acetate, pH 5.2 & 10 mM EDT A, pH 8.0) made with DEPC-treated RNase-free water. The pellet was resuspended with ice cold AE buffer and mixed with SDS, and phenol, pH 4.5. Samples were lysed using a freeze/thaw/vortex cycle twice. First the samples were chilled in a dry ice/ethanol bath slurry mixture (freezing step) for 5 min and then transferred to a 65°C water bath (thawing step) for 5 min, followed by vortex for 30 s. Samples were kept in the dry ice/ethanol bath for a final 5 min before centrifuging at 14, 000 *g* for 7 min at room temperature. RNA was precipitated using the aqueous layer. The isolated RNA was subjected to DNAse treatment followed by cDNA synthesis using the iScript reverse transcriptase (Bio-Rad). Quantitative PCR was performed using primers listed in Table 3S2. mRNA levels were normalized to an internal loading control, 25S rRNA. Error bar represents the standard deviation of three replicates and the asterisks indicate a significant difference with *P*-values less than 0.05 obtained from a Student's *t*-test.

3.4 RESULTS

Cst6 is sumoylated during normal growth

In large-scale proteomics studies, Cst6 was identified as a putative SUMO target [10, 25]. In order to confirm Cst6 sumoylation, we epitope tagged *CST6* with a 6xHA tag using homologous recombination in the common lab yeast strain W303a. The presence of the HA tags had no effect on cell growth as confirmed by a spot assay performed under several growth conditions (Figure 3S1A and B**)**. Cst6-6HA was then immunoprecipitated (IPed) using an HA antibody from cell lysate, prepared in the presence of *N*-ethylmaleimide (NEM), an inhibitor of SUMO proteases, which was then analyzed by SUMO and HA immunoblotting. Supporting that Cst6 is sumoylated, bands were detected on the SUMO immunoblot for the Cst6-6HA IP (Figure 3.1A). As a positive control, Cst6-6HA sumoylation levels were compared alongside another bZIP TF, Sko1, which is known to be polysumoylated during non-stress conditions [20]. In addition to Sko1-6HA or Cst6-6HA-specific bands, a band of variable intensity was detected at ~250 kDa in the SUMO immunoblots of HA IPs (arrow in Figure 3.1A and throughout). Because it is also detected in control HA IPs from lysates lacking HA-fused proteins (Figure 3.1A and see Figure 3.4B), we believe that this band corresponds to a sumoylated protein that non-specifically co-purifies during native IPs. Like for Sko1, multiple SUMO-specific bands were detected for Cst6, including a major band and multiple fainter higher molecular weight bands, which is likely the result of multi- or polysumoylation (Figure 3.1A). To further validate that these bands are due to conjugation of SUMO with Cst6, lysates were prepared in the absence of NEM, in which the level of sumoylation is expected to be reduced significantly due to the uninhibited activity of SUMO proteases. Indeed, in the absence of NEM, Cst6 sumoylation is undetectable (Figure 3S1C, compare lanes 1 and 2). In Cst6-6HA IPs, two bands are observed on long-exposed HA immunoblots: an intense band

slightly below 100 kDa, which corresponds to unmodified Cst6-6HA, and a fainter higher band that co-migrates with the major sumoylated form of Cst6 (~125 kDa) seen on the SUMO immunoblot (indicated by an asterisk on Figures 3.1A and 3S1C). Supporting that this band corresponds to sumoylated Cst6, it is not detected when the IP experiment is performed in the absence of NEM (Figure 3S1C).

Additional experiments were performed to confirm Cst6 sumoylation. To eliminate the possibility that bands observed in the SUMO blot are derived from sumoylated proteins that co-IPed with Cst6, lysates were prepared under denaturing conditions prior to IP. Cellular proteins were extracted with trichloroacetic acid (TCA), treated with sodium dodecyl sulfate (SDS), and boiled before IPing with HA-conjugated beads. Under these conditions, Cst6 sumoylation was still detected, as observed in the SUMO and HA blots in Figure 3.1B, which indicates that the signal observed in the SUMO immunoblot is due to SUMO-conjugated Cst6. As a final method to confirm Cst6 sumoylation, we used a strain in which Ubc9, the sole SUMO conjugating enzyme, is fused to an “anchor away” tag that causes its translocation from the nucleus to the cytoplasm when cells are exposed to rapamycin [31], thereby conditionally blocking nuclear sumoylation events. In the presence of rapamycin, the level of global sumoylation is dramatically reduced, as seen in the SUMO blot of denatured lysate inputs (Figure 3.1C). Cst6-6HA was IPed using an HA antibody from lysates of cultures treated with or without rapamycin and analyzed by HA and SUMO immunoblots. Sumoylation of Cst6 was abolished after rapamycin treatment, further confirming that the TF Cst6 is a target for sumoylation in normally growing yeast (Figure 3.1C).

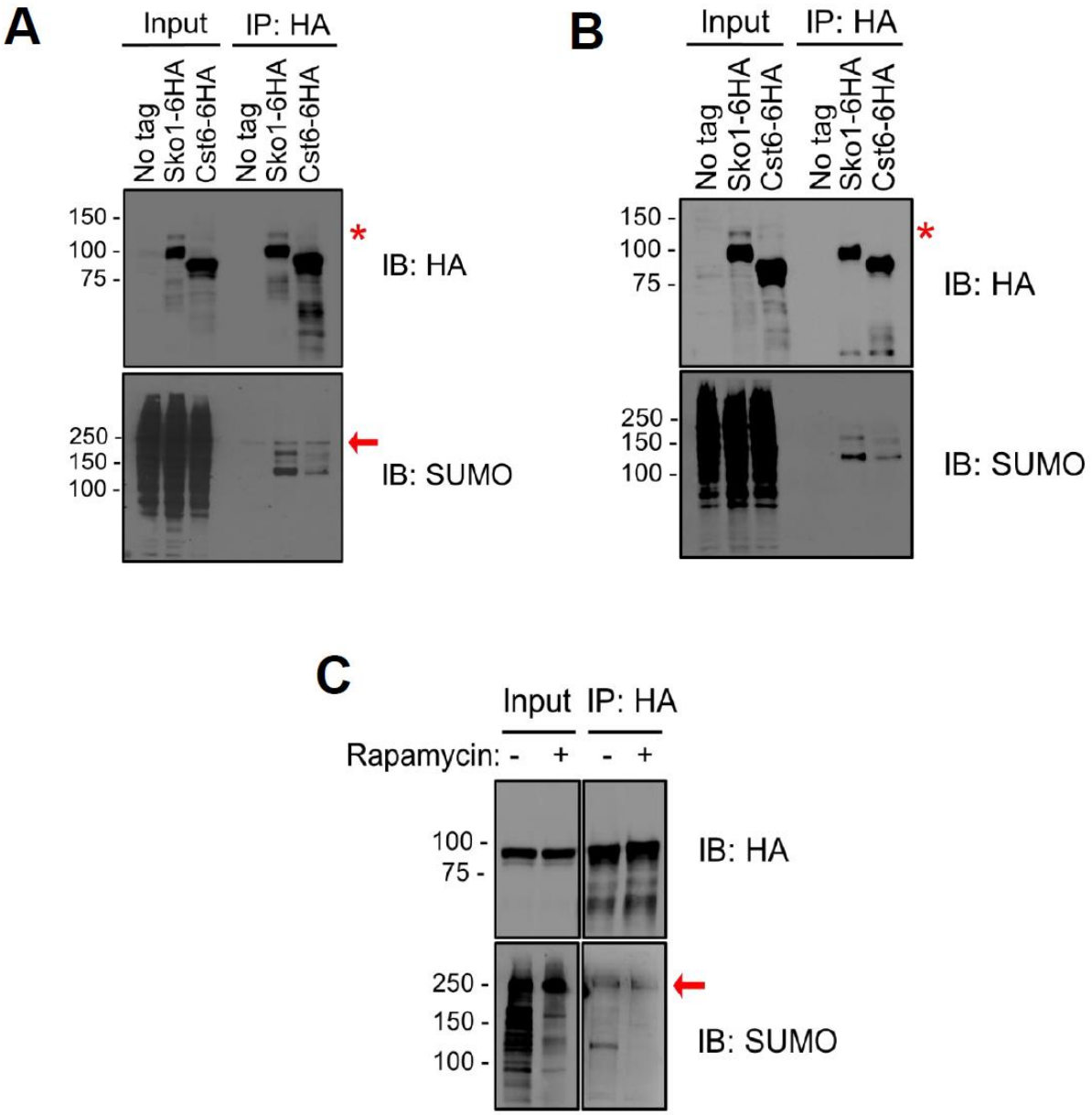

A
Input
IP: HA
No tag
Sko1-6HA
Cst6-6HA
No tag
Sko1-6HA
Cst6-6HA
150 -
100 -
75 -
*
IB: HA
250 -
150 -
100 -
IB: SUMO
B
Input
IP: HA
No tag
Sko1-6HA
Cst6-6HA
No tag
Sko1-6HA
Cst6-6HA
150 -
100 -
75 -
*
IB: HA
250 -
150 -
100 -
IB: SUMO
C
Input
IP: HA
Rapamycin: - + - +
100 -
75 -
IB: HA
250 -
150 -
100 -
IB: SUMO

Figure 3.1: bZIP transcription factor Cst6 is sumoylated. (A) Detection of Cst6 sumoylation using immunoprecipitation (IP) with lysates prepared in non-denaturing conditions. 6HA-tagged Sko1 and Cst6 were IPed using HA-conjugated beads from cell lysates prepared in the presence of N-ethylmaleimide (NEM) and analyzed by immunoblot with HA or SUMO antibodies, as indicated. 'No tag' refers to a sample from the parental strain (*W303a*) expressing no HA-tagged proteins, used as a negative control. Sko1 is a known SUMO target [20] and was used as a positive control. **(B)** Sumoylation of Cst6 detected by IP from protein extracts prepared under denaturing conditions. Extracts were generated by TCA precipitation, treated with SDS, and boiled before IPing with HA conjugated beads. Extracts were analyzed by immunoblot with the antibodies indicated. **(C)** Cst6 sumoylation is abolished when Ubc9 is conditionally removed from the nucleus. Cst6-6HA was IPed from a Ubc9 anchor away strain using an HA antibody from lysates prepared from cells treated with or without rapamycin for 30 min. Sumoylation levels were determined by IP-immunoblot analysis, as in *A*. Red asterisk (*) indicates the position of SUMO-modified Cst6-6HA on HA blots. Arrow indicates the position a non-specific, co-purifying protein detected on SUMO blots.

Cst6 sumoylation increases during oxidative and ethanol stress conditions

Numerous studies have reported the importance of sumoylation in maintaining cell homeostasis during environmental stress (e.g. [32, 33]). Cst6 is part of the stress-responsive transcriptional regulatory network and it was previously shown to be involved in regulating transcription during ethanol, oxidative and heat shock stress [27, 28, 34, 35]. To explore the effect of stress on Cst6 sumoylation, we examined its sumoylation levels by IP-immunoblot in lysates derived from cells grown under different stress conditions (Figures 3.2A and B). Cst6 sumoylation is significantly increased during ethanol and oxidative stress conditions and reduced during osmotic stress, while no change was observed during amino acid starvation or heat shock. Cst6 sumoylation during ethanol stress was further confirmed with additional IP experiments in which NEM was included or excluded. As shown in Figure 3S1C, Cst6 sumoylation increases with ethanol stress in samples prepared in the presence of NEM and it is abolished in its absence (compare lanes 1, 3 and 4). Our data demonstrates that sumoylation of Cst6 increases during ethanol and oxidative stress, and further experiments were performed to investigate the role that sumoylation plays in regulating Cst6 function in these conditions.

Previous studies on Cst6 highlighted its importance for cell growth in the presence of alternative carbon sources [27, 28]. Typically, lab yeast strains are grown in media containing glucose, but other carbon sources, such as glycerol, ethanol, and raffinose, can be used as alternatives. Ethanol, therefore, can serve as a carbon source, or as a stress condition when used at high concentrations in the presence of glucose as a carbon source. In the absence of Cst6, cells grow poorly in alternative carbon sources, including ethanol [27, 28]. We wished to investigate whether the level of Cst6 sumoylation changes with different types of media and carbon sources used for growth.

To test this, we grew the Cst6-6HA strain in SC (Synthetic Complete) or rich medium that was supplemented with either 2% glucose or 1% ethanol as a carbon source, and cells were then treated with or without a stress condition, as indicated on Figure 3.2C. Cells were lysed and Cst6-6HA was then IPed and analyzed by HA and SUMO immunoblots. Strikingly, the level of Cst6 sumoylation was significantly lower when grown in ethanol versus glucose (Figures 3.2C and D), which might reflect the lower levels of global sumoylation observed when ethanol is the sole carbon source (3S2A). As expected, Cst6 sumoylation levels were significantly increased when cells grown in rich, glucose-containing medium were exposed to ethanol stress, but oxidative stress did not show a statistically significant effect on yeast grown in ethanol-containing medium (Figures 3.2C and D). In summary, normally growing yeast display a basal level of Cst6 sumoylation that can be increased by ethanol or oxidative stress, or decreased if ethanol is used as the sole carbon source. Based on these results, we proceeded to investigate what role sumoylation plays in regulating Cst6 function during non-stress and ethanol stress conditions using rich, glucose-containing medium.

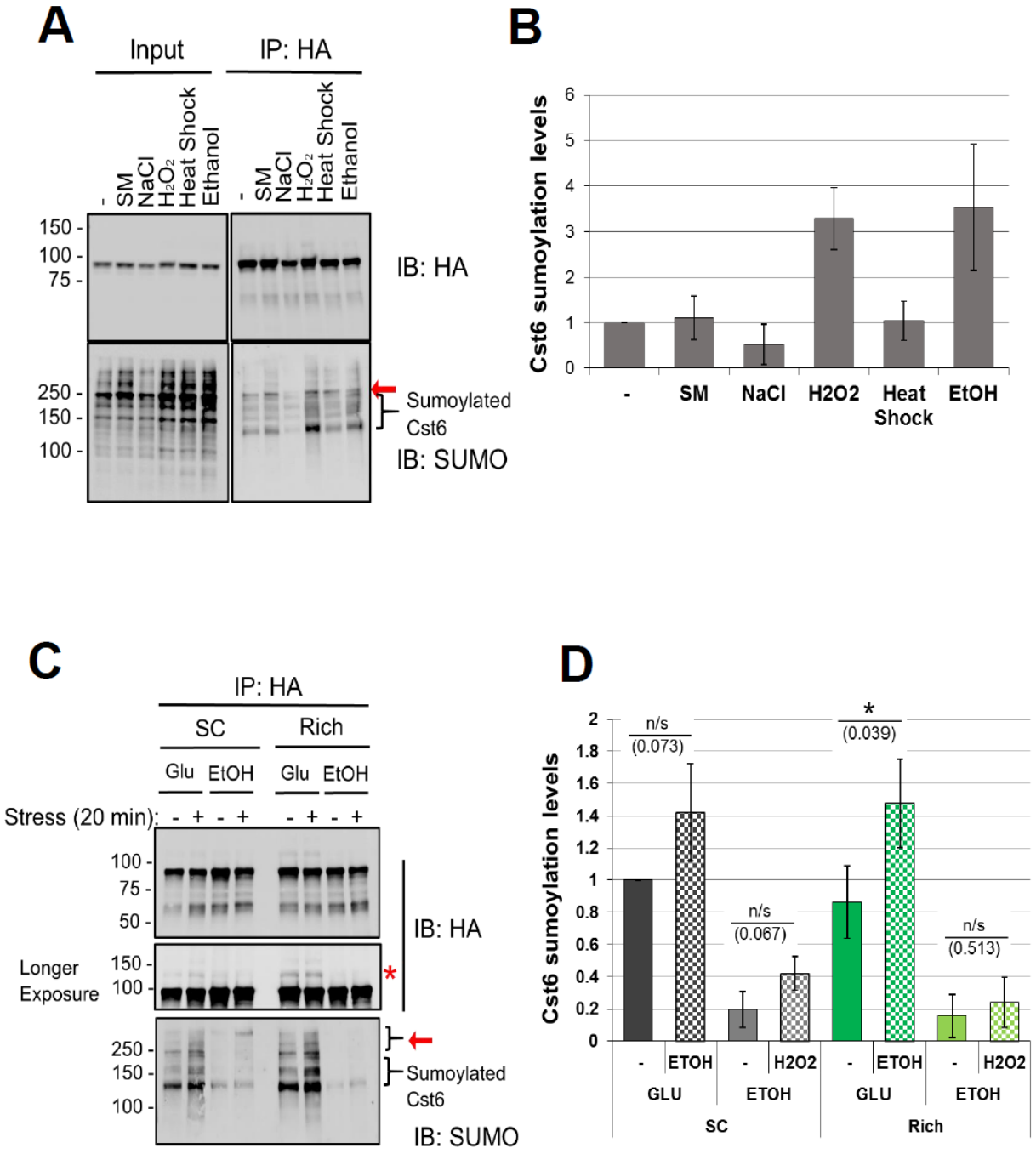
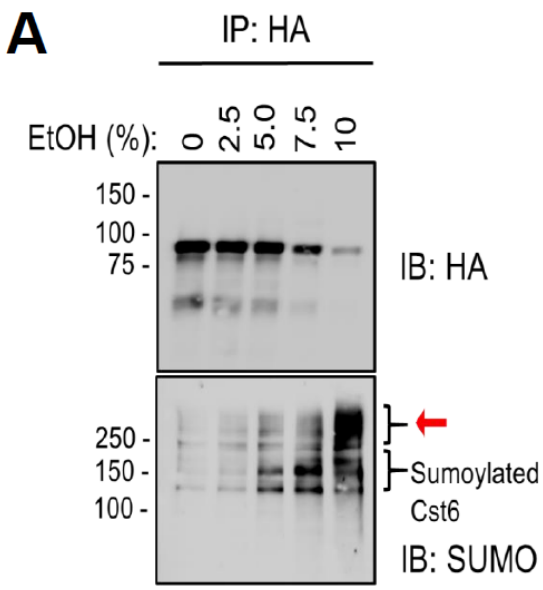
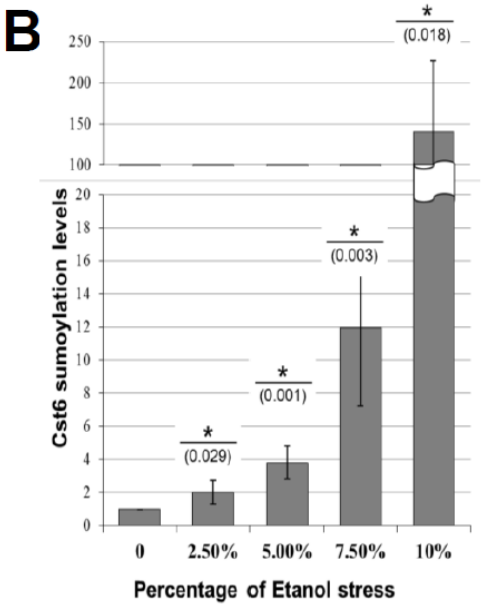
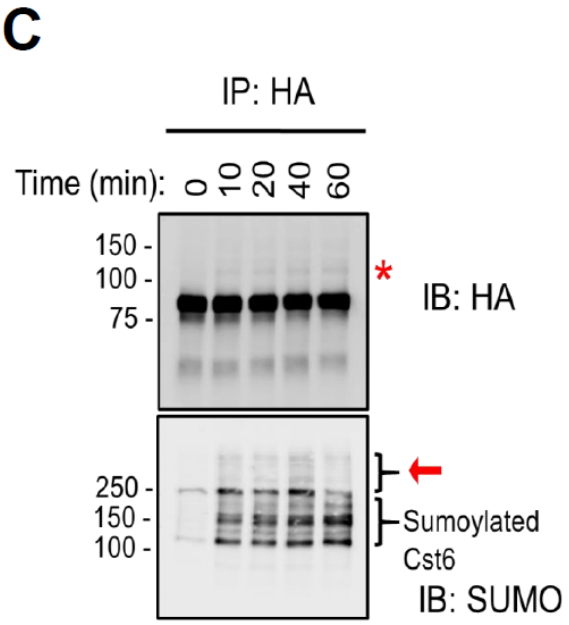

A
Input
IP: HA
-
SM
NaCl
H2O2
Heat Shock
Ethanol
150 -
100 -
75 -
IB: HA
250 -
150 -
100 -
Sumoylated Cst6
IB: SUMO
B
Cst6 sumoylation levels
0
1
2
3
4
5
6
-
SM
NaCl
H2O2
Heat Shock
EtOH
C
IP: HA
SC
Rich
Glu
EtOH
Glu
EtOH
Stress (20 min): - + - + - + - +
100 -
75 -
50 -
IB: HA
Longer Exposure
150 -
100 -
*
250 -
150 -
100 -
Sumoylated Cst6
IB: SUMO
D
Cst6 sumoylation levels
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
n/s
(0.073)
n/s
(0.067)
*
(0.039)
n/s
(0.513)
-
ETOH
-
H2O2
-
ETOH
-
H2O2
GLU
ETOH
GLU
ETOH
SC
Rich

Figure 3.2: Sumoylation level of Cst6 is elevated during oxidative and ethanol stress. (A) Cells expressing Cst6-6HA were treated with the indicated stressors for 20 min and Cst6 sumoylation levels were determined by IP-immunoblot, as in Figure 3.1A. The following stress conditions were used: 0.5 µg/mL of sulfometuron methyl (SM), which induces amino acid starvation; 0.4 M NaCl for osmotic stress; 100 mM H_2O_2 for oxidative stress; 37°C for heat shock; and 5% ethanol (EtOH) for ethanol stress. **(B)** Quantification of Cst6 sumoylation levels for analysis shown in *A*. On the SUMO immunoblot, the intensity of signals below the non-specific bands were quantified using ImageJ, normalized to the HA IP signal intensities, then plotted relative to the first sample. **(C)** Cells were grown in SC or rich media containing either 2% glucose (Glu) or 1% ethanol as a carbon source, then treated with or without stress (100 mM H2O2 for cells grown in ethanol-containing medium or 5% ethanol for cells grown in glucose-containing medium) for 20 min. Sumoylation levels were determined by IP-immunoblot as in Figure 3.1A. **(D)** Quantification of Cst6 sumoylation levels for analysis shown in *C*. Red asterisk (*) indicates the position of SUMO-modified Cst6 on HA immunoblot. Arrows refer to a non-specific band on the SUMO blots. Error bars represent standard deviation of three independent experiments. Significant differences between the non-stress and the stress samples under each media conditions were calculated using Student's t-test (*, $P < 0.05$; n/s, not significant).

To determine whether the effect of ethanol stress on Cst6 sumoylation levels is dose-dependent, we treated the Cst6-6HA strain with different concentrations of ethanol for 20 mins. Cells were then lysed, IPed and analyzed by HA and SUMO immunoblots. Indeed, Cst6 sumoylation was elevated with increased concentrations of ethanol (Figures 3.3A and B). This might at least partly be due to an increase in global sumoylation that occurs with exposure to up to 7.5% ethanol (Figures 3S2B and C). Next, we examined whether Cst6 sumoylation is dependent on the exposure time for treatment with ethanol. Sumoylation levels were determined from Cst6-6HA-expressing cells treated with 5% ethanol for a range of times. As shown in Figures 3.3C and D, Cst6 sumoylation levels increased with prolonged exposure to ethanol stress. Based on these results, and conditions commonly used by others, further ethanol stress treatments were performed at 5% for 20 min.


A
IP: HA
EtOH (%): 0 2.5 5.0 7.5 10
150 -
100 -
75 -
IB: HA
250 -
150 -
100 -
Sumoylated Cst6
IB: SUMO


B
Cst6 sumoylation levels
250
200
150
100
20
18
16
14
12
10
8
6
4
2
0
*
(0.018)
*
(0.003)
*
(0.001)
*
(0.029)
0
2.50%
5.00%
7.50%
10%
Percentage of Etanol stress


C
IP: HA
Time (min): 0 10 20 40 60
150 -
100 -
75 -
*
IB: HA
250 -
150 -
100 -
Sumoylated Cst6
IB: SUMO

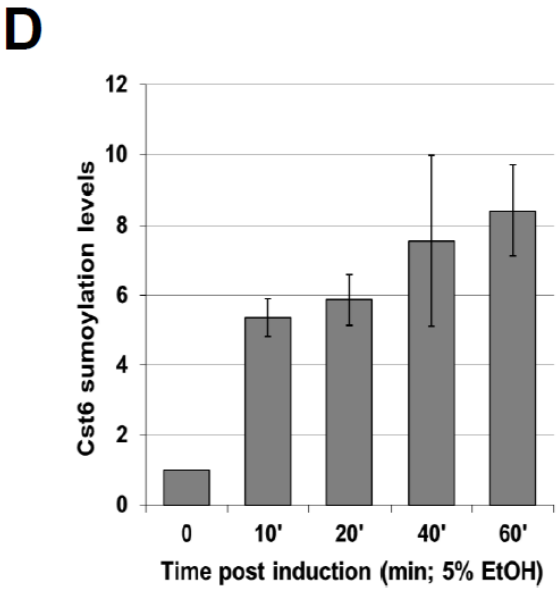

D
Cst6 sumoylation levels
12
10
8
6
4
2
0
0
10'
20'
40'
60'
Time post induction (min; 5% EtOH)

Figure 3.3: Cst6 sumoylation level during ethanol stress is dependent on the dose and duration of stress exposure. (A) Cst6 sumoylation is elevated with increasing concentrations of ethanol exposure. Cells expressing Cst6-6HA were treated with the indicated concentrations of ethanol for 20 mins and then sumoylation levels were determined as in Figure 3.1A. **(B)** Quantification analysis of Cst6 sumoylation levels for *A* as in Figure 3.2B. **(C)** Cst6 sumoylation is increased with prolonged ethanol stress. 6HA-tagged cells were treated with 5% ethanol for indicated time points and then the sumoylation levels were characterized as in Figure 3.1A. **(D)** Quantification analysis of Cst6 sumoylation levels for *C* as in Figure 3.2B. Red asterisk (*) indicates SUMO modified Cst6 on HA immunoblot. Arrows indicate non-specific bands on SUMO blots. Error bars represent standard deviation of three independent experiments. Significant differences between the non-stress sample and with each stress conditions were calculated using Student's *t*-test (*, $P < 0.05$; n/s, not significant).

Cst6 is sumoylated on lysine residues 139, 461 and 547

Using SUMO site prediction software (GPS-SUMO, SUMOplot, and JASSA), we identified three putative SUMO sites for Cst6: K139, K461, and K547, all of which fall within SUMO site consensus motifs (Figure 3.4A) [36, 37]). K139 is situated just upstream of the Aca-specific region (a region identical in Aca1 and Aca2/Cst6 [27]), K461 is found within the bZIP domain, and K547 is at the C-terminus of Cst6. To determine whether these are actual sumoylation sites, we generated yeast strains expressing Lys-to-Arg substitution mutants of all three putative SUMO sites, since Arg cannot be sumoylated (strains Cst6-K139R-6HA, Cst6-K461R-6HA and Cst6-K547R-6HA). We used IP-immunoblot to determine sumoylation levels and found that the individual K139R or K547R mutations decreased Cst6 sumoylation more than the K461R mutation, but they did not completely abolish Cst6 sumoylation (Figures 3.4B and C). Therefore, we generated double- ("D.MT"; Cst6-K461,547R-6HA) and triple-mutant ("T.MT"; Cst6-K139,461,547R-6HA) strains to assess their effects on Cst6 sumoylation. The D.MT mutations partially reduced Cst6 sumoylation, whereas the T.MT mutations abolished sumoylation of Cst6 under normal and ethanol-stress conditions (Figures 3.4B, C, and 3S1C, lanes 5-8). These results strongly suggest that Cst6 is sumoylated at all three lysines and, therefore, Cst6 is multi-sumoylated. The T.MT strain (hereafter referred to as Cst6 MT), alongside the CST6-6HA strain (hereafter referred to as Cst6 WT), were then used to explore roles for Cst6 sumoylation.

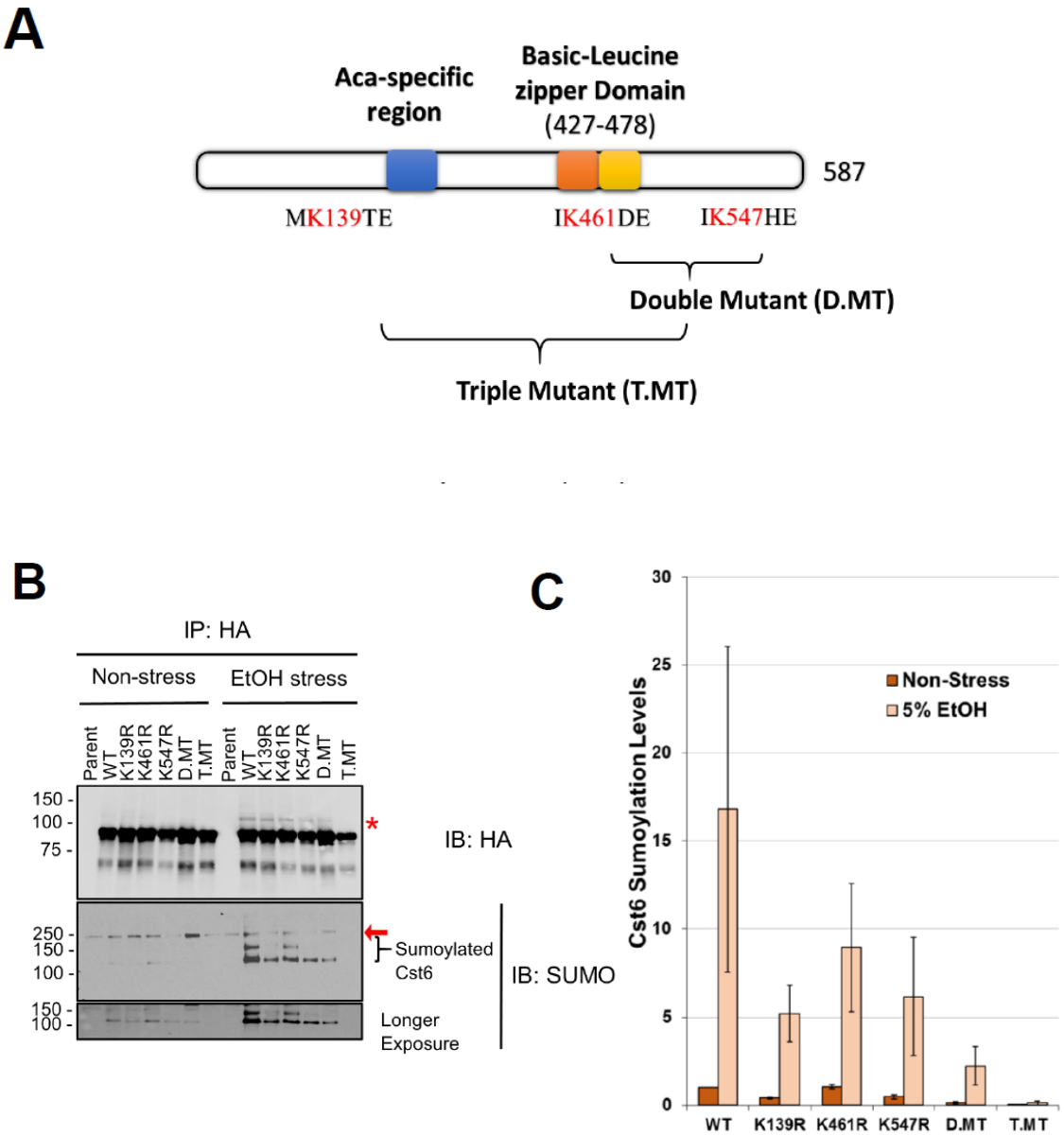

A
Aca-specific region
Basic-Leucine zipper Domain (427-478)
587
MK139TE
IK461DE
IK547HE
Double Mutant (D.MT)
Triple Mutant (T.MT)
B
IP: HA
Non-stress
EtOH stress
Parent
WT
K139R
K461R
K547R
D.MT
T.MT
150
100
75
250
150
100
150
100
IB: HA
Sumoylated Cst6
IB: SUMO
Longer Exposure
C
Cst6 Sumoylation Levels
Non-Stress
5% EtOH
30
25
20
15
10
5
0
WT
K139R
K461R
K547R
D.MT
T.MT

Figure 3.4: Cst6 is multi-sumoylated at K139, K461 and K547 during non-stress and ethanol stress conditions. (A) Schematic diagram of Cst6 indicating the Aca-specific region and basic leucine Zipper (bZIP) domain. Putative SUMO sites on Cst6 were identified as lysine residues 139, 461 and 547 using SUMO site prediction software and are indicated with the encompassing SUMO consensus motifs. **(B)** Cst6 is sumoylated on all three putative SUMO sites during non-stress and ethanol stress conditions. Using site-directed Lys-to-Arg mutagenesis, SUMO-site mutant strains of Cst6 [*Cst6-K139R, Cst6-K461R, Cst6-K547R, Cst6-K461,547R (D.MT), and Cst6-K139,461,547R (T.MT)*] were generated. Sumoylation levels of "No tag", Cst6-6HA (WT), and the mutants were characterized as in Figure 3.1A. **(C)** Quantification of Cst6 sumoylation levels for *B* as in Figure 3.2B. Red asterisk (*) indicates the SUMO modified Cst6 on HA blot. Arrows indicate non-specific bands on SUMO blots. Error bars represent standard deviation of two independent experiments.

Sumoylation of Cst6 is not essential for cell viability but plays a minor role in its stability

To study whether sumoylation of Cst6 has a role in cell fitness, a spot assay was conducted. Supporting previous studies, cells lacking Cst6 showed a growth defect during ethanol stress, when glucose was the carbon source (Figure 3.5A; [27, 28]). However, under either non-stress conditions or when exposed to different concentrations of ethanol, no growth defect was observed for Cst6 MT, implying that sumoylation of Cst6 is not essential for cell viability or survival during ethanol stress (Figure 3.5A). In addition, we compared growth levels on medium in which ethanol (either 1% or 3%) was the carbon source. Deletion of *CST6* led to severe growth defects, but the Cst6 *MT* strain once again grew as well as the Cst6 *WT* strain, and addition of hydrogen peroxide, to induce oxidative stress, had no effect (Figure 3.5B). These results together indicate that sumoylation of Cst6 is not essential for cell viability or fitness under any of the tested growth conditions.

Next, we examined whether ethanol stress or sumoylation affect Cst6 protein abundance. Cell lysates were prepared from cells treated with different concentrations of ethanol for both *WT* and *MT* strains and we analyzed Cst6 levels by HA immunoblot. Increasing ethanol concentration correlated with decreased abundance of Cst6, suggesting that Cst6 is degraded during ethanol stress (Figures 3.5C and D). Interestingly, there appears to be an inverse correlation between the level of Cst6 sumoylation and its abundance (see also Figures 3.3A and B). In conditions where sumoylation of Cst6 increases, protein levels of Cst6 are reduced, suggesting that sumoylation is involved in Cst6 protein stability. To explore this, we compared Cst6 protein levels in the *WT* and *MT* strain after exposure to increasing levels of ethanol. As shown in Figures 3.5C and D, Cst6 MT also becomes less abundant with increased ethanol stress, but the level of Cst6 MT is consistently less than the level of Cst6 WT, ranging from ~25% less in the absence of ethanol to

~49% less in the presence of 10% ethanol. Whereas sumoylation is not needed for the ethanol-dependent reduction in Cst6 protein levels, our data suggests that Cst6 sumoylation plays a role in enhancing its stability in normal and ethanol stress conditions.

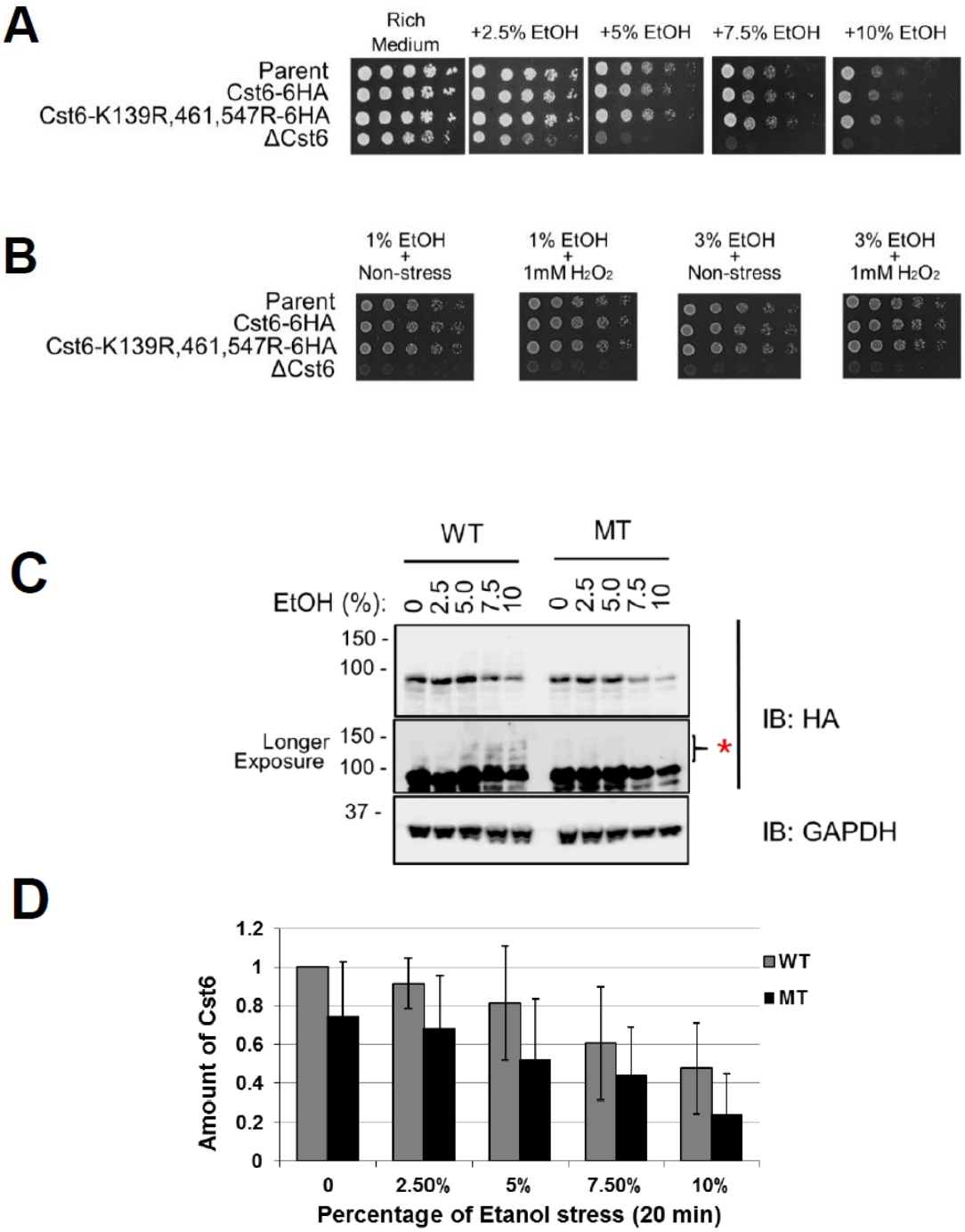

A
Rich Medium
+2.5% EtOH
+5% EtOH
+7.5% EtOH
+10% EtOH
Parent
Cst6-6HA
Cst6-K139R,461,547R-6HA
ΔCst6
B
1% EtOH + Non-stress
1% EtOH + 1mM H2O2
3% EtOH + Non-stress
3% EtOH + 1mM H2O2
Parent
Cst6-6HA
Cst6-K139R,461,547R-6HA
ΔCst6
C
WT
MT
EtOH (%): 0 2.5 5.0 7.5 10 0 2.5 5.0 7.5 10
150 -
100 -
Longer Exposure
150 -
100 -
37 -
IB: HA
IB: GAPDH
D
Amount of Cst6
1.2
1
0.8
0.6
0.4
0.2
0
WT
MT
0
2.50%
5%
7.50%
10%
Percentage of Etanol stress (20 min)

Figure 3.5: Sumoylation of Cst6 is not essential for cell viability but influences its abundance. **(A)** Cst6 sumoylation is not essential for cell viability during ethanol stress on medium containing 2% glucose. Cell growth fitness was assessed on glucose-containing rich-medium plates, under non-stress and indicated ethanol stress conditions, using the spot assay technique with a five-fold serial dilution series. Plates were then incubated for two days at 30°C. **(B)** Cst6 sumoylation is not essential for cell viability when ethanol is the carbon source. Cell growth was assessed on ethanol-containing rich-medium plates under non-stress and oxidative stress conditions, by spot assay. Plates were incubated for two days at 30°C. **(C)** Cst6 abundance is reduced when its sumoylation sites are mutated. Lysates (from cells expressing Cst6-6HA) treated with the indicated percentages of ethanol for 20 min were analyzed by HA and GAPDH immunoblots. **(D)** Quantification analysis of Cst6 levels for *C*. Intensity of HA signals were normalized to corresponding GAPDH signals, then plotted relative to the first sample. Red asterisk (*) indicates the SUMO modified form of Cst6 on the HA blot. Error bars represent standard deviation of three independent experiments.

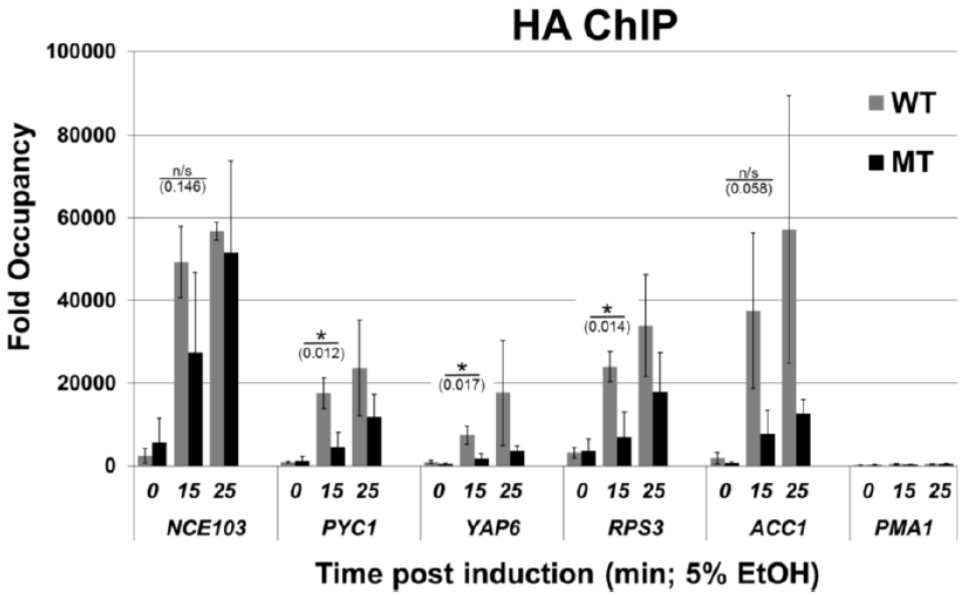
Sumoylation enhances Cst6 promoter binding and restricts expression of its target genes

Sumoylation of bZIP TFs can function in both promoter clearance (e.g. c-Fos, Gcn4 and Sko1; [15, 16, 20]) and enhancing DNA binding (e.g. CREB1; [23]). Therefore, it is possible that sumoylation of Cst6 plays a role in regulating its association or dissociation with promoters of its target genes. To investigate this, chromatin immunoprecipitation (ChIP) was used to compare the occupancy levels of Cst6 WT and Cst6 MT on ethanol-induced target promoters. The occupancy levels of Cst6-WT significantly increased during ethanol stress on all the target genes tested, except on the control gene *PMA1*, which is not a target of Cst6 (Figure 3.6A). Cst6 MT was also recruited to Cst6 target genes during ethanol stress but showed significantly reduced occupancy compared to WT after 15 min of ethanol stress at three of the tested genes (*PYC1*, *YAP6*, and *RPS3*). Although efforts were made to match conditions and timing between replicate experiments, there were significant deviations in Cst6 occupancy levels among the replicates, which hampered determination of statistical significance between Cst6 WT and MT occupancy for other genes and time points. Nonetheless, comparing the occupancy patterns of WT and MT suggests that recruitment of non-sumoylatable Cst6 is generally delayed and/or attenuated on target genes, which implies that sumoylation is important for the timely association of Cst6 with target promoters during ethanol stress.

To further study the effect of Cst6 sumoylation on gene expression, we investigated how sumoylation might regulate transcription of Cst6 target genes. The majority of initial studies on roles for sumoylation in regulating TFs pointed to roles for the modification in transcription silencing [12-16], whereas other studies then demonstrated roles for SUMO modification in transcriptional activation (e.g. CREB1 and Pax6 [22, 23]). To determine whether sumoylation of Cst6 is important for repression or activation of target genes, quantitative RT-PCR (qRT-PCR)

analysis was performed on RNA isolated from the *Cst6 WT* and *Cst6 MT* strains. As seen in Figure 3.6B, the expression of various Cst6 target genes differs during ethanol stress. For example, although transcription of *NCE103, YAP6,* and *HAP4* was induced, the transcription of *ACC1* and *RPS3* was reduced, and no changes were observed for *ROX1*. In the *Cst6 MT* strain, expression patterns for most genes paralleled the patterns seen for *Cst6 WT*, with notable exceptions. Some genes showed elevated mRNA levels during later time points after exposure to ethanol stress (*NCE103, ROX1* and *ACC1*), some showed elevated levels during non-stress conditions (*ACC1* and *RPS3*), and some did not show significant differences (*YAP6,* and *HAP4*) (Figure 3.6B). Strikingly, *RPS3* showed dramatically elevated mRNA levels at all the indicated time points, suggesting that Cst6 sumoylation plays a major role in repressing expression of this gene. Overall, these results indicate that blocking Cst6 sumoylation affects expression of target genes and suggests that Cst6 sumoylation has a general repressive effect that is observed differentially at different targets.

A


HA ChIP
Fold Occupancy
100000
80000
60000
40000
20000
0
WT
MT
n/s (0.146)
* (0.012)
* (0.017)
* (0.014)
n/s (0.058)
0 15 25
NCE103
PYC1
YAP6
RPS3
ACC1
PMA1
Time post induction (min; 5% EtOH)

B

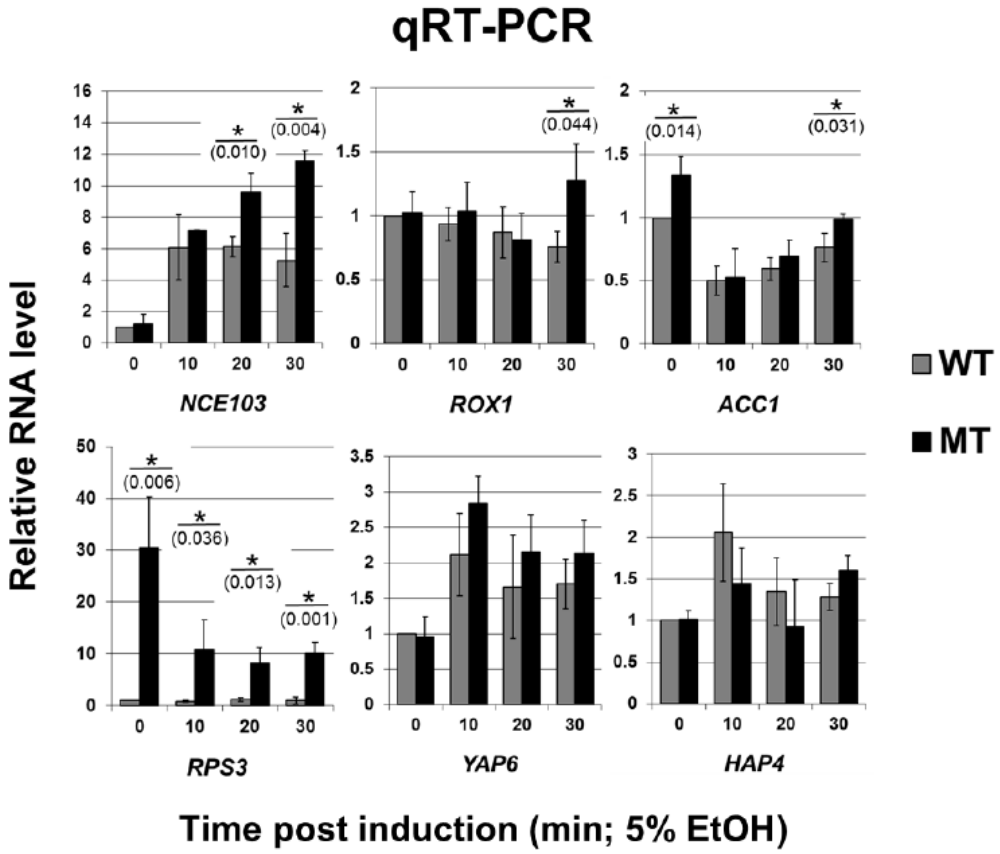

qRT-PCR
Relative RNA level
* (0.010)
* (0.004)
* (0.044)
* (0.014)
* (0.031)
* (0.006)
* (0.036)
* (0.013)
* (0.001)
0 10 20 30
NCE103
ROX1
ACC1
RPS3
YAP6
HAP4
WT
MT
Time post induction (min; 5% EtOH)

Figure 3.6: Sumoylation enhances Cst6 promoter binding and restricts expression of some target genes. (A) Quantitative HA ChIP analysis of Cst6 WT and Cst6 MT occupancy levels on its ethanol stress-regulated target gene promoters after induction with 5% ethanol for indicated times. Gene occupancy signals were normalized to an internal control, an untranscribed region of Chromosome V. **(B)** Quantitative RT-PCR analysis was performed on RNA isolated from *CST6 WT* and *Cst6 MT* strains. Cells were treated with 5% ethanol and harvested after the indicated time points. mRNA levels were normalized to levels of the *25S* rRNA. Error bars represent standard deviation of three replicates. Significant differences between Cst6 WT and Cst6 MT under each tested condition were determined by Student's *t*-test (*, $P < 0.05$).

3.5 DISCUSSION

Extending our previous work on Gcn4 and Sko1, we have now shown that another bZIP TF, Cst6, is also regulated by sumoylation in budding yeast [16, 20]. Gcn4 expression (and consequently, its sumoylation) is dependent on amino acid starvation, but like Sko1, a fraction of Cst6 molecules is sumoylated during normal growth conditions, suggesting that the modification regulates properties of the TF not related to stress. However, Cst6 sumoylation increases with exposure to stress, specifically ethanol and oxidative stress, which we did not observe for Sko1. Environmental stressors such as osmotic stress, oxidative stress, heat shock and ethanol stress lead to a dramatic increase in the level of protein sumoylation in mammalian, yeast and plant cells, but it is not fully understood how elevated sumoylation of specific target proteins affects their stress-related functions [38-41]. As the level of Cst6 sumoylation during ethanol stress was dependent on the dose and the duration of the stress, this provided us with an opportunity to examine how increased sumoylation might regulate properties of Cst6 during stress. Indeed, our analysis indicates that elevated sumoylation after ethanol stress likely promotes Cst6 recruitment or accumulation at target gene promoters and ensures that expression of its targets is not excessive.

Controlling the association of TFs with chromatin is a frequently reported function for sumoylation [11]. For some TFs, sumoylation was shown to occur specifically after they bind to target promoters and the modification facilitates their subsequent removal from DNA, thereby enabling gene deactivation or restricting expression levels (e.g. Gcn4, Ikaros, and Sko1; [16, 20, 42]). As mentioned above, however, in other cases, sumoylation promotes the interaction of TFs with DNA (e.g. CREB1 and Pax6 [22, 23]). As our ChIP analyses demonstrate that a sumoylation-deficient form of Cst6 can be recruited to DNA, sumoylation is not required for Cst6 to bind to its target promoters. However, our results support a role for sumoylation in enhancing the association of

Cst6 with its binding sites. For one, as Cst6 sumoylation increases during exposure to ethanol stress, the levels of DNA-bound Cst6 also increase. Secondly, sumoylation-blocking mutations in Cst6 result in reduced chromatin occupancy at some target promoters.

As one possible mechanism for promoting the association of Cst6 with chromatin, sumoylation might increase its affinity for DNA through conformational changes or by promoting homodimerization or interactions with other proximal DNA binding transcriptional factors. In support of this, one of the Cst6 sumoylation sites that we identified, K461, is predicted to lie adjacent to the hydrophobic face of the leucine zipper region of its bZIP domain, which enables dimerization [43]. Phosphorylation of an analogous position of the leucine zipper of C/EBPβ is believed to enhance homodimerization, but more analysis is needed to explore how addition of a SUMO moiety at this residue affects pairing of Cst6 units, or heterodimerization with its paralog, Aca1 [27, 44]. In an alternative mechanism for promoting increased Cst6 occupancy on DNA, sumoylation might enhance Cst6 stability, as has been shown for TFs Delta-Lactoferrin and NPHP7 [45, 46]. Indeed, our finding that Cst6 protein levels are reduced when its sumoylation sites are mutated supports this idea. In any case, whether through an active role in promoting its interaction with DNA, or through a less direct role in inhibiting its degradation, our study points to an important function for sumoylation in ensuring that high levels of Cst6 occupy its target gene promoters rapidly after exposure to ethanol stress.

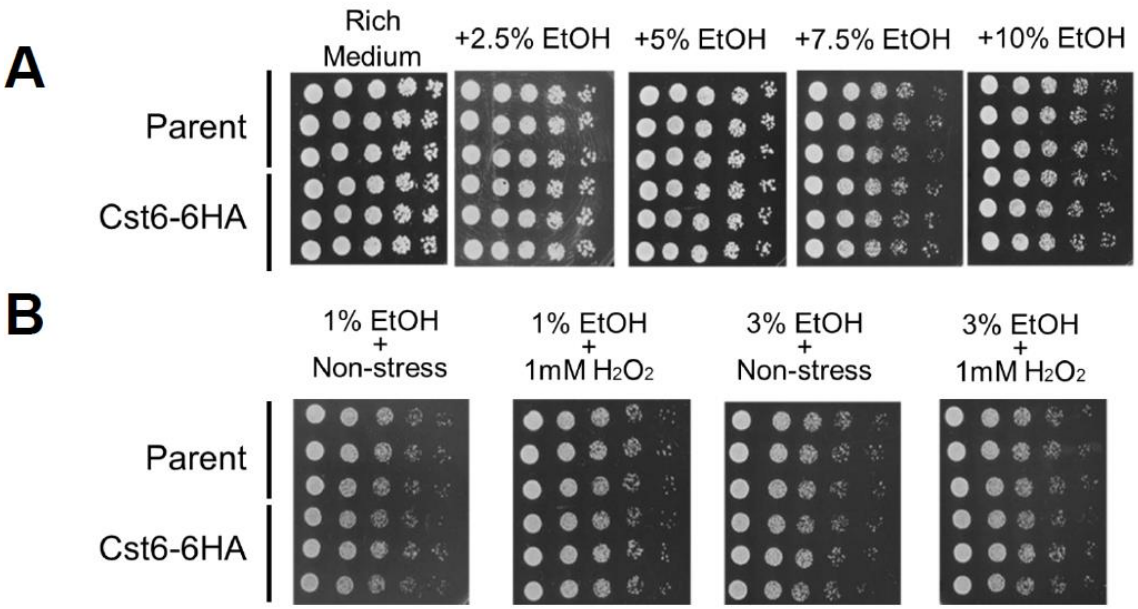
Although Cst6 binds dozens of gene promoters in the presence of ethanol, the effect of Cst6 binding on their expression is gene dependent. For example, during ethanol stress, compared to wild-type cells, *NCE103* expression is dramatically reduced while *RPS3* expression is elevated in cells lacking Cst6 [28]. Our analysis demonstrates, however, that impairing Cst6 sumoylation has

a consistent effect on elevating transcription levels, wherever an effect was observed. This might reflect reduced occupancy levels for the sumoylation-deficient Cst6 mutant, suggesting that appropriate levels of Cst6 are normally needed to prevent excessive expression of targets. However, in normal conditions (i.e. time zero for the ethanol stress time course), we detected approximately equal levels of Cst6 WT and MT on the *RPS3* promoter, but expression of the gene was dramatically derepressed in a strain in which Cst6 cannot be sumoylated (Figure 3.6A and B). This points to a more direct role for sumoylation of Cst6 in inhibiting transcription. As with multiple metazoan TFs that are sumoylated, SUMO can directly impair transcription by promoting interactions with transcriptional corepressors, particularly histone deacetylase complexes [11, 47]. Future studies will be key for understanding how sumoylation affects the molecular properties of Cst6, including its dimerization and interactions with other proteins and DNA, that lead to its transcriptionally repressive effects. Taken together, our study on Cst6 sumoylation provides further evidence that the modification controls the association of TFs with chromatin and fine-tunes transcription levels of target genes.

3.6 ACKNOWLEDGEMENTS

This work was supported by a grant from the Canadian Institutes of Health Research (CIHR; grant number MOP-142282) to E.R.

3.7 SUPPLMENTARY DATA



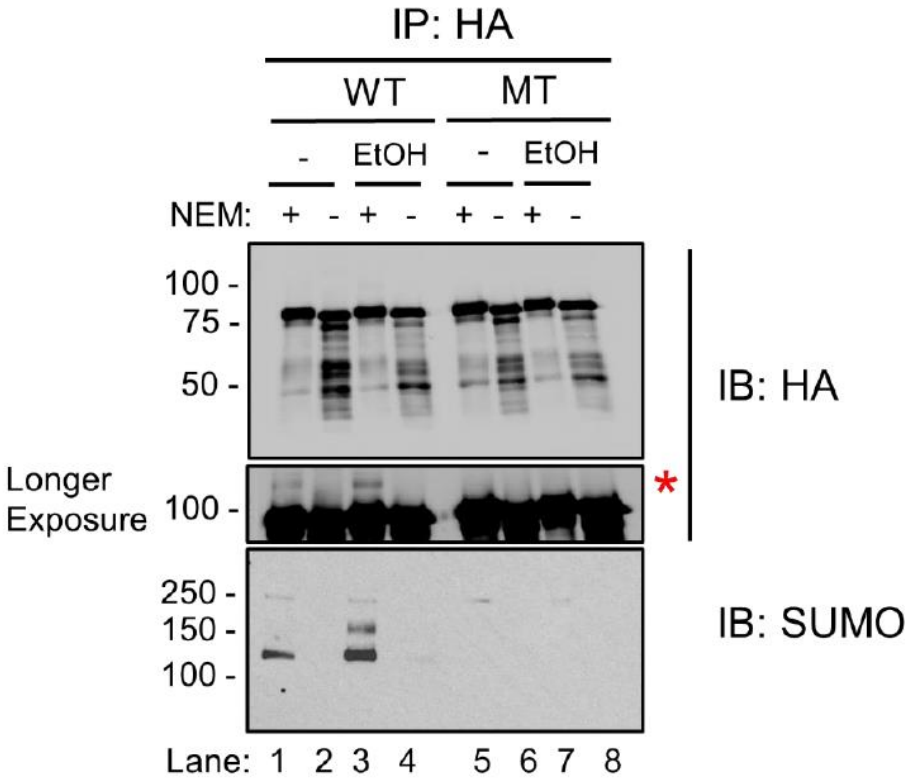


Figure 3 S 1: (A) Presence of HA tag does not affect growth in rich media containing glucose as the carbon source. Cell growth of Cst6-6HA and parent strains was assessed on glucose-containing, rich medium plates under non-stress and indicated ethanol stress conditions by spot assay. Plates were incubated two days at 30°C. **(B) Presence of HA tag does not affect growth in rich media containing ethanol as the carbon source.** Growth fitness was assessed by spot assay on media containing either 1% or 3% ethanol as the sole carbon source, in the absence or presence of oxidative stress (1 mM H_2O_2). Plates were incubated for two days at 30°C. **(C) Cst6 sumoylation is detected in samples prepared with NEM and with intact lysine residues 139, 461 and 547.** Cst6-6HA WT and Cst6-6HA MT were IPed using HA-conjugated beads from cell lysates prepared with or without NEM and analyzed by HA and SUMO immunoblots. The experiment was also performed in the presence and absence of 5% ethanol stress, where indicated. Red asterisk (*) indicates the SUMO modified form of Cst6 on the HA blot.

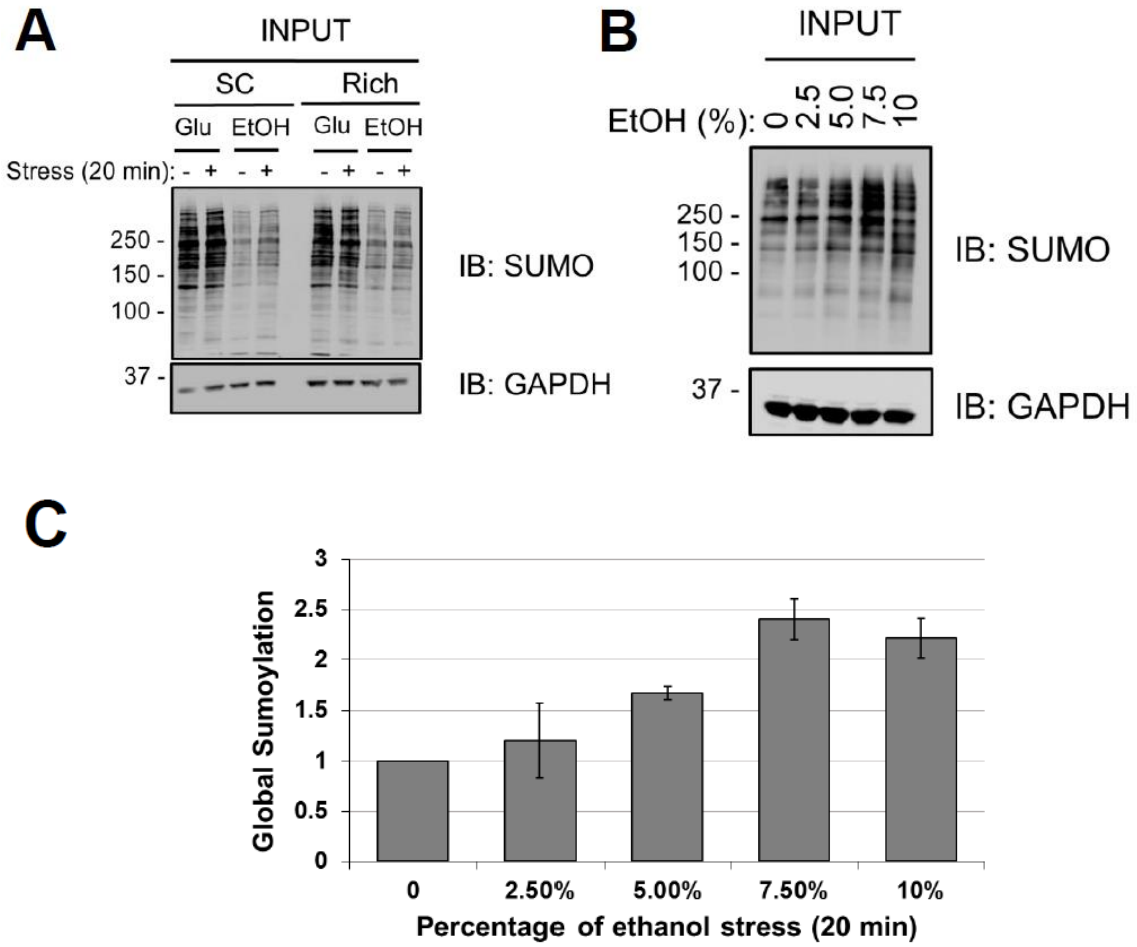


Figure 3 S 2: Global levels of sumoylation are influenced by carbon source and ethanol stress. (A) Yeast cells show significantly higher levels of sumoylation when grown in media containing glucose compared to ethanol. Cells were grown in SC or rich media containing either 2% glucose (Glu) or 1% ethanol (EtOH) as a carbon source, then treated with or without a stressor (100 mM H_2O_2 for ethanol-containing medium, or 5% ethanol for glucose-containing medium) for 20 min. Lysates were prepared and analyzed by SUMO and GAPDH immunoblots. **(B)** Global sumoylation during ethanol stress is dose dependent. Lysates from cells treated with the indicated concentrations of ethanol for 20 min were analyzed by SUMO and GAPDH immunoblots. **(C)** Quantification of sumoylation levels for *B.* SUMO signals were normalized to corresponding GAPDH signals, and the results were plotted relative to the first sample. Error bars represent standard deviation of three replicates.

Table 3 S 1: Yeast strains used in this study

Strain	Parental	Genotype
Background Strain		
W303a (YER023)		*MAT a ura3-52 trp1Δ2 leu2-3_112 his3-11 ade2-1 can1-100*
Derived Strains		
YVS003C	W303a	*SKO1-6HA::kl TRP1*
YVS004A1	W303a	*CST6-6HA::kl TRP1*
YVS064G	W303a	*cst6-K139R-6HA:: kl TRP1*
YVS060C	W303a	*cst6-K461R-6HA:: kl TRP1*
YVS061D	W303a	*cst6-K547R-6HA:: kl TRP1*
YVS063C	W303a	*cst6-K461,547R-6HA:: kl TRP1*
YVS065C	W303a	*cst6-K139,461,547R-6HA:: kl TRP1*
YVS062A	W303a	*cst6Δ::kanMX*

Table 3 S 2: Oligonucleotide primers used in this study.

Gene	**Oligonucleotide sequence(s)**
Primers for quantitative PCR analysis of ChIP samples	
NCE103 promoter	Forward: 5′-CGTTCGTTCCCGTTTCGATG-3′
	Reverse: 5′-CGGCTTGATAAGCGTCATGG-3′
PYC1 promoter	Forward: 5′-CTTAGACAAGCGCCAGTTGC-3′
	Reverse: 5′-CACTGCGGAAAAGGCCAAAA-3′
YAP6 promoter	Forward: 5′-CTCCCACAGTCACGATGAGC-3′
	Reverse: 5′-TAGTCTCAACGGCTGTGCAT-3′
RPS3 promoter	Forward: 5′-GCAGGCATAAGATGCATAGCG-3′
	Reverse: 5′-TCAAAAGCATCATGCGGCG-3′
ACC1 promoter	Forward: 5′-GCATGCATCGACGTCACAGT-3′
	Reverse: 5′-CTAGTCGCAGTCGTGGTGAC-3′
PMA1 promoter	Forward: 5′-CAATTATGACCGGTGACGAAAC-3′
	Reverse: 5′-AATCGAAACTAATGGAGGGGAG-3′
CHRV untranscribed region	Forward: 5′-CATTATCCGTAACGCCACTTT-3′
	Reverse: 5′-CGATCTTAGTTCCAATGGTGAAA-3′
Primers for quantitative RT-PCR analysis	
NCE103	Forward: 5′-ACCCTACTGTGCAAACTGCT-3′
	Reverse: 5′-GCAGTAGACCGTCCTCTACG-3′
ROX1	Forward: 5′-GTCCACAACTACCCCTACGC-3′
	Reverse: 5′-TAGCGGTGACCTCAGTGTTG-3′
ACC1	Forward: 5′-TCCAGAAGATGTCGAAGCCG-3′
	Reverse: 5′-TCAAGGCACGCAATGGTACT-3′
RPS3	Forward: 5′-TCTGGTCAACCAGTCAACGAC-3′
	Reverse: 5′-AGCCTTTGGACCAGTTCTGC-3′
YAP6	Forward: 5′-GGACTTCCGAACCAGAGCAT-3′
	Reverse: 5′-GGTATTGCGAGATGGGAGGG-3′
HAP4	Forward: 5′-GTATTGACGGTAGTGCCGGT-3′
	Reverse: 5′-TGGTGGCAGTTGCATCATTG-3′
25S	Forward: 5′- TCTAGCATTCAAGGTCCCATTC-3′
	Reverse: 5′- CCCTTAGGACATCTGCGTTATC-3′

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

4.1 SUMMARY

Since the discovery of sumoylation as a post-translational modification, multiple studies have observed abnormal levels of sumoylation in cells from patients with cancer, Huntington's, and Alzheimer's diseases [1]. In an attempt to study the functions of sumoylation, researchers have found that TFs are one the largest classes of SUMO substrates [2]. Recently, multiple studies have focused on identifying the role sumoylation plays in regulating TFs in mammals and yeast cells. My investigation focused on finding the function of sumoylation in regulating two gene-specific bZIP TFs Sko1 and Cst6, in *S. cerevisiae*.

4.1.1 Yeast transcription factor Sko1 is regulated by sumoylation

Recent studies on TF sumoylation suggested that sumoylation is involved in transcriptional repression, partly by reducing the association of TFs with chromatin [2, 3]. To investigate whether this was a general role of sumoylation in regulating TFs, part of my dissertation focused on identifying roles for sumoylation in regulating Sko1 function.

Sko1 is a bZIP TF that binds to promoters containing CRE-like sequences as a homodimer [4-6]. It plays a unique bi-functional role in transcription: during normal growth, it represses the transcription of some osmotic and oxidative stress-inducible genes and upon stress, it activates the transcription of stress defense genes. Its transcriptional repressor function is dependent on the recruitment of Tup1 and Cyc8 co-repressor complex to its target promoters [4-6]. The switch from being a repressor to an activator during osmotic stress relies specifically on Hog1 MAP kinases-

dependent recruitment of the co-activator SAGA, SWI/SNF nucleosome remodeling complexes and RNA polymerase II (RNAPII) to induce transcription of target genes [4-6]. Sko1 was identified as a SUMO substrate in large scale proteomic studies, but the relevance of this modification on Sko1 has not been reported [7-9]. Considering the function of Sko1 during non-stress and osmotic stress conditions, we studied the functional importance of Sko1 sumoylation under these growth conditions.

In this study, we discovered that Sko1 is poly-sumoylated at Lys 567 and the sumoylation levels were unchanged when cells were exposed to different stress conditions, including osmotic stress. This indicates that sumoylation of Sko1 was involved in functions that are unrelated to stress response. To investigate whether Sko1 sumoylation plays a role in cell viability, we performed growth test under non-stress and with different osmotic stressors. Under tested conditions, the SUMO mutant form of Sko1 (*sko1-MT: sko1-K567R*) strain grew similar to the wildtype (*SKO1-WT)* strain indicating that sumoylation of Sko1 is not essential for yeast cell viability. Moreover, Sko1 sumoylation is not important for the protein stability as the protein levels of both Sko1-WT and Sko1-MT were approximately the same, even in the presence of 0.4M NaCl.

Apart from being sumoylated, Sko1 was previously known to be phosphorylated by PKA and Hog1 kinases [4-6]. Numerous studies have shown the codependence or interference between post-translation modifications [2]. Therefore, we next attempted to study the possible cross-talk between Sko1 phosphorylation and its sumoylation. Sko1, with phospho-site mutant forms of either PKA or Hog1, was modified by SUMO under the tested growth conditions, indicating that sumoylation of Sko1 occurs independent of its prior phosphorylation. Next, we tested whether Sko1 phosphorylation is dependent on its prior sumoylation and to test this, a mobility shift

experiment was conducted. The Sko1 migration pattern on SDS-PAGE gel was slightly higher in samples collected after the addition of 0.4 M NaCl, due to its phosphorylation by Hog1 [5]. This shift in migration was compared between Sko1-WT and Sko1-MT during non-stress and osmotic stress conditions. It was noted that the migration patterns of Sko1-MT were similar to Sko1-WT, even during osmotic stress, suggesting that prior sumoylation of Sko1 is not important for its phosphorylation by Hog1. Together, these results indicates that post-translational modifications on Sko1 are independent of each other.

To investigate whether sumoylation plays a role in regulating Sko1 association with DNA, we first tested whether DNA binding is a requirement for Sko1 sumoylation. To do this, we generated DNA-binding mutant forms of Sko1 and checked their ability to get sumoylated. The DNA-binding mutant forms of Sko1 were unable to get sumoylated, implying that DNA binding is necessary, and that Sko1 sumoylation takes place after binding to its target promoters. Next, we constructed a plasmid that generates Sko1 DNA-binding mutant fused to DNA binding domain of the transcription activator Gal4 to investigate whether DNA binding is sufficient for Sko1 sumoylation. When this plasmid was introduced into a yeast strain that contains multiple Gal4 binding sites on reporter genes, the fusion protein showed high levels of sumoylation, implying that DNA binding is sufficient for Sko1 modification by SUMO.

To further study the role sumoylation plays in regulating Sko1 DNA binding, we performed ChIP experiments followed by next-generation sequencing (ChIP-seq) for both *SKO1-WT* and *sko1-MT* strains, during non-stress and osmotic stress conditions. There were four main results acquired from the ChIP-seq experiment: (i) Sko1-MT bound more promoter regions than Sko1-WT during non-stress and osmotic stress conditions; (ii) During non-stress growth conditions, Sko1-MT

recognized Sko1 binding motif-like sequences, but with less stringency than Sko1-WT; (iii) During osmotic stress, Sko1-MT was still able to bind to non-target promoters but binding sites appeared to be more stringent compared to non-stress; and (iv) the occupancy levels of Sko1-MT was greater than Sko1-WT on Sko1 target gene promoters, during tested growth conditions. Taken together, our analysis suggests that sumoylation functions in preventing the association of Sko1 with non-specific binding sites that show some sequence similarity to its consensus binding motif. In addition, sumoylation also appears to function in preventing excessive occupancy of Sko1 on its target genes.

However, the higher occupancy by Sko1-MT on target genes did not have a general effect on the expression levels of most of the target genes tested. Although bound by Sko1-MT at non-specific sites, it was not able to induce the expression of these genes. These results suggests that Sko1 binding alone is not sufficient to alter the expression of these genes. Finally, we investigated the relationship between Sko1 sumoylation and Hog1 recruitment on Sko1 target promoters, using ChIP experiments. In the absence of sumoylation, the recruitment of Hog1 was significantly impaired on target genes, implying that sumoylation facilitates the binding of Hog1 on target promoters. Collectively, our results suggest important roles for Sko1 sumoylation in regulating target specific binding and in enhancing the recruitment of Hog1 kinase.

4.1.2. Yeast transcription factor Cst6 is regulated by sumoylation

To continue the investigation on TF sumoylation, I next focused on finding roles for Cst6 sumoylation. Cst6 is a gene-specific bZIP TF that is essential for yeast cell viability during ethanol stress and in non-optimal growth conditions [10]. Similar to Sko1, Cst6 recognises and binds to CRE-like promoter sequences. A recent study showed that the Cst6 binding site has an additional

guanine nucleotide at the 5′ end of its binding motif, when compared with the consensus CRE motif, implying that it has a somewhat different binding site preference than other bZIP TFs [11]. Cst6 binds to DNA as a homodimer or a heterodimer together with Aca1, another yeast bZIP TF [10, 12]. Cst6 was also identified as a SUMO substrate in large scale proteomic studies, but the functional importance of this modification was not studied [8, 9]. In this study we focused on finding roles for Cst6 sumoylation during non-stress and ethanol stress conditions.

We demonstrated that Cst6 is multi-sumoylated on three lysine residues (K139, K461, and K547), and that the level of its SUMO modification increases during ethanol and oxidative stress. Because Cst6 is essential during ethanol stress and in the presence of ethanol as a carbon source, we next looked at the level of Cst6 sumoylation under these growth conditions. Its sumoylation was elevated with an increased duration and dose of ethanol stress, but it was significantly reduced when ethanol was used as the sole carbon source in the medium.

Next, we investigated the importance of Cst6 sumoylation in cell viability. A yeast strain with SUMO-deficient Cst6 (*Cst6-MT*: *Cst6-K139,461,547R*) was able to grow as well as the wild type strain (*CST-WT*) under the following growth conditions: (i) normal growth condition with glucose as a sole carbon source; (ii) when exposed to different concentrations of ethanol stress; (iii) growth condition with different concentrations of ethanol as a sole carbon source; and (iv) when exposed to oxidative stress along with condition iii. These results suggests that sumoylation of Cst6 is not important for cell viability under the tested conditions. Following the cell viability test, we then examined whether sumoylation plays a role in Cst6 stability. Although Cst6 sumoylation increased with ethanol stress, the abundance of Cst6 was reduced compared to non-stress conditions, suggesting a possible correlation between sumoylation and Cst6 stability. Interestingly,

the Cst6-MT was less abundant compared to Cst6-WT even during ethanol stress. This implies that Cst6 sumoylation plays a role in enhancing its stability in normal and ethanol stress conditions.

Sumoylation of TFs has been shown to be involved in the regulation of promoter association [2, 3]. To investigate any possible correlation between Cst6 sumoylation and its DNA binding level, the occupancy levels of both Cst6-WT and Cst6 MT, on ethanol-induced promoters, were compared during non-stress and ethanol stress conditions using ChIP experiments. The occupancy level of Cst6-WT significantly increased with ethanol stress. Although Cst6-MT was also recruited to the target promoters, there was a reduced or delayed association with these promoters during ethanol stress. These results suggest that sumoylation of Cst6 is important for the timely recruitment of Cst6 to its target genes during ethanol stress.

To further study the effect of Cst6 sumoylation on gene expression, we investigated how sumoylation might regulate transcription of Cst6 target genes. For this, we measured the transcription level of ethanol-induced genes from *CST-WT* and *cst6-MT* strains, using RT-qPCR. In most of the genes tested, the mutant strain showed elevated levels of mRNA, at least at one point during transcription. The acquired results in the absence of sumoylation was gene specific because some genes showed elevated mRNA levels during later time points after exposure to ethanol stress (*NCE103*, *ROX1* and *ACC1*), some showed elevated levels during non-stress conditions (*ACC1* and *RPS3*), and some did not show significant differences (*YAP6*, and *HAP4*). *RPS3* showed elevated mRNA levels at all the indicated time points. Overall, these results suggest that Cst6 sumoylation has a general repressive effect that is observed differentially at different targets. Taken together, the data implies that Cst6 sumoylation enhances Cst6 association with ethanol-induced target promoters, partly by increasing Cst6 stability, to limit the transcription of its target genes.

4.2 FUTURE DIRECTIONS

4.2.1 Further examining the regulation of Sko1 by sumoylation

One of our main findings was that sumoylation of Sko1 prevents its association with non-target promoters. Based on the results, we propose that sumoylation of promoter bound Sko1 might loosen its interaction with chromatin, to ensure binding specificity. To confirm this model, future experiments, using nuclear magnetic resonance (NMR) spectroscopy, should focus on finding how sumoylation changes Sko1 conformation that will prevent non-specific binding. In fact, a previous study has already shown that SUMO1 interaction with thymidine DNA glycosylase (TDG), which is involved in base excision repair, changed the structure of TDG that led to its removal from the damaged DNA [13].

Although our ChIP-seq analysis showed that sumoylation prevents non-specific binding of Sko1 to DNA, it does not provide us the quantitative analysis of DNA binding affinity. It is still not clear whether Sko1-MT binds to DNA with similar affinity to Sko-1 WT or not. Therefore, future studies using protein binding microarrays (PBM) should focus on finding DNA binding affinity of Sko1 in the presence and absence of sumoylation will be an asset in finding the mechanism to explain how SUMO prevents or limits Sko1 interaction with promoters.

We also discovered that a SUMO-deficient form of Sko1 has reduced interaction with Hog1 on target promoters. However, important insights can also be gained by examining the interaction between the co-repressor complex (Tup1-Cyc8) and Sko1. In fact, for some target genes, Sko1 acts as a repressor during normal growth due to its association with Tup1-Cyc8 complex and this association is continued even during osmotic stress [4-6]. Therefore, Tup1 ChIP experiments can be conducted to investigate the occupancy levels of Tup1 on Sko1 target genes in the presence and

absence of sumoylation. This might also assist in finding additional roles for Sko1 sumoylation during normal growth.

4.2.2 Further examining the regulation of Cst6 by sumoylation

In our Cst6 study, we found that sumoylation is important for enhancing the association between Cst6 and its target promoters. To date, there have only been four genome-wide studies, including Sko1, conducted to find the function of sumoylation in regulating TF association with DNA [14-16]. Contrasting to Cst6, these genome-wide studies have suggested that sumoylation prevents non-specific binding of TFs with promoters. Therefore, it will be interesting to do a ChIP-seq experiment for Cst6 sumoylation to find out what other roles SUMO might play in regulating Cst6 DNA binding. We could also use the genome-wide studies to learn whether SUMO differentially regulates other Cst6 target genes. In addition, future studies focusing on Cst6 conformation and DNA binding affinity will also shed light on finding the mechanism of how SUMO might regulate the association of Cst6 with promoters.

In an attempt to find the function of Cst6 sumoylation in regulating gene expression, we discovered that in the *Cst6-MT* strain, transcriptional levels of its target genes were elevated later during ethanol stress compared to the wildtype strain. Although it suggests a repressive role for sumoylation, it is important to note that this repression was observed mostly around the time the target gene gets shutdown. Therefore, future studies must focus on elucidating the mechanism of how Cst6 sumoylation functions in deactivating the transcription of Cst6 target genes. It is possible that sumoylation of Cst6 might be important for the recruitment of co-repressor complexes to the promoters. Supporting this prediction, sumoylation of Tup1, a co-repressor protein, is required to deactivate the transcription of the amino acid starvation gene *ARG1* [17]. Sumoylated Tup1

interacts with sumoylated Gcn4, another yeast bZIP TF, to assist in Gcn4 removal from promoters thereby deactivating transcription [17]. It is possible that the sumoylation of Cst6 maintains its interaction with promoters so that co-repressor proteins, similar to Tup1, can gain access to these promoters through Cst6, to fine-tune transcriptional deactivation. Interestingly, both the SUMO mutants of Tup1 and Cst6 show similar transcription level profiles, with significantly higher levels of transcription specifically during later time points, suggesting a possible correlation. To test this, ChIP experiments can be conducted to check the occupancy levels of Cst6 and Tup1 on Cst6 target promoters in the presence and absence of sumoylation.

In addition, it is also possible that sumoylation might influence the homodimerization between Cst6 proteins, or heterodimerization with Aca1 TF. Supporting this, one of the SUMO sites on Cst6, K461, lies within its leucine zipper region, a basic region in bZIP TFs used for protein dimerization. Future investigations, using electrophoretic mobility shift assay (EMSA), should focus on Cst6 homo- or herterodimerization in the presence and absence of sumoylation will be informative.

4.3 Conclusions

A number of TFs interact with each other to regulate gene expression for the proper functioning of the cell. These TFs are also regulated by post translational modifications such as phosphorylation, ubiquitination and sumoylation. Due to the fact that TFs are one of the largest classes of SUMO targets, and altered sumoylation levels were observed in diseases, there are a number of studies being performed to understand the mechanisms of how sumoylation can regulate them.

In this study, I found that two yeast gene-specific bZIP TFs, Sko1 and Cst6, are sumoylated to appreciable levels during non-stress and stress conditions. Sumoylation is important for preventing Sko1 from binding to non-target promoters, and to limit the occupancy level of Sko1 on its target promoters. In addition, sumoylation is also important to enhance the interaction between Sko1 and Hog1 kinase during osmotic stress. Alternatively, Cst6 sumoylation enhances the association of Cst6 with its targets promoters to facilitate the timely deactivation of gene transcription during ethanol stress. Collectively, both our studies suggests a general role for sumoylation; regulating the interaction between TFs and DNA in order to maintain proper gene expression.

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Chapter 1

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Chapter 2

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Chapter 4

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APPENDIX

COPYRIGHT PERMISSIONS FOR FIGURES:

Figure 6.1: Structural comparison between Ubiquitin and the human SUMO1 isoform.

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Figure 1.3: The sumoylation pathway in mammals and S. cerevisiae

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Figure 1.4: Types of sumoylation and the function of poly-sumoylation in ubiquitin-mediated proteasomal degradation.

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Figure 1.5: Schematic representation of how SUMO alters the binding site on the target surface.

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Figure 1.6: Methods explaining how sumoylation can have a prolonged effect on transcriptional repression even after de-sumoylation of the transcription factor.

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Figure 1.7 A&B: Schematic representation of the cis-regulatory sequences involved in transcription.

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Figure 1.8 A&B: Schematic representation of the formation of transcription machinery.

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Figure 1.9: bZIP transcription factor structures and comparisons.

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